Electronic Supplementary Information

Synthetic copolymer conjugates of docetaxel and *in vitro* assessment of anticancer efficacy

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1. Characterisation methods

a. NMR

^{1}H and ^{13}C NMR spectra were recorded at 298 K on a Bruker Avance IIIHD 500 MHz (^{1}H at 500.10 MHz, ^{13}C at 125.75 MHz) or 600 MHz (^{1}H at 600.13 MHz, ^{13}C at 150.90 MHz) spectrometer. The data were processed with Bruker's TopSpin 3.5 software. ^{1}H chemical shifts, δ, are given in ppm relative to TMS. Diffusion NMR experiments were performed on a Diff30 probe, with the gradient strength calibrated against the diffusion coefficient of HDO in D_2O (1.9×10^{-5} cm^2 s^{-1}). Diffusion experiments were performed using the Bruker pulse sequence *diffSteBp*, using a linear ramp (5–95% gradient strength) with pulse lengths of 1 ms, diffusion time of 20 ms. Diffusion data was processed using Bruker's DynamicsCentre software package. NOESY experiments were performed on a standard broadband probe, using a mixing time of 300 ms. DTX loading of conjugates was calculated by comparing the relative intensities of their corresponding ^{1}H NMR spectroscopic signals with those of precisely weighed 3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP) as an internal standard. Quantitative NMR spectroscopy was carried out using a 30° pulse and relaxation delays of at least 20 s, which were found sufficient to allow for complete relaxation of all signals. Integration of the TSP peak at 0.00 ppm (9H, s) was compared with DTX aromatic integrals (10H, m).

b. GPC

Dimethylacetamide (DMAc) was used as the eluent. The GPC system was a Shimadzu modular system with an autoinjector, a Phenomenex™ 5.0 μm bead sizeguard column (50 × 7.5 mm) followed by four Phenomenex™ 5.0 μm bead size columns (10^5, 10^4, 10^3, and 10^2 Å), and a differential refractive index detector. Calibration used narrow M_w distribution polystyrene and poly(methyl methacrylate) standards ranging from 2×10^2 to 10^6 g mol^{-1}. Analysis software was Cirrus™ GPC Offline GPC/SEC from Varian Polymer Laboratories.

c. DLS

Dynamic light scattering and zeta potential measurements were performed with a Malvern ZetaSizer Nano ZS instrument using a polystyrene fluorescence cuvette (Sarstedt No. 67.754) and DTS clear disposable folded zeta cell (DTS1070). Samples were dissolved in MilliQ water and measurements were recorded in triplicate at 25 °C. Zeta measurements were taken at pH ~ 6.

d. HPLC

Purification of DTX-2′-Suc-NHS was performed with a Waters 2698 Separations Module, Waters 2498 UV/Visible detector (230 nm), and reverse phase silica column (Grace Apollo C18, No. 36543, 5 μm, 250 × 10 mm) with the mobile phase consisting of a gradient of water + TFA 0.1% v/v and acetonitrile.

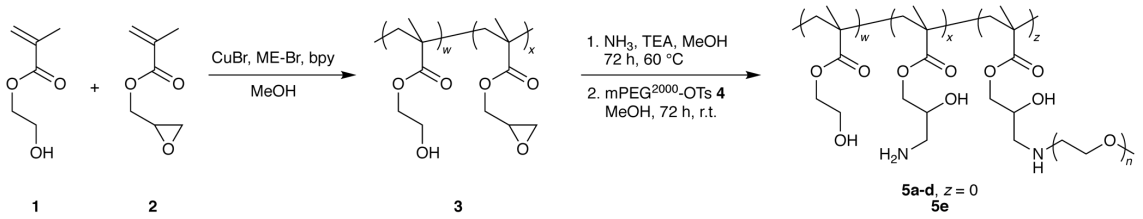
DTX present in drug release samples was quantified using a Waters 2698 Separations Module, Waters 2498 UV/Visible detector (232 nm), and reverse phase silica column (Phenomenex Luna C18(2), 5 μm, 150 × 5 mm) maintained at 40 °C with the mobile phase consisting of a gradient of water/acetonitrile (72:28 ⟶ 0:100 over 10 min).

e. Mass spectrometry

Mass spectrometry was performed on a Waters LCT premier XE in ESI mode. Samples were dissolved in acetonitrile, and formic acid provided the reference ion.

