A Small Molecule Drug Conjugate (SMDC) of DUPA and a Duocarmycin Built on the Solid Phase

Andrew Michael Beekman,*[a] Marco M. D. Cominetti,[a] Oliver Charles Cartwright,[a] Dale L. Boger[b] and Mark Searcey*[ac]

[a]School of Pharmacy & [c]School of Chemistry, University of East Anglia, Norwich Research Park, Norwich, Norfolk, NR47TJ, United Kingdom.

[b]Department of Chemistry, The Scripps Research Institute, La Jolla, California 92037, United States*

E-mail: A.Beekman@uea.ac.uk, M.Searcey@uea.ac.uk.

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General procedures

Reagents and Solvents

All chemicals were reagent grade and were purchased from Sigma Aldrich. Fmoc-amino acids and coupling reagents were purchased from Novabiochem or AGTC Bioproducts. Anhydrous solvents were bought from Sigma Aldrich and assumed to conform to specification.

Physical Characterisation and Spectroscopic Techniques

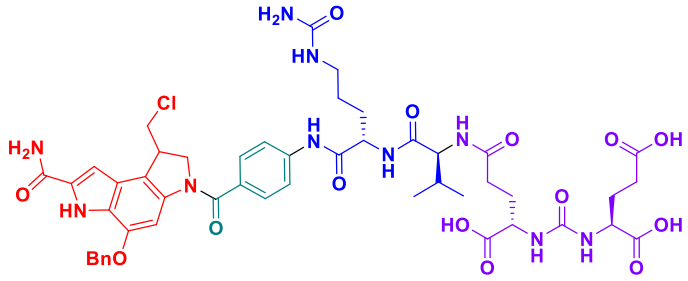
^{1}H-spectra were recorded on a Bruker spectrometer operating at 400 MHz (^{1}H) using the specified deuterated solvent. The chemical shifts for ^{1}H were recorded in ppm and were referenced to the residual solvent peak of DMSO at 2.50 ppm (^{1}H). Multiplicities in the NMR spectra are described as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, and combinations thereof; coupling constants are reported in Hz. MALDI was performed on Kratos Analytical Axima MALDI-TOF using α-cyano-4-hydroxycinnamic acid as a matrix. Low resolution mass spectra were recorded using a Shimadzu LCMS 2010EV operated under electrospray ionisation in positive (ES+) mode. Accurate mass spectra were recorded at the EPSRC National Mass Spectroscopy Service Centre, Swansea or at The John Innes Centre, Norwich Research Park.

Chromatographic Techniques

Analytical RP-HPLC was performed on an Agilent 1200 using an Agilent eclipse XDB-C18 column, 4.6 x 150 mm, 5 µM and a flow rate of 1 mL/min. Solvent A = Water + 0.05% TFA and Solvent B = MeOH + 0.05% TFA. Gradient 5% B up to 95% B over 20 minutes. Detection wavelength 214 nm and 254 nm. Semi-preparative RP-HPLC was performed on an Agilent 1200 using an Agilent eclipse XDB-C18 column, 9.4 x 250 mm, 5 µM and a flow rate of 4 mL/min. Solvent A = water + 0.05% TFA and Solvent B = MeOH + 0.05% TFA. Gradient 5% B up to 95% B over 20 minutes. Detection wavelength 214 nm and 254 nm. Preparative RP-HPLC was performed on an Agilent 1200 using an Agilent eclipse XDB-C18 column, 21.2 x 150 mm, 5 µM and a flow rate of 20 mL/min. Solvent A = 95% H_2O + 5% MeOH + 0.05% TFA and Solvent B = 95% MeOH + 5% H_2O + 0.05% TFA. Gradient 5% B up to 95% B over 20 minutes.

