Electronic Supporting Information

for

Combating metastasis of breast cancer cells with a carboplatin analogue containing all-trans retinoic acid ligand

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Experimental Section

Materials: Cisplatin was purchased from Shandong Boyuan pharmaceutical Co., Ltd. (Jinan, China). All-trans retinoic acid (ATRA) was purchased from D&B Chemical Technology Co., Ltd (Shanghai, China). Trypsin-EDTA and fetal bovine serum (FBS) were obtained from Gibco-BRL (Burlington, Canada). Dulbecco's high glucose modified eagles medium (DMEM) was purchased from HyClone (Logan, UT). Gelatin (from porcine skin, Type A) was purchased from Sigma-Aldrich (St. Louis, MO). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and crystal violet were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Ultra-purified water was prepared using a Milli-Q Synthesis System (Millipore, Bedford, MA). All other solvents and reagents were used as received.

Cell culture: 4T1 cell line we purchased from Cell Bank of Shanghai, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM containing 10% FBS at 37°C in a humidified and 5% CO₂ incubator.

Animals: Balb/c mice (5 weeks old) were purchased from the Shanghai slack laboratory animal Co., LTD (Shanghai, China). All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. The procedures were approved by the University of Science and Technology of China Animal Care and Use Committee.

![Scheme S1](attachment:image.png)

**Scheme S1.** Synthetic route of Pt-ATRA (compound 4) and compound 5.

**Synthesis of compound 1:** Firstly, compound 1 was synthesized as described in the literature. The mixture of 1,6-dibromotexane (5.00 g, 20.49 mmol), TEBA (0.025 g, 0.11 mmol) and potassium carbonate (8.49 g, 61.47 mmol) was stirred in a flask. Then diethyl malonate (9.84 g, 61.47 mmol) was slowly added into above mixture at 100 °C. After that, the reaction for another 2 h at 100 °C, the solution was filtered.
and the resulting filtrate was separated primarily by silica gel column chromatography to obtain diethyl 2-(6-bromohexyl)malonate, a colorless oil of 12.1 g. Then to a solution of diethyl 2-(6-bromohexyl)malonate (2.0 g, 6.19 mmol) in DMF, NaN₃ (1.0 g, 15.47 mmol) was added and stirred for 12 h at room temperature. After the reaction was over, 10-fold volume of water was added into reaction solution, and then the resulted solution was extracted three times with ethyl ether (3×50 mL). The combined organic layer was further washed with saturated salt water (3×30 mL) and distilled water (3×30 mL), respectively. Finally, the organic layer was dried over sodium sulfate. Solvent was removed under reduced pressure to obtain another colorless oil, diethyl 2-(6-azidohexyl)malonate with a 1.37 g. All resulting diethyl 2-(6-azidohexyl)malonate without further purifying was dissolved in 20 mL ethanol, followed addition of 15 mL 2N NaOH. The mixture was heated to reflux at 80 °C, monitored by TLC (EtOAc: CH₃OH: CH₃COOH 95: 5: 0.1). After the reaction was over, ethanol was removed in vacuum with rotary evaporator and the aqueous layer was washed with dichloromethane (3×20 mL). The resulted aqueous layer was adjusted to pH value 1-2, followed extraction with dichloromethane (3×20 mL). The organic phases were combined, dried with MgSO₄, and evaporated under reduced pressure to obtain compound 1, a white solid with a yield of 92%. ¹H NMR (300 MHz, Chloroform-d) δ 1.39 (s, 6H), 1.55 – 1.66 (t, J = 6.8 Hz, 2H), 1.88 – 2.04 (t, J = 7.4 Hz, 2H), 3.22 – 3.33 (t, J = 6.8 Hz, 2H), 3.39 – 3.51 (t, J = 7.5 Hz, 1H), 11.94 (s, 2H).