2. Synthetic methods and characterisation

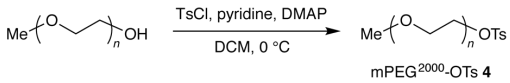
a. Poly(HEMA-*ran*-GMA) copolymer



Copolymer backbones **3** were synthesised from 2-hydroxyethyl methacrylate (HEMA, **1**) and glycidyl methacrylate (GMA, **2**). Inhibitors were first removed from GMA by passage through a basic alumina column. Atom transfer radical polymerisation was achieved in methanol with 2,2′-bipyridine, CuBr as the catalyst, and 2-(4-morpholino)ethyl 2-bromoisobutyrate as the initiator. The resulting poly(HEMA-*ran*-GMA) was collected by dialysis over 5 days, first against 5% methanol, then against pure water. The products were lyophilised, collected and confirmed by ^{1}H NMR spectroscopy and GPC. Amination of the copolymer was achieved by dissolving poly(HEMA-*ran*-GMA) (1 g) in methanol (50 ml), adding aqueous ammonia (30%, 2 ml, 33 mmol) and triethylamine (2 ml, 14 mmol) and reacting at 60 °C for 72 h. The aminated product was again collected by dialysis and lyophilisation.

Poly[(2-hydroxyethyl methacrylate)-*ran*-(3-amino-2-hydroxypropyl methacrylate)] 3: ^{1}H NMR (CD_3OD, 500 MHz) δ (ppm): 0.94 (2H, br), 1.11 (1H, br), 2.00 (2H, br), 3.78 (br), 4.05 (br).

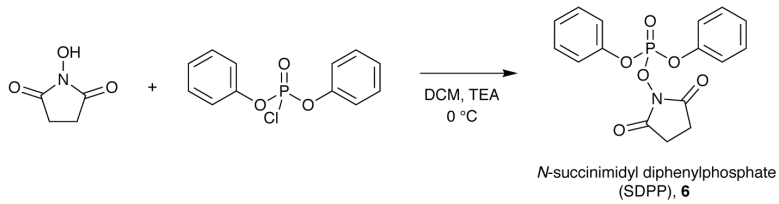
b. Monomethyl polyethylene glycol tosylate



Prior to use, monomethyl polyethylene glycol 2000 ($mPEG^{2000}$) was dried by reflux in toluene using a Dean–Stark apparatus. $mPEG^{2000}$ (10.9 g, 5.45 mmol) and 4-dimethylaminopyridine (DMAP, 34 mg, 0.28 mmol) were dissolved in a 1:1 mixture of dry pyridine/CH_2Cl_2 (32 ml) and cooled to 0 °C. Tosyl chloride (1.90 g, 9.8 mmol) in dry CH_2Cl_2 (10 ml) was added dropwise with stirring. The reaction mixture was washed with dilute HCl and brine, extracted with CH_2Cl_2, dried over magnesium sulfate, and the solvent removed under vacuum to produce a waxy white solid.

Monomethyl polyethylene glycol tosylate 4: ^{1}H NMR (DMSO, 600 MHz) δ (ppm): 2.42 (3H, s, Ar-C*H*$_3$), 3.24 (3H, s, -OC*H*$_3$), 3.51 (172H, br, -C*H*$_2$-), 3.57 (2H, t, -SO_3C*H*$_2$-), 4.11 (2H, t, -SO_3CH_2C*H*$_2$-), 7.48 (2H, d, *ortho*-Ar-SO_3-), 7.78 (2H, d, *meta*-Ar-SO_3-).