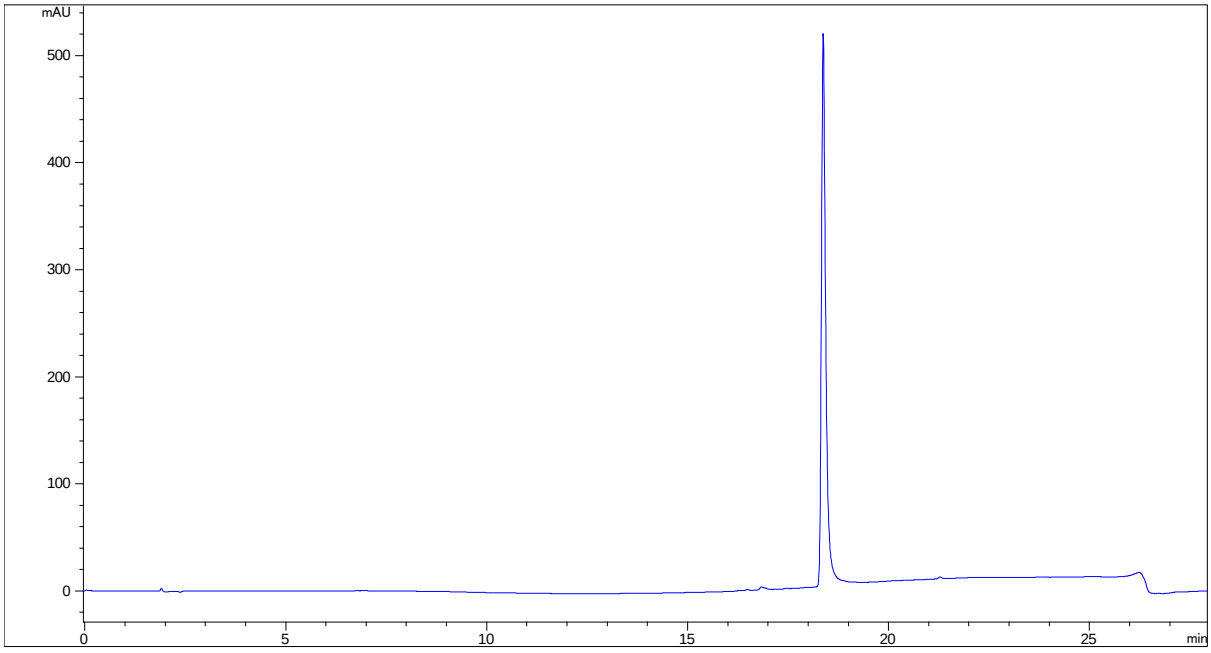
Synthesis

Synthesis of SMDC 3-OBn



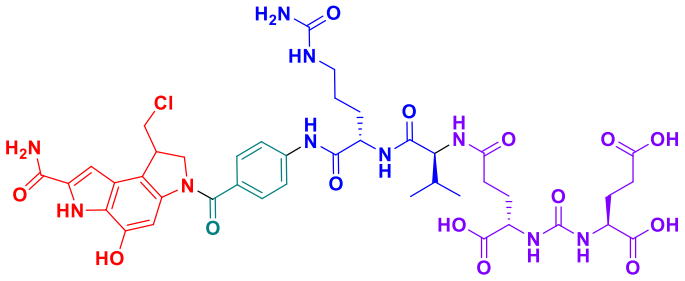
NovaPEG Rink amide resin (100 mg, 0.049 mmol, manufacturer's resin loading 0.49 mmol/g) was prepared for coupling by swelling in dichloromethane (DCM) for 30 mins, followed by N,N-dimethylformamide (DMF) for 30 mins. The swelled resin was treated with Fmoc-DSA(OBn)-OH (1.2 equiv., 0.059 mmol, 34 mg), HATU (1.2 equiv., 0.059 mmol, 23 mg) and DIPEA (3 equiv., 0.147 mmol, 26 µL) and reacted overnight, followed by Fmoc deprotection (2 x 2 mL 20% piperidine in DMF, 10 min) and finally the resin was washed with DMF. The SMDC was synthesised on NovaPEG Rink amide

