Supporting Information

A novel docetaxel derivative exhibiting potent anti-tumor activity and high safety in preclinical animal models

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1. Materials

Docetaxel (DTX, 98%, Jiangsu Yew Pharmaceutical Co., Ltd), triethylchlorosilane (TESCl, 99%, Sigma-Aldrich), 4-(dimethylamino) pyridine (DMAP, 99%, Sigma-Aldrich), 4-ketonphenyl isocyanate (98%, Sigma-Aldrich), DTX injection (Xicun, Shenzhen Main Luck Pharmaceuticals Inc.) and ammonium chloride (NH_4Cl, 99%, Innochem) were used directly from purchase. Medium and long chain fat emulsion injection (fat emulsion for short, 25 g medium chain triglycerides, 25 g soybean oil, 3 g egg phospholipids, 6.25 g glycerin; Sino-Swed Pharmaceutical Corp. Ltd) was used directly from purchase. N, N-Dimethylformamide (DMF), toluene, dichloromethane (DCM), and pyridine were dried with calcium hydride for more than 48 h and then distilled before use. Roswell Park Memorial Institute (RPMI) 1640 medium, Phosphate-buffered saline (PBS), Minimum Essential Media (MEM), Ham's F-12K medium, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin, streptomycin, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were provided by Sigma-Aldrich (St. Louis, MO). 6- and 96-well polystyrene plates were purchased by Chinese Sangon Biotech. Dead Cell Apoptosis Kit with Annexin V Alexa Fluor™ 488 & Propidium Iodide (PI) was purchased by Invitrogen™. Cell Cycle Kit was provided by Beyotime Biotechnology. Unless mentioned above, all other compounds, solvents and reagents were received from the domestic companies without additional processing.

2．Measurements

^{1}H and ^{13}C NMR spectra were registered on a Varian MERCURY plus-400 spectrometer (400 MHz) or a Varian MERCURY plus-600 spectrometer (600 MHz) with dimethyl sulfoxide-d_6 (DMSO-d_6) as solvent at 298 K. The chemical shifts were referenced to residual peaks of deuterated solvents: DMSO-d_6 (2.48 ppm). The purity of final compound was determined by high performance liquid chromatography (HPLC). The column was a Unitary C18, 5 μm particle size (250 mm × 4.6 mm).

High resolution mass spectrometry (HRMS) was performed on an electrospray mass spectrometer (Waters Q-TOF Premier, MA, USA). The high-performance liquid chromatography & triple quadrupole mass spectrometer (UPLC-3Q MS) analysis was performed on an AcquityTM Ultra Performance LC (Waters, USA) and AB SCIEX Triple QuadTM 5500 (AB, USA) System. An Acquity UPLC BEH C8 column (50 mm × 2.1 mm, 1.7 μm) was used for analysis.

3. Cell Cultures

L929 cells (mouse fibroblast cells), HeLa cancer cells (human cervical cancer cells), and MCF-7 cells (human breast adenocarcinoma cell line) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin under a humidified atmosphere of 5% CO_2 at 37 °C. HeLa/PTX cancer cells (Paclitaxel-resistant HeLa cells) were maintain in RPMI 1640 medium supplemented with 10% FBS, 2 mM Glutamine and 1% penicillin/streptomycin. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. A549 cancer cells (human non-small cell lung cancer cell) were cultured in Ham's F-12K medium supplemented with 1% penicillin/streptomycin and 10% FBS. Cells were incubated in an incubator maintained at 37 °C with 5% CO_2 under a humidified condition. MRC-5 cells (Human embryo lung cells) were cultured in MEM under a humidified atmosphere containing 5% CO_2 at 37 °C. The culture medium consisted of 2 mM Glutamine, 1% Non Essential Amino Acids (NEAA), penicillin, 10% FBS and 1% streptomycin.