c. *N*-Succinimidyl diphenylphosphate

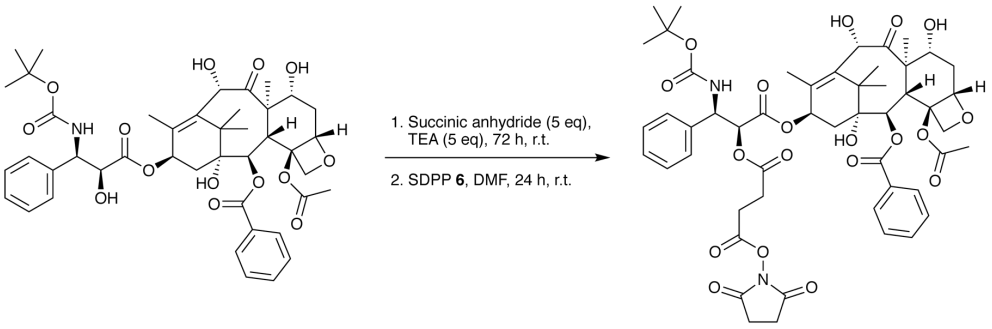


N-Succinimidyl diphenylphosphate (SDPP) was synthesised by the method of Ogura in 68 % yield.[1] Diphenylphosphoric chloride (2.1 ml, 2.7 g, 10 mmol) and *N*-hydroxysuccinimide (1.2 g, 10 mmol) were dissolved in dichloromethane (6 ml, distilled) under Schlenk conditions in an ice bath. Triethylamine (1.4 ml, 10 mmol) was added slowly to the reaction mixture while stirring. After 90 min, the product was dried under vacuum,

then triturated with diethyl ether. The product was dissolved in ethyl acetate, washed with water, dried over magnesium sulfate, and dried under vacuum producing a waxy white solid. The product was confirmed by MS and NMR in accordance with the literature.[1]

***N*-Succinimidyl diphenylphosphate (SDPP):** mp 59-62 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ (ppm): 2.79 (4H, s, NHS), 7.24 (2H, m, aromatic *para*), 7.36 (8H, m, aromatic *ortho* and *meta*); HRMS (ESI, *m/z*): $[SDPP + H]^+$ calcd for $C_{16}H_{15}NO_6P$, 348.06; found, 348.06.

d. Docetaxel-2′-hemisuccinate *N*-hydroxysuccinimide ester



Docetaxel-2′-hemisuccinate NHS ester (DTX-2′-Suc-NHS) **7**

DTX-2′-hemisuccinate (DTX-2′-Suc) was synthesised as an intermediate to DTX-2′-Suc-NHS. DTX (25 mg, 30.9 μmol) and succinic anhydride (1.23 eq, 3.81 mg, 38.1 μmol) were combined in a dry multineck flask with a septum and stirrer, which was evacuated and back filled with argon. DCM (distilled, 1.5 ml) was added, followed by pyridine (dry, 5 μl). The solution was protected from light and stirred for 72 h. The crude product was dried under vacuum and purified on silica gel, washed with hexanes and eluted with ethyl acetate. The ethyl acetate fractions were collected and dried under vacuum. Successful reaction was confirmed by ^{1}H NMR. The product was used to prepare DTX-2′-Suc-NHS immediately after drying.

DTX-2′-Suc-NHS was synthesised from DTX-2′-Suc (20 mg, 22 mmol) using SDPP (1.5 eq, 11 mg, 33 mmol). Acetonitrile (1.5 ml) was added, followed by triethylamine (approximately 5 eq, 15 μl). The flask was protected from light and left stirring for 12 h. The solvent was removed *in vacuo* and the product was dissolved in ethyl acetate and hexanes (2.5:1). The product was precipitated onto a silica gel column with hexanes, then washed twice with hexanes, followed by elution with ethyl acetate/hexanes (2.5:1). Fractions were dried under vacuum, triturated with diethyl ether, and checked by TLC ($CHCl_3$/MeOH 9:1) and HPLC (ACN/water 1:1). HPLC fractions were collected for NMR spectroscopy. The product was characterised by ESI-MS and NMR.