resin (resin loading 0.49 mmol/g) using an automated peptide synthesiser. Preloaded DSA-NovaPEG Rink Amide resin (0.049 mmol) was suspended in DMF (2 mL) and was allowed to swell for 30 minutes. The DMF was drained from the peptide vessel and the resin was treated with a solution of Fmoc-PABA-OH (4 equiv. compared to resin loading, 0.20 mmol, 70 mg), to which HBTU (3.9 equiv., 0.20 mmol, 72 mg), HOBt (4 equiv., 0.20 mmol, 30 mg) and DIPEA (8 equiv., 0.39 mmol, 69 µL) in DMF were added. The mixture was then vortexed for 30 min. The vessel was drained and the resin washed with DMF (3 x 2 mL). The coupling reaction was then repeated followed by Fmoc deprotection (2 x 2 mL 20% piperidine in DMF, 10 min) and finally the resin was washed with DMF. Subsequent amino acids were coupled in an identical fashion. After the final amino acid coupling reaction (Val) and Fmoc deprotection, the resin was treated with tert-butyl protected DUPA **4** (prepared exactly as described by Cushman and co-workers – *J. Med. Chem.* **2015**, *58*, 3094-3103) (1.5 equiv., 0.074 mmol, 36 mg), HATU (1.5 equiv., 0.074 mmol, 29 mg) and DIPEA (3 equiv., 0.147 mmol, 26 µL) and reacted overnight. The resin was washed with DMF (6 x 10 mL) followed by DCM (6 x 10 mL). The peptide was cleaved from the resin using 95% TFA, 2.5% TIPS, 2.5% H_2O (5 mL) and shaken for 3 h after which the cleavage cocktail was drained into a round bottom flask. The resin was washed with TFA (x 3) and the solutions combined and concentrated under reduced pressure. The peptide was precipitated using cold diethyl ether and filtered. SMDC **3-OBn** was purified using automated reversed phase preparative HPLC and lyophilised from water to yield a brown solid (9 mg, 18 % based on manufacturer's resin loading). This was subsequently analysed using RP-HPLC (RT = 18.374 min) and MALDI mass spectrometry (calcd for **3-OBn** ($C_{48}H_{58}ClN_{10}O_{14}$) (M + H)+, 1033.3817; found, 1033.3829).