4. Synthesis of DTX-AI

4.1. Synthesis of 2'-O-(triethylsilyl)-7-O-(triethylsilyl)docetaxel (DTX-2TES)
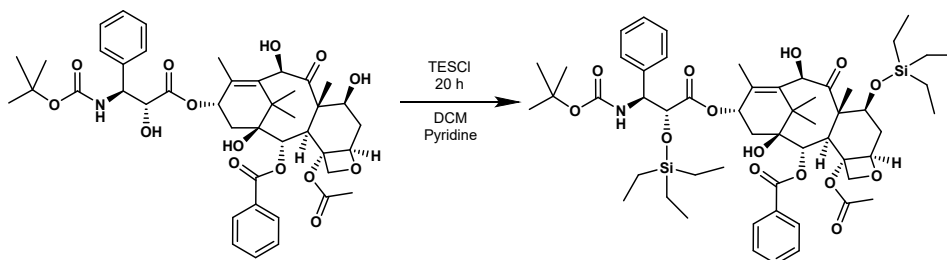
TESCl in 4 portions (4.96 mmol) was added dropwise (1 mL/min) into the reaction flask with DTX (400 mg, 0.496 mmol) in 8 mL DCM and 8 mL pyridine under argon in an ice bath. After that, the reaction system was kept in the ice bath for another 40 min. The reaction system was then placed in an oil bath at 35 °C for 24 h. The reaction was monitored by thin layer chromatography (TLC) (silica, 30% ethyl acetate (EtOAc)/hexane). When the starting materials disappeared, the reaction was quenched with aq. NH_4Cl (3 mL). The reaction mixture was extracted with DCM. The organic layer was separated, washed with aq. NH_4Cl (6 mL × 2) and brine (6 mL), dried over anhydrous magnesium sulfate (Mg_2SO_4), and concentrated under vacuum. The residue was purified by silica gel flash chromatography (20-50% EtOAc/hexane) to give DTX-2TES as a white powder (433 mg, 89% yield). ^1H NMR (400 MHz, DMSO-d_6) δ 10.24 (s, 1H), 8.02-7.94 (d, 2H), 7.93-7.85 (d, 2H), 7.74-7.67 (t, 1H), 7.65-7.52 (m, 5H), 7.39-7.30 (m, 4H), 7.17 (tt, J = 5.8, 3.0 Hz, 1H), 6.26 (s, 1H), 5.78 (t, J = 9.1 Hz, 1H), 5.44 (d, J = 7.1 Hz, 1H), 4.95 (d, J = 9.1 Hz, 1H), 4.89 (dd, J = 9.9, 7.1 Hz, 1H), 4.66 (s, 1H), 4.48-4.33 (m, 2H), 4.05-4.01 (s, 2H), 3.62 (d, J = 7.0 Hz, 1H), 2.49 (s, 3H), 2.36 (s, 3H), 1.97-1.95 (m, 1H), 1.79 (s, 3H), 1.74-1.59 (m, 1H), 1.54 (s, 3H), 1.33 (s, 9H), 1.21 (s, 1H), 1.12 (s, 3H), 0.98 (s, 3H), 0.85 (td, J = 7.9, 6.2 Hz, 18H), 0.52 (hept, J = 7.9 Hz, 12H).^{13}C NMR (101 MHz, DMSO-d_6) δ 208.64, 172.38, 170.65, 165.91, 155.75, 139.30, 137.77, 136.33, 134.08, 130.67, 130.23, 129.29, 128.83, 128.44, 84.01, 80.61, 78.76, 77.43, 76.19, 75.28, 74.41, 73.28, 71.07, 58.56, 57.66, 46.49, 43.48, 37.48, 35.51, 28.84, 27.19, 23.34, 21.26, 14.58, 10.47, 7.30, 7.20, 5.40, 4.79. ESI-MS m/z calcd. [M+H$^+$]: 1036.520. Found: 1036.544.