DTX-2′-hemisuccinate: ^{1}H NMR ($CDCl_3$, 500 MHz) δ (ppm): 1.11 (s), 1.22 (m), 1.34 (m), 1.50 (m), 1.61 (m), 1.75 (s), 1.83 (d), 1.85 (m), 1.88 (m), 1.94 (m), 2.071 (s), 2.076 (s), 2.09 (s), 2.18 (m), 2.32 (m), 2.38 (br), 2.42 (br), 2.57 (m), 2.61 (s), 2.65 (br), 2.74 (m), 3.91 (m), 4.16 (m), 4.30 (m), 4.85 (q), 4.95 (m), 5.21 (m), 5.26 (m), 5.41 (m), 5.45 (m), 5.68 (m), 6.22 (br), 7.30 (d), 7.38 (m), 7.50 (t), 7.62 (t), 8.10 (d).

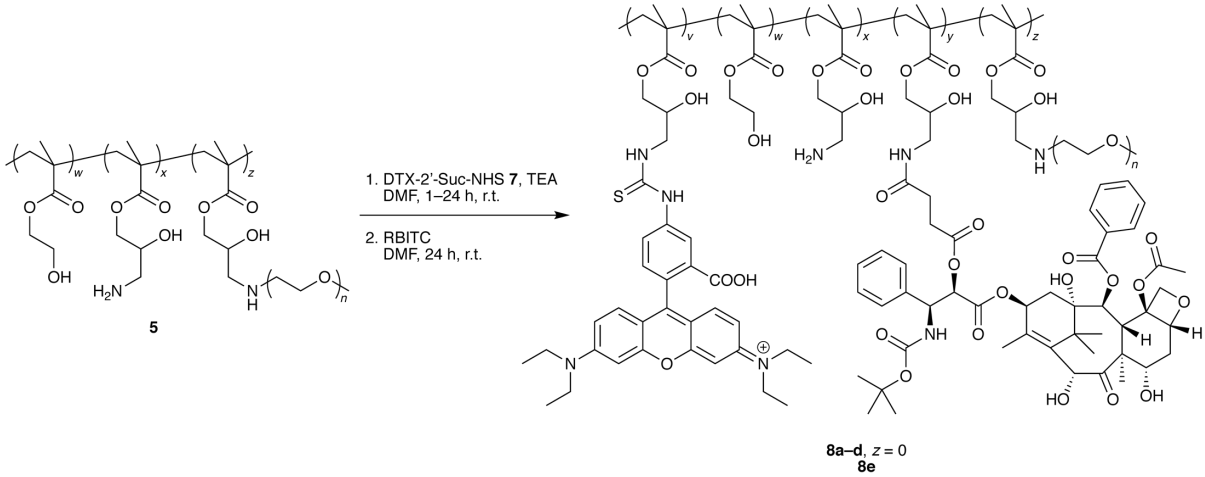
DTX-2′-hemisuccinate NHS ester 7: ^{1}H NMR (DMSO-d_6, 600 MHz) δ (ppm)[2]: 0.97 (3H, s, H16/H17), 0.98 (3H, s, H16/H17), 1.37 (9H, s, Boc), 1.50 (3H, s, H19), 1.60 (1H, m, H14), 1.64 (1H, m, H6), 1.68 (3H, s, H18), 1.85 (1H, m, H14), 2.23 (3H, s, OAc), 2.28 (1H, t, H6), 2.78 (m, H2″), 2.80 (s, NHS), 2.98 (2H, t, H3″), 3.63 (m, 3H), 3.89 (1H, m, H20), 4.00 (m, H20), 4.00 (m, H7), 4.45 (1H, s, OH1), 4.9 (1H, d, H5), 4.94 (1H, s, OH10), 5.04 (1H, br, OH7), 5.08 (1H, s, H10), 5.1 (1H, s, H2′), 5.1 (1H, s, H3′), 5.40 (1H, d, H2), 5.80 (1H, t, H13), 7.18 (1H, t, *para* Ar2), 7.36 (2H, d, *ortho* Ar2), 7.41 (2H, t, *meta* Ar2), 7.64 (2H, t, *meta* Ar1), 7.72 (1H, t, *para* Ar1), 7.87 (1H, br, NH), 7.98 (2H,

[1] Ogura, H.; Nagai, S.; Takeda, K., A Novel Reagent (*N*-Succinimidyl Diphenylphosphate) for Synthesis of Active Ester and Peptide. *Tetrahedron Letters* **1980,** *21* (15), 1467-1468.