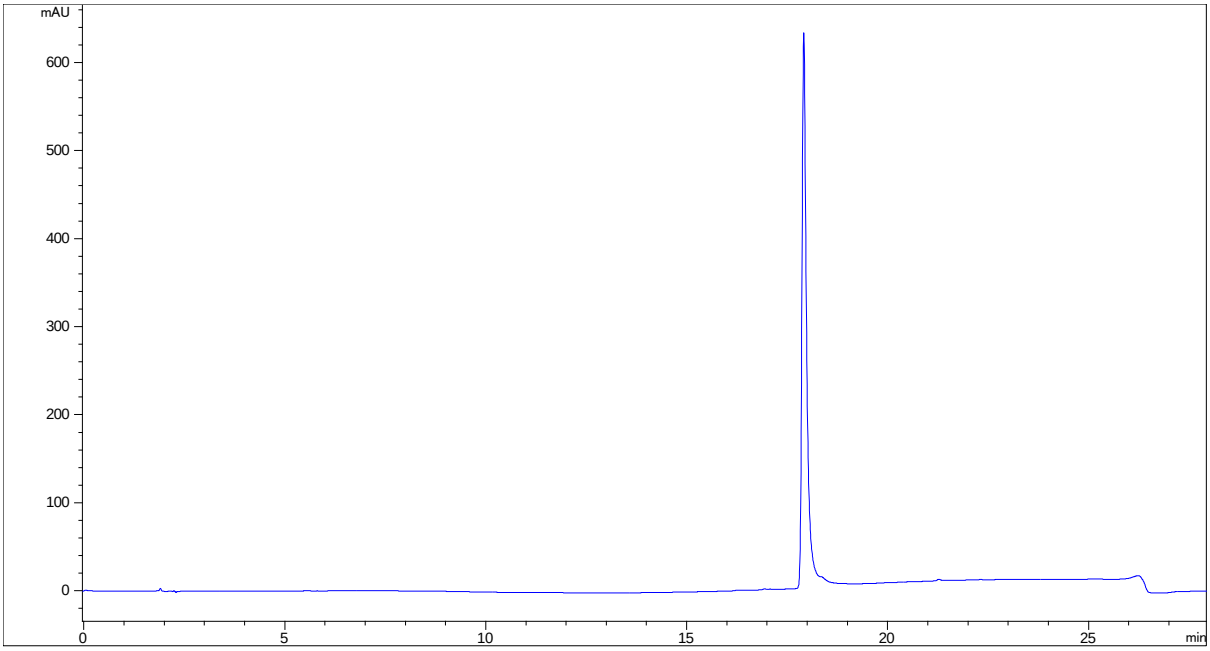


HPLC Trace of 3-OBn at 214 nm

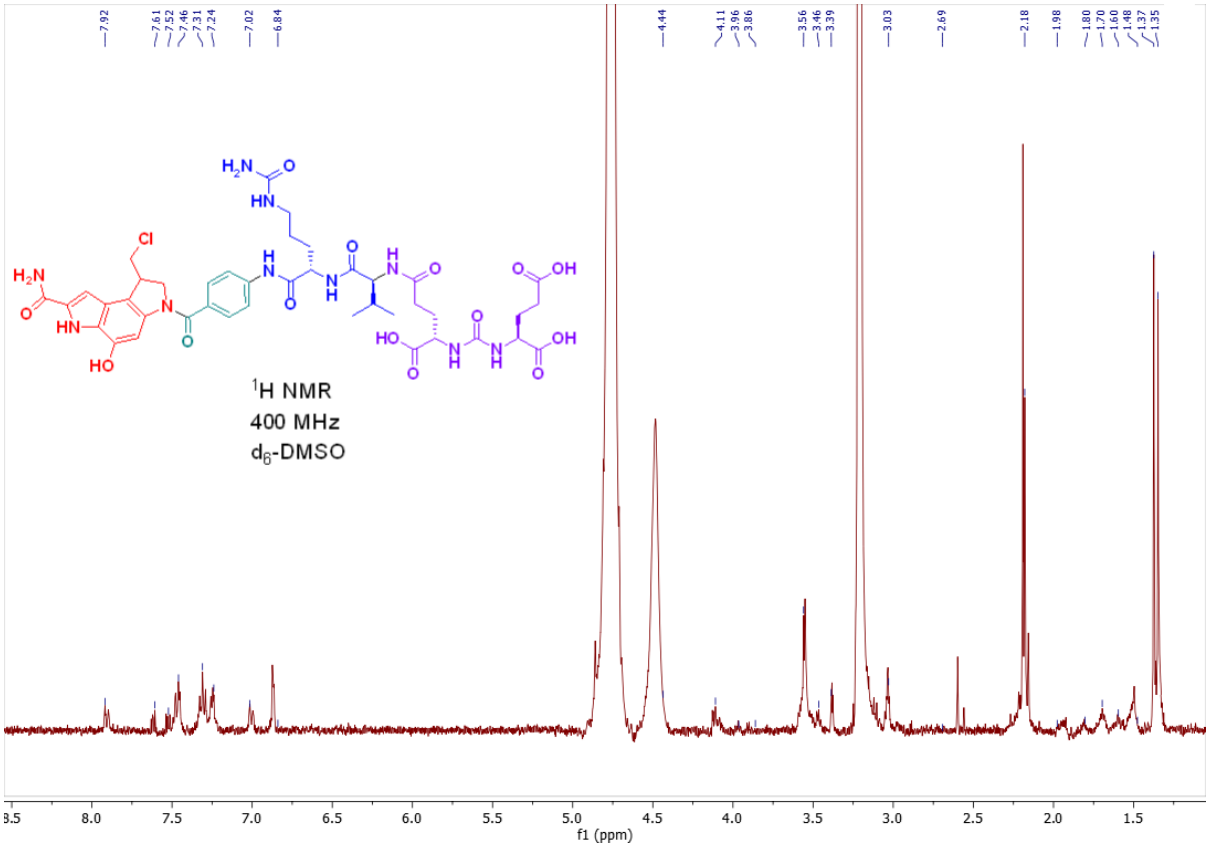
Synthesis of SMDC 3



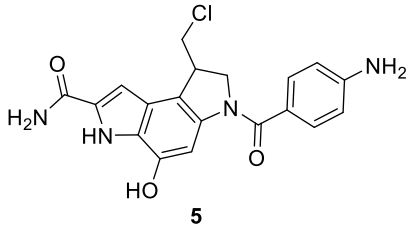
SMDC **3-OBn** (50 mg, 0.048 mmol) was dissolved in MeOH (1 mL) and treated with a slurry of Pd/C (20 mg) in MeOH (5 mL) under H_2. After 1 hour the Pd/C was removed by filtering through a plug of celite. Purification was achieved by reverse-phase preparative HPLC. This yielded SMDC **3**, (38 mg, 0.040 mmol, 84%) as a light brown solid which was analysed using RP-HPLC (RT = 17.914 min) and MALDI mass spectrometry (calcd for **3** ($C_{41}H_{52}ClN_{10}O_{14}$) (M + H)+, 943.3348; found, 943.3336). The obtained proton NMR appears below for reference. The NMR spectra of the SMDC shows high complexity, and in many places frustrating convolution, most likely due to the combination of overlapping diastereomers and atropisomerism. We have observed this in other conjugates of Fmoc-DSA-OH and work continues to fully understand this phenomenon