4.2. Synthesis of 2'-O-(triethylsilyl)-7-O-(triethylsilyl)-10-O-4-ketonephenylcarbamatedocetaxel (DTX-2TES-AI)
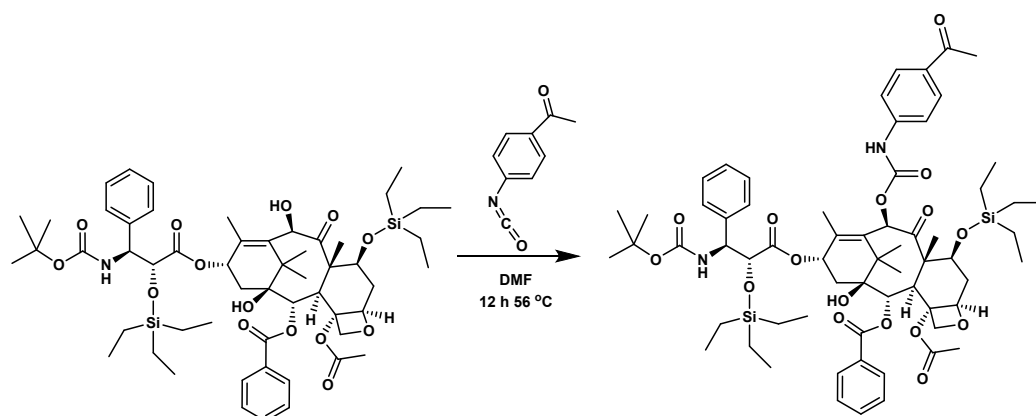
To a solution of DTX-2TES (400 mg, 0.386 mmol) in anhydrous DMF (10 mL) was added 4-acetylphenyl isocyanate (187 mg, 1.16 mmol) and DMAP (5 mg, 0.04 mmol) at room temperature. The reaction mixture was stirred for 12 h at 56 °C prior to quenching with aqueous sodium bicarbonate (aq. NaHCO$_3$). The aqueous phase was extracted with EtOAc twice and the combined organic phases were dried sodium sulphate (Na$_2$SO$_4$) and concentrated in vacuum. The residue was purified by silica gel flash chromatography (30% EtOAc/hexane) to give DTX-2TES-AI (361 mg, 78%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.01-7.94 (m, 2H), 7.72-7.66 (m, 1H), 7.59 (t, J = 7.6 Hz, 2H), 7.48 (d, J = 9.9 Hz, 1H), 7.38-7.30 (m, 4H), 7.16 (tt, J = 6.0, 2.5 Hz, 1H), 5.86-5.74 (m, 1H), 5.39 (d, J = 7.1 Hz, 1H), 4.92 (tt, J = 12.6, 5.0 Hz, 4H), 4.50 (s, 1H), 4.45 (d, J = 7.1 Hz, 1H), 4.29 (dd, J = 10.5, 6.6 Hz, 1H), 4.03 (s, 2H), 3.65 (d, J = 7.1 Hz, 1H), 2.34 (s, 4H), 1.91 (dd, J = 15.3, 9.2 Hz, 1H), 1.71-1.57 (m, 4H), 1.53 (s, 3H), 1.34 (s, 9H), 0.96 (d, J = 6.4 Hz, 6H), 0.87 (td, J = 7.9, 2.2 Hz, 18H), 0.60-0.39 (m, 12H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 202.52, 196.93, 172.19, 170.80, 170.53, 165.72, 155.59, 152.57, 143.89, 139.93, 139.03, 133.95, 131.75, 130.41, 130.02, 129.14, 128.67, 128.25, 118.03, 83.71, 80.41, 78.60, 77.08, 75.94, 75.86, 74.83, 72.59, 70.82, 60.22, 58.36, 58.15, 46.50, 43.44, 37.22, 34.94, 28.64, 26.87, 23.18, 21.44, 21.23, 14.55, 10.31, 7.23, 7.01, 5.32, 4.56. ESI-MS m/z calcd [M+H$^+$]: 1197.567. Found: 1197.570.