**Synthesis of compound 2:** Compound 2 was synthesized as described in the literature.³ To a solution of cisplatin (120 mg, 0.4 mmol) in 30 mL water, AgNO₃ (136 mg, 0.8 mmol) was added. The mixture was stirred for 24 h at 40 °C, then filtered to obtain aqueous solution of Pt(NH₃)₂(ONO₂)₂ for the next step reaction. Meanwhile, to a solution of compound 1 (92 mg, 0.4 mmol) in 5 mL water, NaOH (32 mg, 0.8 mmol) was added, then the resulted aqueous solution was added the above aqueous solution of Pt(NH₃)₂(ONO₂)₂ and stirred overnight at room temperature. After the reaction was over, a large amount of water was removed from the reaction solution. The remaining solution was placed at 4 °C to crystallize for compound 2, a white solid with a yield of 35%. ¹H NMR (300 MHz, DMSO-d₅) δ 1.20 – 1.36 (m, 6H), 1.44 – 1.58 (q, J = 7.1 Hz, 2H), 1.72 – 1.86 (q, J = 7.0 Hz, 2H), 3.26 – 3.41 (t, J = 6.7 Hz, 2H), 3.45 – 3.57 (t, J = 7.0 Hz, 1H), 4.15 (brs, 6H).

**Synthesis of compound 3:** To a solution of ATRA (150 mg, 0.4 mmol) in 50 mL THF, cesium carbonate (300 mg, 0.8 mmol) and propargyl bromide (230 μL, 2.0 mmol) were added, and the reaction mixture was stirred at room temperature. Progress of the reaction was monitored by TLC. After the reaction was over, the crude product was subjected to column chromatography (eluent: ethyl acetate/hexane 5:95) to obtain pure compound 3 with yield of 89%.⁴ ¹H NMR (300 MHz, Chloroform-d) δ 1.00 – 1.09 (s, 6H), 1.41 – 1.55 (m, 2H), 1.56 – 1.70 (m, 2H), 1.68 – 1.80 (s, 3H), 1.98 – 2.13 (m, 5H), 2.34 – 2.43 (d, J = 1.1 Hz, 3H), 2.42 – 2.53 (t, J = 2.4 Hz, 1H), 4.65 – 4.80 (d, J = 2.5 Hz, 2H), 5.75 – 5.88 (s, 1H), 6.06 – 6.22 (d, J = 16.2 Hz, 2H), 6.24 – 6.39 (d, J = 14.7 Hz, 2H), 6.92 – 7.19 (dd, J = 15.0, 11.4 Hz, 1H).

**Synthesis of Pt-ATRA (compound 4), compound 5:** To a solution of compound 2 or propargyl alcohol (0.06 mmol) in t-BuOH/H₂O mixture (2:1, 2 mL), sodium ascorbate (1.2 mg, 0.006 mmol) and CuSO₄ (1.2 mg, 0.0045 mmol) were added at room temperature and under a nitrogen atmosphere. To this mixture, compound 3 (0.06 mmol) was added and the reaction mixture was stirred at 45 °C, monitored by TLC. The crude mixture was removed in vacuum with rotary evaporator and lyophilized. The resulted light yellow solid was dissolved in mixed solution of acetone and methanol (5:1). After centrifugation, supernatant was dried in vacuum, and washed with ethyl ether to obtain compound 4 and 5 in 50–60% yields. Compound 4, a light yellow solid, ¹H NMR (300 MHz, DMSO-d₆) δ 0.95 – 1.03 (s, 6H), 1.13 – 1.30 (m, 6H), 1.38 – 1.46 (m, 2H), 1.50 – 1.60 (m, 2H), 1.63 – 1.69 (s, 2H), 1.72 – 1.83 (s, 4H), 1.87 – 2.09 (d, J = 4.7 Hz, 6H), 2.25 – 2.37 (s, 3H), 3.46 – 3.55 (t, J = 6.9 Hz, 1H), 4.02 – 4.23 (m, 6H), 4.25 – 4.45 (t, J = 7.0 Hz, 2H), 4.98 – 5.26 (s, 2H), 5.78
- 5.89 (s, 1H), 6.09 – 6.31 (dd, J = 21.0, 14.1 Hz, 3H), 6.34 – 6.48 (d, J = 15.1 Hz, 1H), 6.96 – 7.15 (dd, J = 15.1, 11.4 Hz, 1H), 8.07 – 8.23 (s, 1H). ESI-MS: m/z = 794.8 (calculated 794.3) ; Compound 5, a white solid, \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 1.17 – 1.31 (m, 6H), 1.45 – 1.58 (q, J = 6.8 Hz, 1H), 1.75 – 1.82 (s, 3H), 3.45 – 3.57 (td, J = 7.0, 4.2 Hz, 1H), 4.06-4.24 (brs, 6H), 4.26 – 4.37 (t, J = 6.8 Hz, 2H), 4.46 – 4.53 (s, 2H), 7.92 – 8.00 (d, J = 2.9 Hz, 1H).