HPLC Trace of 3 at 214 nm



Synthesis of 8



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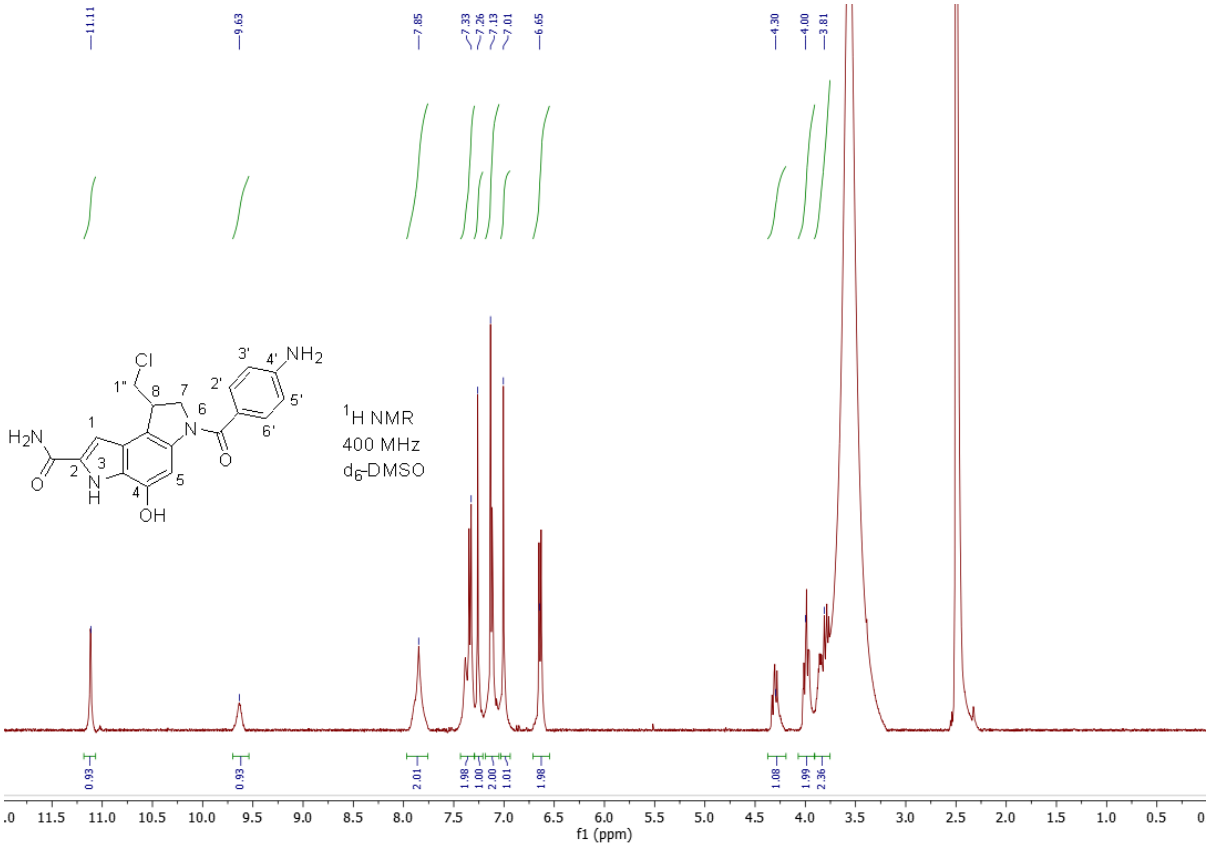
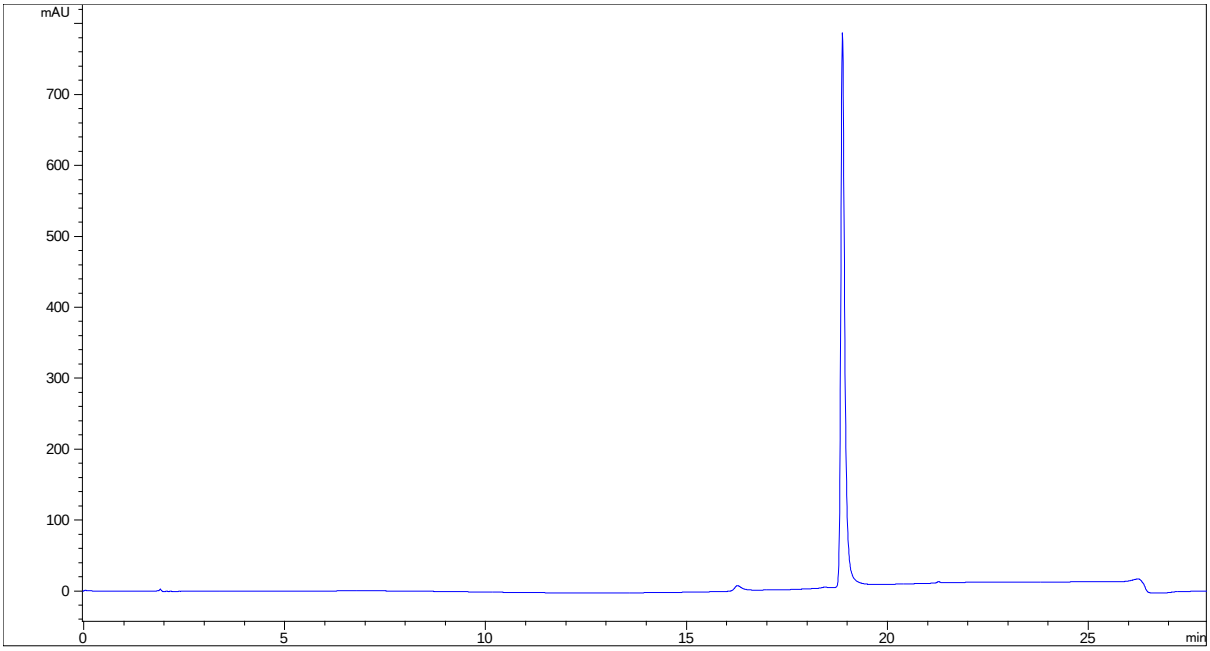
NovaPEG Rink Amide resin (150.0 mg, 0.0735 mmol, manufacturer's resin loading 0.49 mmol/g) was prepared for coupling by swelling in dichloromethane (DCM) for 30 mins followed by N,N-dimethylformamide (DMF) for 30 mins. Fmoc-DSA(OBn)-OH (64 mg, 0.110 mmol) was dissolved in 2 mL of DMF and treated with HATU (42 mg, 0.110 mmol) and DIPEA (39 µL, 0.220 mmol). After 10 secs the resulting solution was added to the resin and the mixture shaken overnight. Subsequently, the resin was washed with DMF (6 x 10 mL) before removal of the indoline Fmoc using piperidine in DMF (6 mL 20 % 10 mins, 6 mL 20 % 5 mins twice). Following Fmoc deprotection the resin was washed with DMF (6 x 10 mL). Next, Fmoc-PABA-OH (132 mg, 0.368 mmol) was dissolved in 2 mL of DMF and treated with HATU (140 mg, 0.368 mmol) and DIPEA (129 µL, 0.735 mmol). After 10 secs the resulting solution was added to the resin and the mixture shaken for 2 h. The resin was subsequently washed with DMF (6 x 10 mL) and the coupling with Fmoc-PABA-OH repeated. Next, the Fmoc group on the PABA was removed using piperidine in DMF (6 mL 40 % 10 mins, 6 mL 20 % 5 mins twice). The resin was then washed again with DMF (6 x 10 mL) and then with DCM (6 x 10 mL) before cleavage from the resin using 95% TFA, 2.5% TIPS, 2.5% H_2O (10 mL) for 3 h. **5-OBn** was