[2] Assignments are given according to the numbering scheme shown in Figure 2a.

d, *ortho* Ar1). ^{13}C NMR (150 MHz, DMSO-d_6, δ): 9.9 (C19), 13.7 (C18), 22.56 (CH_3, OAc), 27.5 (CH_3, Boc), 55.1 (C3′), 57.0 (C8), 70.8 (C7), 74.8 (C2), 75.0 (C2′), 78.5 (*spiro*, Boc), 83.8 (C5), 127.4 (*ortho*, Ar2), 128.6 (*meta*, Ar2), 128.7 (*meta*, Ar1), 136.8 (*tert*, Ar2), 165.4 (*tert* C=O, Ar1); HRMS (ESI, *m/z*): [DTX-2′-Suc-NHS + Na]$^+$ calcd for $C_{51}H_{60}N_2O_{19}Na$, 1027.37; found, 1027.37. A physical transition (sample expansion) was observed at 145-147 °C, but melting was not observed below 200 °C.

e. DTX–polymer conjugate



DTX–polymer conjugate **8** was synthesised by combining dry aminated copolymer **5** (29 mg) and DTX-2′-Suc-NHS (30 mg), and adding DMF (dry, 1.5 ml) and triethylamine (~20 µl). The solution was protected from light and left stirring at room temperature for 1 h, then washed twice with CH_2Cl_2 and diethyl ether. The product was dissolved in DMF, precipitated in diethyl ether and collected, or dialysed against MeOH/water.

Conjugates were labelled with fluorescent Rhodamine B isothiocyanate for imaging *in vitro*. DTX–polymer conjugate (*ca.* 5 mg) was dissolved in DMF (2 ml). A solution of rhodamine B isothiocyanate (0.20 mg, 0.37 mmol dissolved in DMF) was added and the mixture left stirring at room temperature for 12 h. The product was dialysed against water (3 × 4 L) to remove unbound RBITC, and recovered by lyophilisation.

3. Additional characterisation of DTX–polymer conjugates

a. DTX release by NMR

DTX release from conjugates by hydrolysis of the ester bond was demonstrated by successive Carr-Purcell-Meiboom-Gill (CPMG) NMR spectroscopy experiments as has been previously described.[3] A sample of conjugate dissolved in CD_3OD was analysed over a 20 day interval. On day 1, significant peak attenuation was evident, and no well resolved peaks characteristic of free DTX were visible, indicating a high degree of covalent conjugation. By day 20, resonances consistent with free DTX were clearly visible. These free DTX resonances were not significantly attenuated during the CPMG NMR experiment, again consistent with their release from the polymer conjugate.

b. DTX release profile by HPLC

Dialysis membrane (Spectra/Por G235029, 3.5–5 kDa cutoff) was equilibrated in a buffered sink solution (40 ml) consisting of PBS pH 7.4 containing 0.5% v/v Tween 80. The sink solution was maintained at 37 °C.

[3] Bain, A. D., *Modern NMR Techniques for Synthetic Chemistry*. 1st ed.; Chapman and Hall: Florida, 2014; Vol. 13, p 349.

Polymer conjugate **8** (5 mg) was dissolved in 100 µl methanol and 900 µl sink buffer was added. This mixture was transferred to the dialysis tube and immersed in the sink solution. At each timepoint, 5 ml aliquots were withdrawn in duplicate and replaced with fresh buffer. Aliquots were carefully lyophilised and redissolved in methanol (200 µl) prior to HPLC analysis.