**Cellular platinum uptake and NDA platination**

4T1 cells were seeded in 6-well plates overnight and treated with 2 mL fresh medium containing 100 μM of Pt-ATRA or carboplatin at 37°C for 3 h. The cells were washed with PBS (pH7.4) for 3 times and collected. After the cell numbers were counted by a hemacytometer, the harvested cells were digested by HNO\(_3\) for ICP-MS assay. For DNA platination, cellular DNA in 4T1 cells was isolated by using Genomic DNA Extraction Kit (Beyotime, China) and analysed using ICP-MS.

**In vitro cytotoxicity assays**: 4T1 cells were seeded in 96-well plates at 4000 cells/well in 100 μL of DMEM containing 10% FBS. Cells were incubated overnight in a humidified and 5% CO\(_2\) incubator at 37°C. The culture medium was replaced with 100 μL fresh medium containing drugs of different concentrations. After further incubation for 72 h, the medium was replaced with fresh culture medium containing 1 mg/mL MTT. The cells were incubated for another 4 h to ensure that viable cells could reduce the yellow tetrazolium salt (MTT) into dark blue formazan crystals. Finally, to very cells, 100 μL of lysis buffer was added, followed incubation for another 4 h at 37°C. The absorbance was measured at 490 nm using a Bio-Rad 680 microplate reader. The IC\(_{50}\) values were calculated using GraphPad Prism software (version 6.01) based on data from three parallel experiments.

**The wound healing assay**: 4T1 cells were seeded in 6-well plates until 95-100% of confluency. A linear wound was created using a plastic tip and washed with PBS to remove cell debris. After that, fresh serum-free DMEM medium containing drugs of 50 μM were added and the cells were further incubated for 24 h. The wound healing was analyzed by photoimaging at 0 h and 24 h. The healed areas in individual group of cells were calculated. Experiment was confirmed for three independent times.

**In vitro transwell assay**: The migration and invasion of cells were accessed using transwell assays. For migration assays, \(1 \times 10^5\) cells were plated in the top chamber with a non-coated membrane (24-well insert, 8 mm pore size). For invasion assays, \(2 \times 10^5\) cells were plated in the top chamber with a Matrigel-coated membrane (24-well insert, 8 mm pore size). In both assays, cells were plated in medium without serum, pre-incubated with drugs for 24 h. Serum supplemented medium was used as a chemoattractant in the lower chamber. After 24 h incubation, cells that did not cross the pores (non-migration or non-invasion cells) were removed by a cotton swab. Cells on the lower surface of the membrane were stained with 0.1% crystal violet solution and were counted by microscopic examination. Then the dye in the migrating and invading cells on the lower surface of the membrane were dissolved using 10% acetic acid for 10 min and the absorbance of the solution was measured at 550 nm.

**In vivo antitumor efficacy**: For in vivo antitumor experiment, \(3 \times 10^5\) 4T1 cells were injected to the right mammary gland of female mice. Tumors were allowed to grow to a volume of 100-200 mm\(^3\). The mice bearing metastatic 4T1 breast cancer were randomly assigned to 5 groups (n=5) for the treatment of PBS, carboplatin, compound 5, ATRA or Pt-ATRA. Drugs were administrated through tail vein injection every other day for 6 times in a dosage of 1.5 mg/kg platinum or 5.4 mg/kg ATRA. Tumor growth was monitored...
by measuring the perpendicular diameter of the tumor using calipers every two days. The tumor volume was calculated according to the formula: tumor volume (mm$^3$) = 0.5×length×width$^2$. Animals were sacrificed for humane reasons and the lung tissues were immediately excised for image and H&E staining.