4.3. Synthesis of 10-O-4-ketonephenylcarbamatedocetaxel (DTX-AI)
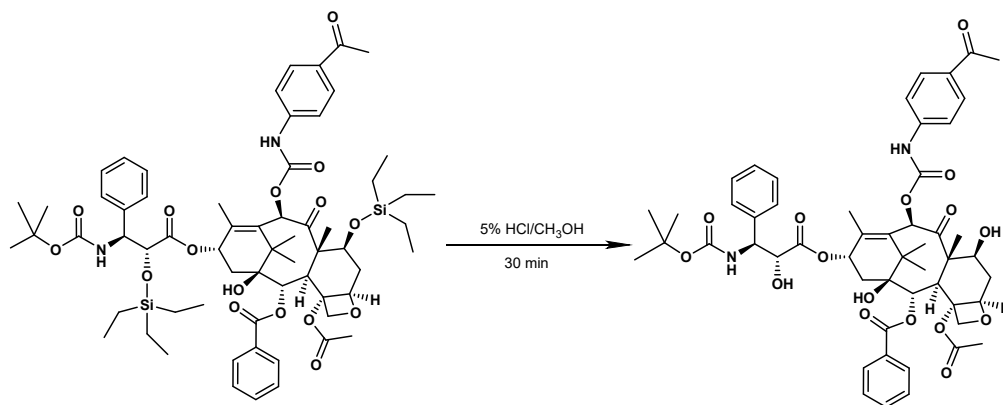
DTX-2TES-AI (200 mg, 0.167 mmol) was dissolved in freshly prepared methanol/HCl (5% v/v, 2 mL) and the solution stirred at 26 °C for 30 min. The solution was then diluted with EtOAc (10 mL) and washed with water, dilute NaHCO$_3$, and finally with brine. The organic layer was dried over Na$_2$SO$_4$ and evaporated under reduced pressure. The crude product was purified further by silica gel flash chromatography (EtOAc:hexane, 1:1) to yield DTX-AI as a white solid (142 mg, 88%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.19 (s, 1H), 7.97 (d, J = 7.6 Hz, 2H), 7.91 (d, J = 8.9 Hz, 2H), 7.70 (t, J = 7.2 Hz，1H), 7.66-7.57 (m, 4H), 7.41 (d, J = 9.4 Hz, 1H), 7.35 (t, J = 7.6 Hz, 2H), 7.29 (d, J = 7.6 Hz, 2H), 7.19 (t, J = 7.3 Hz, 1H), 6.31 (s, 1H), 5.87 (t, J = 8.0 Hz, 2H), 5.45 (d, J = 7.1 Hz, 1H), 4.97 (d, J = 7.0 Hz, 1H), 4.94 - 4.84 (m, 2H), 4.66 (s, 1H), 4.34 (t, J = 7.2 Hz, 1H), 4.13 (dt, J = 11.0, 6.8 Hz, 1H), 4.012 (s, 2H), 3.63 (d, J = 7.1 Hz, 1H), 2.50 (s, 3H), 2.37-2.27 (m, 1H), 2.22 (s, 3H), 1.95-1.89 (m, 1H), 1.87 (s, 3H), 1.80 (m, 1H), 1.64 (t, J = 12.8 Hz, 1H), 1.51 (s, 3H), 1.33 (s, 9H), 1.13 (d, J = 6.0 Hz, 3H), 1.04 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 203.81, 197.12, 173.49, 170.51, 165.89, 155.77, 152.71, 144.17, 140.44, 140.19, 134.08, 131.85, 130.64, 130.24, 129.37, 128.87, 127.91, 118.17, 84.29, 80.96, 78.81, 77.44, 76.07, 75.19, 74.59, 71.22, 70.54, 58.16, 58.00, 55.59, 46.86, 43.69, 37.27, 35.37, 28.83, 26.90, 23.10, 21.93, 14.64, 10.47. ESI-MS m/z calcd. [M+H^{+}]: 969.3943. Found: 969.4338. HPLC analysis: retention time = 23.93 min; peak area, 99.66% (Purity); eluent A, water; eluent B, CH$_3$CN; flow rate of 1 mL/min; detection wavelength, 220 nm; column temperature, 30 °C.