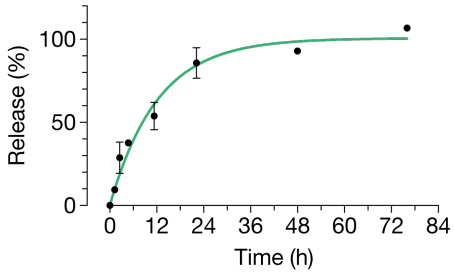


Figure S1. DTX release from polymer over time, as quantified by HPLC. The curve of best fit (R^2 = 0.9327) yields $t_{1/2}$ = 8.4 h and τ = 12.2 h. Data were fitted using GraphPad Prism v8.

c. Diffusion Ordered Spectroscopy (DOSY) NMR

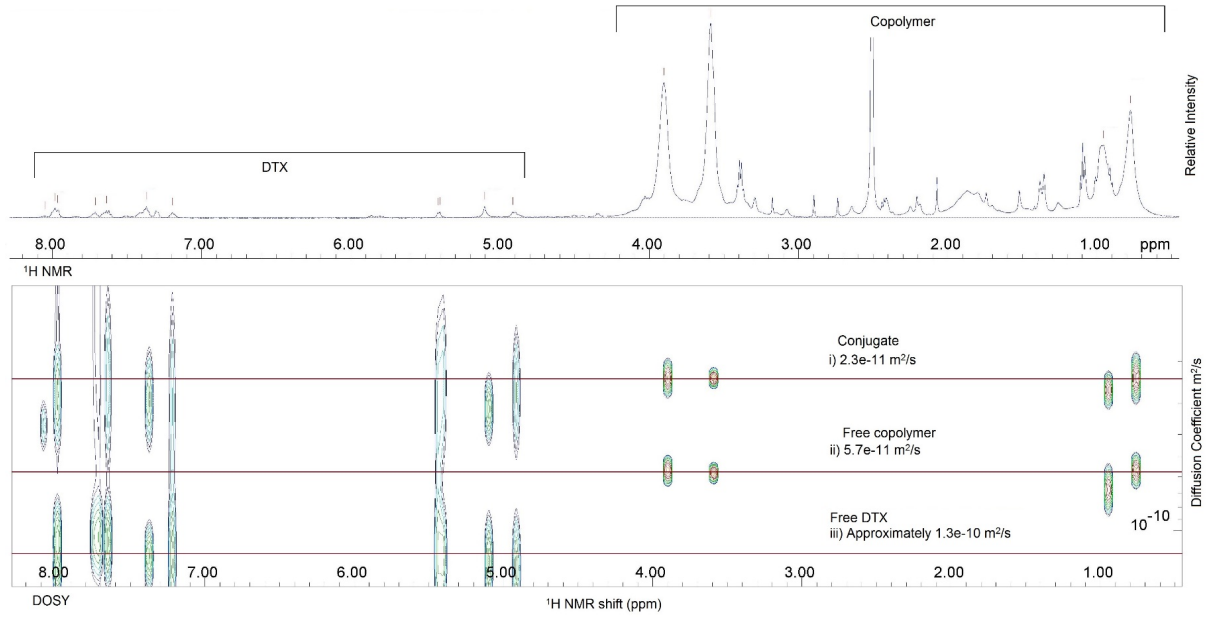


Figure S2. DOSY plot of conjugate **8d**. Free DTX (iii) and free copolymer **5d** (ii) have diffusion coefficients (*D*) of approximately 1.3×10^{-10} and 5.7×10^{-11} m^2 s^{-1} respectively. Conjugation is demonstrated by the slower diffusing set of peaks (i) at approximately 2.3×10^{-11} m^2 s^{-1}. DMSO-d_6, 500 MHz, two-component curve fitting. Diffusion coefficients were assigned by manually picking ^{1}H peaks, then assigning *D* for the resulting DOSY peaks that had good agreement and small errors. Polymer DOSY peaks were generally precise and in good agreement due to high signal strength and small ^{1}H peak overlap. DTX DOSY peaks were less precise and/or distributed over a range of *D* values, owing to overlap and low signal strength. DTX *D* values should therefore be read as approximations only.