**Real-time quantitative PCR:** The expression of CSC-associated genes was measured as described in the literature. 4T1 cells were seeded at a density of 1×10$^6$ cells per well onto 6-well tissue culture plates in complete DMEM culture medium. After 12 h incubation, cells were treated with various formulations of carboplatin, compound 5, ATRA or Pt-ATRA and incubated for 24 h. The total RNA in the cells was then collected using RNAiso Plus (TaKaRa, Dalian, China). 500 ng of total RNA was transcribed into cDNA using the PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China). The mRNA levels of Oct4, Nanog and Sox2 were measure using real-time quantitative PCR (qPCR) on a LightCycler®480 SYBR Green I Master (Roche Applied Science, Indianapolis). The mRNA levels were normalized against the housekeeping gene GAPDH. The qPCR starting with 10 min of preincubation at 95°C followed by 40 amplification cycles. Sox2, Oct4 and Nanog mRNA levels were finally normalized to cells with PBS treatment alone. The following primers were used in the analysis:

- **Sox2** forward: 5’-GCTGGACCTGGAACCTGGAGAAG-3’;
- **Sox2** reverse: 5’-TTGCGTTAAATTTGGATGGGATTTGTG-3’;
- **Oct4** forward: 5’-GTGTAGGATGGATGGGATTTGTG-3’
- **Oct4** reverse: 5’-GAGTAGAGTGTTGGAAGTG-3’
- **Nanog** forward: 5’-CTTGTTGTGTTAGGTATTGTCTTAG-3’;
- **Nanog** reverse 5’-CTGGTTCCTTGTCTTCATCCTC-3’;
- **GAPDH** forward: 5’-AAGGTGGTGAAGCAGGCATC-3’;
- **GAPDH** reverse 5’-GAAGGTGGAAGAGTGGGAGTTG-3’.

**Reference:**
Figure S1. $^1$H NMR spectrum of compound 1 in CDCl$_3$.

Figure S2. $^1$H NMR spectrum of compound 2 in DMSO-d$_6$ (The inset shows the full NMR spectra of compound 2).
Figure S3. $^1$H NMR spectrum of compound 3 in CDCl$_3$ (The inset shows the full NMR spectra of compound 3).

Figure S4. $^1$H NMR spectrum of Pt-ATRA in DMSO-d$_6$ (The inset shows the full NMR spectra of Pt-ATRA).
**Figure S5.** ESI-MS spectrum of Pt-ATRA.

**Figure S6.** $^1$H NMR spectrum of compound 5 in DMSO-$_d_6$. The inset shows the full NMR spectra of compound 5.
Figure S7. Cellular platinum uptake and DNA platination. Platinum in 4T1 cells was determined using ICP-MS after the treatment of the 100 μM platinum complex for 3 h. (A) Platinum in whole cells. (B) Platinum in DNA.

Figure S8. The effect of compounds on lateral migration of cancer cells measured using scratch test. 4T1 cells were pre-incubated for 24 h with drugs at dose of 50 μM. Analyses of lateral migratory cells were obtained by measuring wound closure rate.
Figure S9. The effect of compounds on migration and invasion of cancer cells measured using the transwell assay. 4T1 cells were pre-incubated for 24 h with drugs at dose of 50 μM. Quantitative analyses of migratory cells and invasive cells were obtained by measuring wound closure rate and the UV absorbance at 550 nm of the crystal violet dye that was extracted from the migration or invasion cells on the lower surface of the membrane.

Figure S10 The number of metastatic tumor nodules on the lungs of mice at the end of the treatment. Balb/c mice-bearing 4T1 tumors were injected through the tail vein with PBS, carboplatin, compound 8, ATRA and Pt-ATRA. The Pt(II) dose per injection was equivalent to 1.5 mg kg\(^{-1}\) body weight and the ATRA dose per injection was equivalent to 5.4 mg kg\(^{-1}\) body weight, (n = 5). PBS was used as a control. Mice were treated every-other-day 6 times. Error bars denote standard deviations.