5. Characterization of DTX-AI

The ^{1}H and ^{13}C NMR spectra with number-labeled chemical structure of DTX-AI were shown in Fig. S2. For comparison, the ^{1}H NMR spectra of AI and DTX were also displayed in Fig. S2B and the ^{13}C NMR spectrum of DTX was in Fig. S2C. In ^{1}H NMR spectrum of DTX-AI, the integral area of the proton signals (25, 26) at 7.96 - 7.98 ppm was equal to that of the proton signals (5'', 6'') at 7.90 - 7.92 ppm, which confirmed the molar ratio of DTX and AI was accord with 1:1 in DTX-AI. Moreover, after the nucleophilic addition reaction, two hydroxyl proton signals (C2'-OH, C7-OH) of DTX appeared at 5.00 - 5.02 ppm and 5.83 - 5.88 ppm remained nearly unchanged. But C10 hydroxyl proton signal (C10-OH) of DTX at 4.93 - 4.94 ppm disappeared in the ^{1}H NMR spectrum of DTX-AI. This indicated that AI was covalently attached to DTX only at the C10 position. In addition, the proton signals of C10 in DTX at 5.08 - 5.09 ppm shifted to 6.31 ppm in DTX-AI, which also confirmed the above result. On the other hand, comparing with the ^{13}C NMR spectra of DTX and DTX-AI in Fig. S2C, the signal of C9 at 209.84 ppm in DTX was shifted to 203.81 ppm in DTX-AI, while the signal of C10 at 75.28 ppm in DTX shifted to 75.19 ppm in DTX-AI, which further confirmed AI was only attached to the C10 position of DTX by nucleophilic addition reaction. The purity and molecular weight of DTX-AI was characterized by the high-performance liquid chromatography (HPLC) and high-resolution mass spectrometry (HRMS), respectively. The HPLC profile and HRMS spectrum of DTX-AI were shown in Fig. S3 and Fig. S4. Only the signal of DTX-AI was found at 23.93 min (retention time) and the purity of DTX-AI was 99.66%. The molecular ion peak (*m/z*, M+H$^+$) of DTX-AI was observed at 969.4338, which agreed with the calculated value 969.3943 (Δ *m/z* = 0.0395 < 0.05). All above results confirmed the successful synthesis of DTX-AI.

6. Formulation of DTX and DTX-AI

6.1 DTX injection

DTX injection used was commercial formulation named Xicun: DTX powder (20 mg) was stored in 0.5 mL Tween 80 (40 mg/mL). Firstly, a 10 mg/mL stock was received by diluting DTX/Tween 80 with 13% (w/w) injectable ethanol (1.5 mL). Afterwards, different concentrations of DTX injection were yielded with the dilution by PBS.