4. Cell culture and IC_{50} determinations

a. Tissue culture methods

MCF-7 cells were cultured in Minimum Essential Medium α with 10% v/v fetal bovine serum (FBS), 1 × GlutaMAX and 0.15% sodium bicarbonate in a humidified incubator at 37 °C and 5% CO_2. Cells were cultured

without antibiotics or antimycotics. Cells were washed with PBS, trypsinised, collected by centrifugation (1000 × *g*, 5 min), and subcultured before reaching 100% confluence. Passage numbers were kept low. Cells were plated in 96-well plates at densities of 5000 cells/well in 50 µl of media. Plates were incubated for 24 h and checked visually for consistent cell density before treatments were applied.

The DTX wt % in conjugates was calculated by ^{1}H NMR. The requisite dilutions for use *in vitro* were calculated from these values. Docetaxel and conjugate stock solutions were prepared in a minimum of 7 concentrations between 10 mg ml^{-1} and 0.0001 mg ml^{-1} in DMSO. Copolymer without DTX was prepared at 100, 10, and 1 mg ml^{-1} in DMSO. These solutions were sterilised with ultraviolet light, then diluted 1:250 in media. Treatments in media were applied in triplicate at 50 µl per well then thoroughly agitated. Treatments containing DMSO were diluted 1:1 by addition to media in wells, for a total of 0.2% v/v DMSO in 100 µl of media per well. The outer wells of each plate were filled with PBS to maintain even evaporation in treated cells. Each plate also contained three wells of cells with 100 µl of media, and three with 100 µl of media + 0.2% v/v DMSO as controls. After the designated treatment time had elapsed, media were removed, cells were washed with PBS (100 µl per well) for 1 min, then the PBS was removed and prewarmed media were applied (100 µl per well). Plates were incubated for a total of 72 h from the beginning of treatment. Assays were performed using warmed CellTiter 96® AQueous One Solution Cell Proliferation Assay ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), MTS) solution at 20 µl per well. Plates were read 3 h after the reagent was applied with a PerkinElmer EnSpire™ Multimode plate reader. Absorbance at 490 nm was measured in triplicate and averaged. IC_{50} curves were constructed from triplicate assay data in GraphPad Prism. Datasets were normalised, then analysed by four-parameter nonlinear regression with an inhibition model.

For 72 h treatment, the polymer backbones without DTX (three sets of triplicate data) were compared with controls (no treatment triplicate and 0.2% v/v DMSO triplicate) by one-way ANOVA. For other treatments, IC_{50} curves were compared with an extra sum of squares *F* test. For $p < 0.05$, the null hypothesis ($\log IC_{50}$ is the same) was rejected. 4 h, 24 h, and 72 h data were compared. The DTX/polymer mixture was compared to DTX. Comparisons were made between each tested conjugate and DTX. To investigate the effect of polymer M_w, **8a–c** were compared to each other. To investigate DTX loading, **8b** and **8d** were compared.

b. Cytotoxicity of copolymer alone

Aminated polymer backbone **5** was tested for cytotoxicity prior to drug conjugation at up to 200 µg ml^{-1}, which was higher than any individual conjugate treatment. No measurable inhibition of cellular metabolic activity was detected from the backbones alone ($F(4, 10) = 0.6006$, $p = 0.67$).

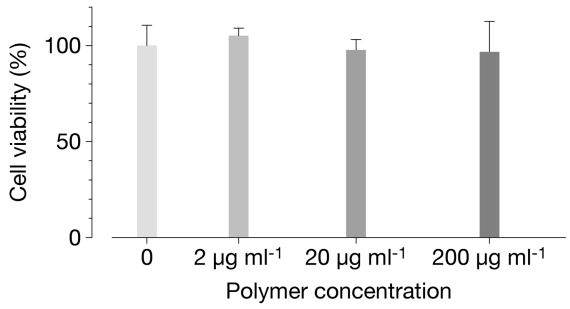


Figure S3. Cell viability as measured by MTS assay for aminated polymer **5**.