isolated by filtration of the cleavage mixture and subsequent washing of the resin with DCM (3 x 5 mL). The combined filtrates were evaporated to dryness under reduced pressure to give crude **5-OBn** as a dark green solid. Crude **5-OBn** (10 mg, 0.021 mmol) was dissolved in MeOH (1 mL) and treated with a slurry of Pd/C (20 mg) in MeOH (5 mL) under H_2. After 1 hour the Pd/C was removed by filtering through a plug of celite. Purification by preparative HPLC yielded **5** (3 mg, 38%) as a light brown solid. RP-HPLC (RT = 18.882 min). ^{1}H NMR (d_6-DMSO, 400 MHz) δ 11.12 (1H, s, 3-NH), 9.63 (1H, s, 4-OH), 7.85 (2H, s, 2-$CONH_2$), 7.33 (2H, d, *J* = 8.1 Hz, 2',6'-H), 7.26 (1H, s, 1-H), 7.12 (2H, m, 4'-NH_2), 7.01 (1H, s, 5-H), 6.64 (2H, d, *J* = 8.1 Hz, 3',5'-H), 4.33-4.24 (1H, m, 8-H), 4.01-3.93 (2H, m, 1"-CH_2), 3.87-3.81 (2H, m, some obfuscation from water, 7-CH_2). HRMS (ES+) calcd. for $C_{19}H_{17}O_3N_4Cl$ $(M + H)^+$, 385.1067; found, 385.1058.