6.2 DTX or DTX-AI delivered by fat emulsion

DTX or DTX-AI was initially solubilized in injectable ethanol and then fat emulsion was added to yield a stock solution with different concentrations. Collectively, mice were treated i.v. with DTX or DTX-AI prepared fresh in 5% ethanol and 95% fat emulsion.[1]
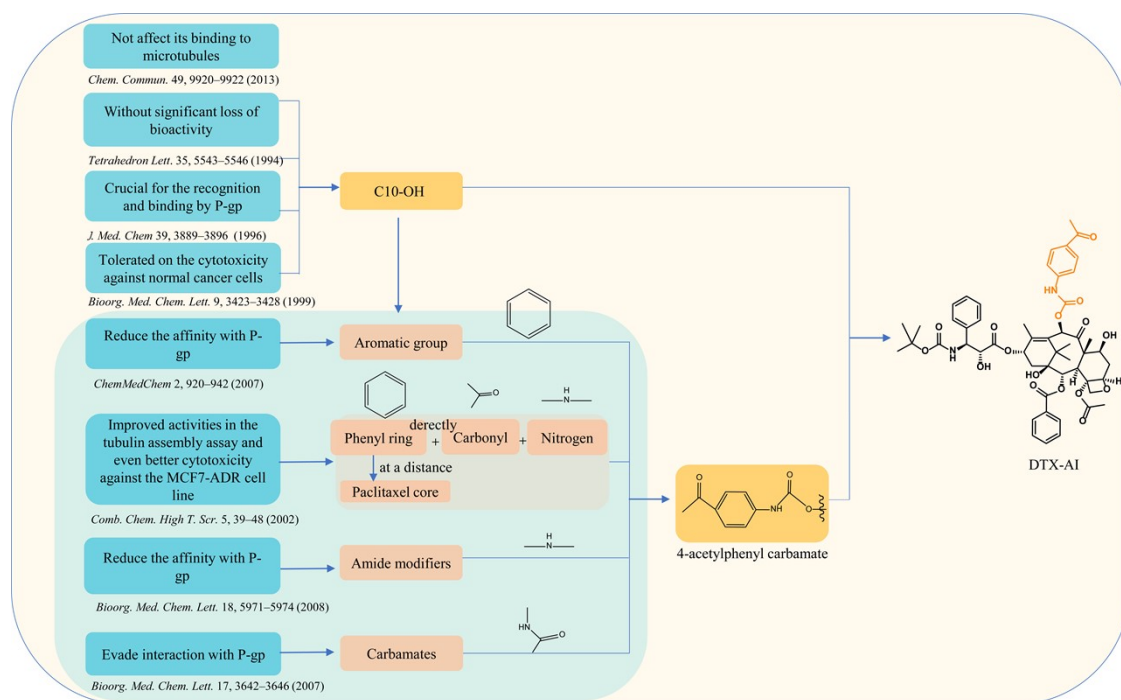
7. Figures

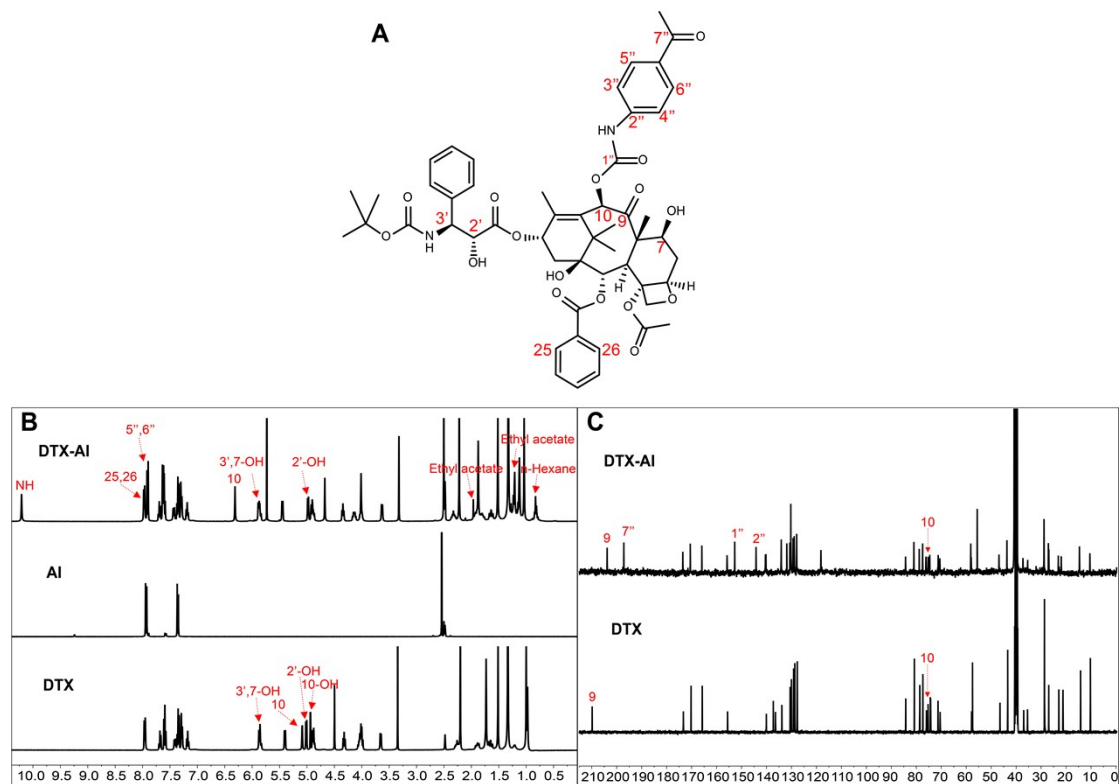Fig. S1. The optimization process of DTX-AI