c. Statistical comparison of DTX–polymer conjugate efficacy

The following discussion applies to Figure 4 of the article. At 4 h, **8a** and DTX had no significant difference in efficacy ($p = 0.71$), but at 24 h ($p < 0.0001$), and 72 h ($p < 0.0001$), DTX was more effective than **8a**. At 4 h ($p = 0.62$), **8b** and DTX had no significant difference in efficacy, but at 24 h ($p < 0.0001$) and 72 h ($p < 0.0001$), DTX was more effective than **8b**. At 4 h ($p = 0.25$), there was no significant difference in efficacy between **8c**

and DTX, however at 24 h ($p < 0.0001$) and 72 h ($p < 0.0001$), DTX was more effective. At 72 h, the IC_{50} values for **8a**, **8b**, and **8c** were 6.0, 7.4, and 5.3 ng ml^{-1} respectively. These values are similar to IC_{50} of 1.2 ng ml^{-1} for DTX at 72 h. DTX and **8d** had no significant difference at 4 h ($p = 0.52$). However **8d** was relatively less effective at 24 h and 72 h ($p < 0.0001$). Despite statistically lower efficacy at 72 h, **8d** gave IC_{50} of 4.1 ng ml^{-1}, similar to DTX (1.4 ng ml^{-1}).

d. Immunohistochemistry and additional confocal images

MCF-7 human breast adenocarcinoma cells were seeded on glass coverslips and treated for 2 h with RBITC-labelled DTX–polymer conjugates **8b** and **8e** (25 μg ml^{-1}) diluted in OptiMEM media, and fixed with 4% paraformaldehyde. Coverslips were washed thrice with TBST, incubated with CytoPainter Phalloidin-iFluor 488 (ab176753) diluted 1:1000 in TBST + BSA 1% for 45 min, washed thrice with TBST, and counterstained with Hoechst 34580 (Sigma, 1 μg ml^{-1}) in TBST for 5 min. Coverslips were rinsed thrice with TBST, and mounted using Fluoromount G (Southern BioTech) on SuperFrost glass microscope slides. Images were recorded on a Nikon Ti-E inverted confocal microscope with a Nikon A1Si spectral detector system (40×/1.30 Plan Fluor oil immersion objective; 405, 488, and 561 nm excitation).

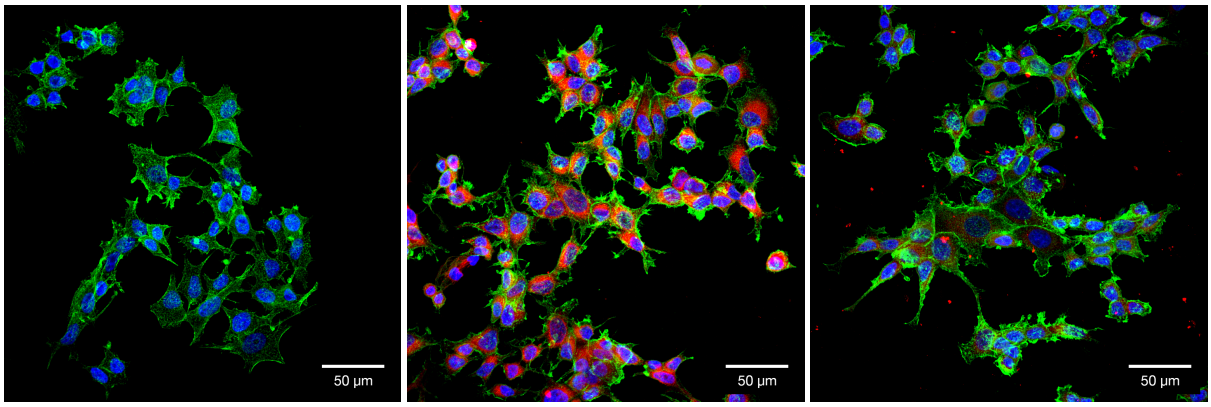


Figure S4. Lower magnification confocal microscopy images. (L-R) control cells, cells incubated with DTX–polymer conjugate **8d**, and cells incubated with PEGylated DTX–polymer conjugate **8e**. Confocal images are maximum intensity projections, 40×/1.30 oil immersion, red = RBITC, DTX–polymer conjugate; green = phalloidin-iFluor 488, cytoskeleton; blue = Hoechst 34580, nuclei.