Fig. S2. Characterization of DTX-AI. (A) Number-labeled chemical structure of DTX-AI. (B) ^{1}H NMR spectra of DTX, AI and DTX-AI in DMSO-d_6. (C) ^{13}C NMR spectra of DTX and DTX-AI in DMSO-d_6.
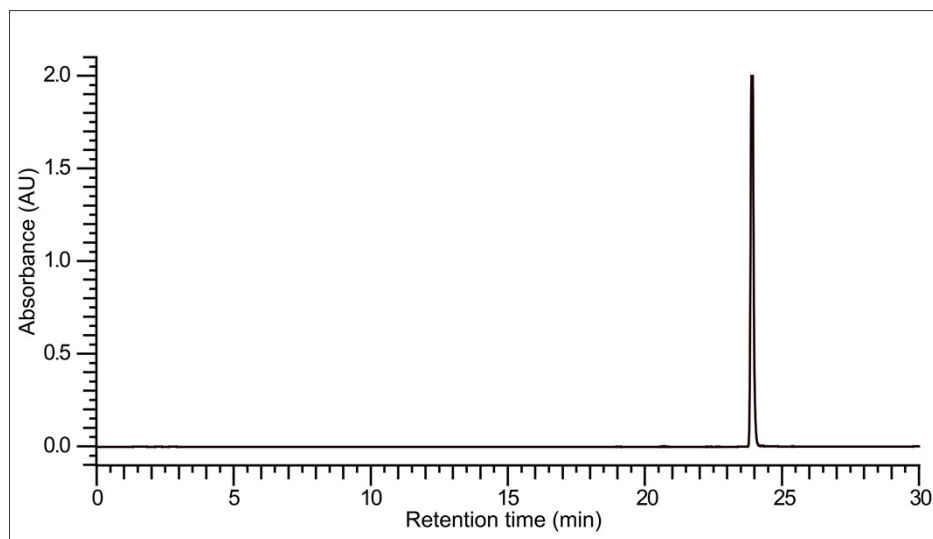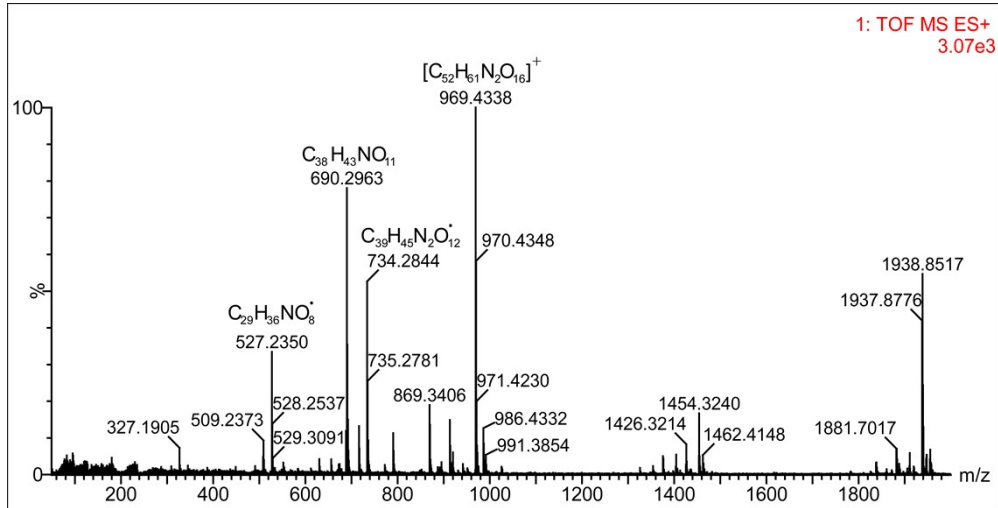

Fig. S3. HPLC profile of DTX-AI.
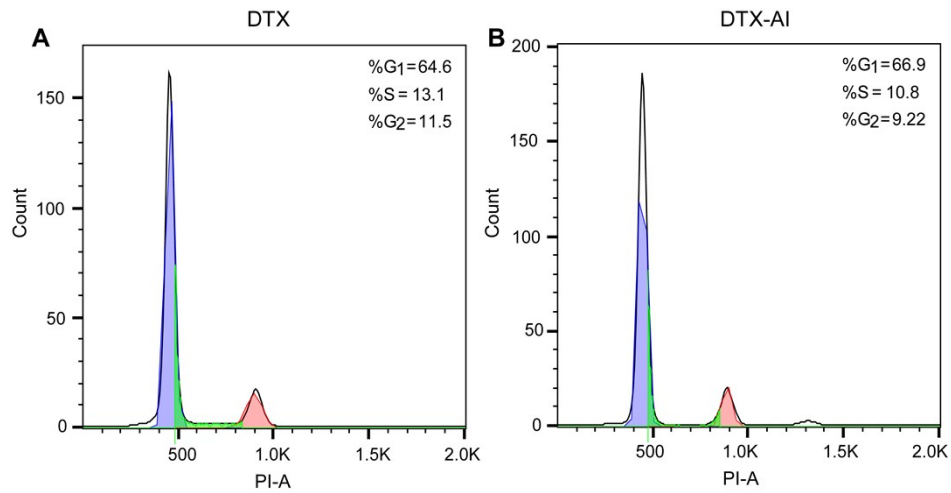
Fig. S4. HRMS spectrum of DTX-AI.

Fig. S5. Effects of DTX and DTX-AI on the cell cycle distribution in HeLa/PTX cells determined by analyzing 10000 gated cells. Content of DNA is represented on the X-axis; Number of cells counted is represented on the Y-axis.

Fig. S6. The mice were treated with DTX/Fat emulsion and the HeLa tumor size was real-time monitored during the evaluation period.
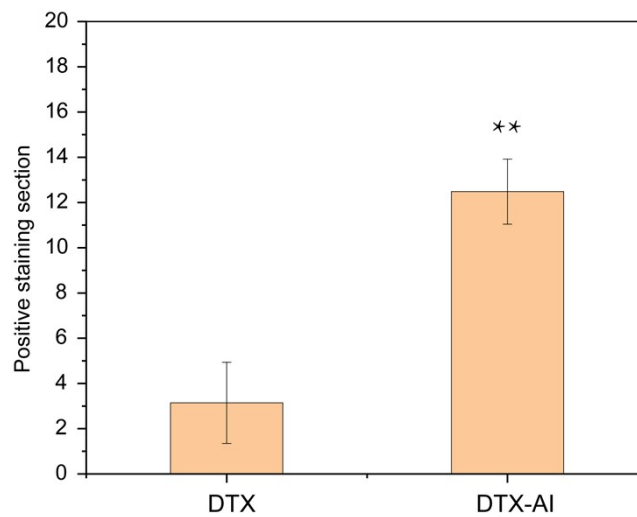

Fig. S7. Immunohistochemical analysis of tumor cells treated with mouse anti-Ki67 monoclonal antibody. The proliferation index by Ki-67 was evaluated using ImageJ analysis software[2], **P < 0.01.

8. Reference

1. D. Sampath, L. M. Greenberger, C. Beyer, M. Hari, H. Liu, M. Baxter, S. Yang, C. Rios and C. Discafani, *Clin. Cancer Res.*, 2006, **12**, 3459–3469.

2. J. I. Kuroda, J. Kuratsu, M. Yasunaga, Y. Koga, H. Kenmotsu, T. Sugino and Y. Matsumura, *Clin. Cancer Res.*, 2010, **16**, 521–529.