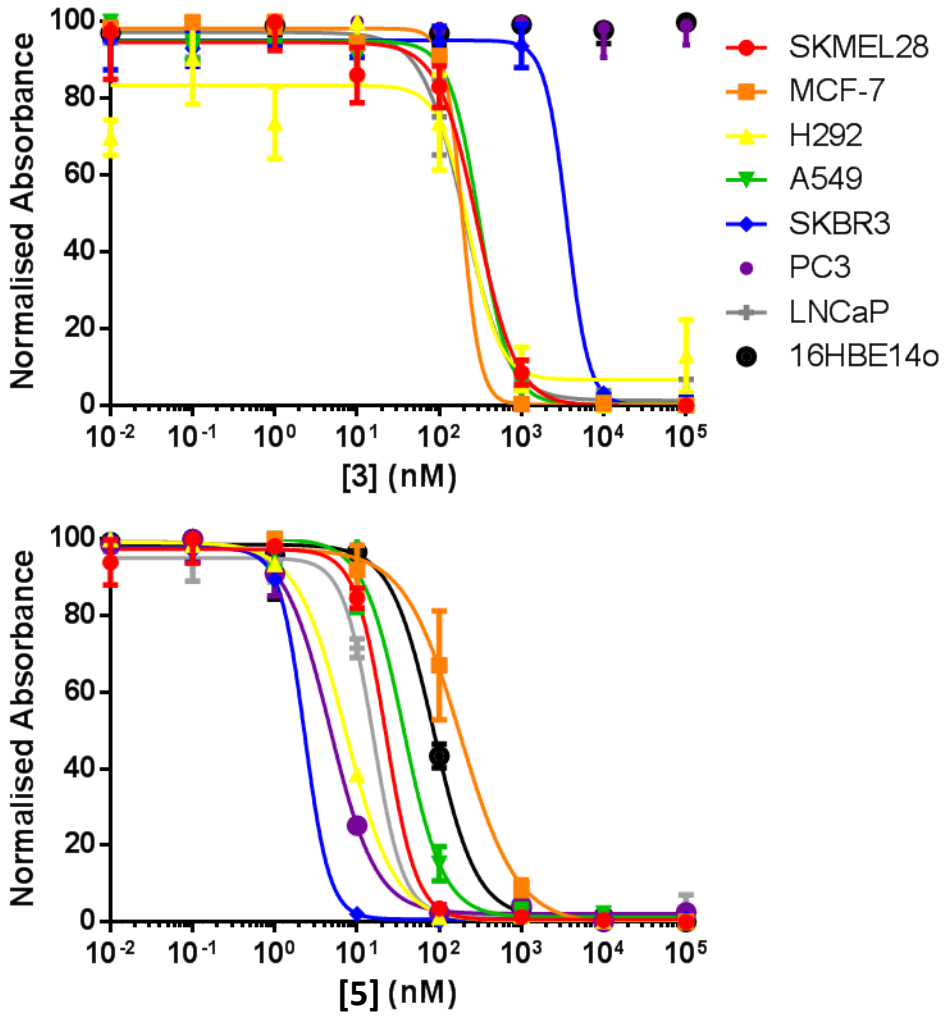
HPLC Trace of **5** at 214 nm

Biological Evaluation

Antiproliferative assays

The LNCaP, PC3, H292, A549, MCF-7, SKBR3, SKMEL28 and 16HBE14o cell lines was purchased from ECACC (Porton Down, UK). Cells were cultured in the media suggested by the supplier supplemented with 10% foetal calf serum and 2 mM L-glutamine. Cells were passaged twice weekly and maintained between 1-9 x 10^5 cells/mL at 37 $^{\circ}$C and 5 % CO_2.

Antiproliferative activity was determined by MTS assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) and following the manufacturer's instructions. Stock solutions were prepared in DMSO the same day as experiments were performed. Briefly, cells (3 x 10^4 /100 µL) were seeded in 96-well plates and left untreated or treated with DMSO (vehicle control), compounds (100 µM, 10 µM, 1 µM, 0.1 µM, 0.01 µM, 0.001 µM, 0.0001 µM, 0.00001 µM), or doxorubicin hydrochloride (10 µM) as a positive control, in triplicate, and allowed to incubate for 72 hr at 37 °C with 5 % CO_2. The final DMSO concentration in wells was 1%. Following this, MTS assay reagent was added for 3 hrs and the absorbance measured at 490 nm using the Polarstar Optima microplate reader (BMG Labtech). Doxorubicin demonstrated toxicity to all cell lines and this was used as the 100% inhibition of cell proliferation value. Experiments were performed in triplicate, with different cell passages and freshly prepared compound dilutions. IC_{50} values were calculated using GraphPad Prism Version 5.0 software.



Normalised absorbance at 490 nm compared to concentration of SMDC **3** or payload **5**. Representative example of three different experiments.

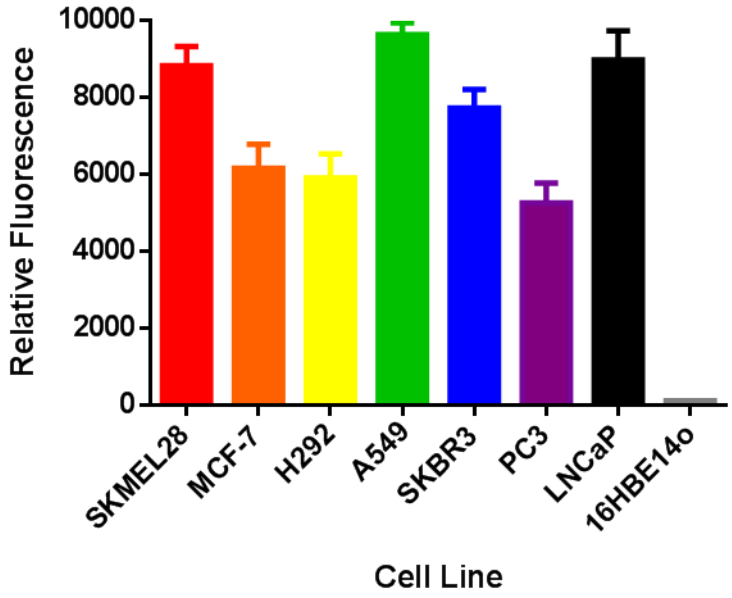
Competition Assay

Briefly, cells (3 x 10^4 /100 µL) were seeded in 96-well plates and were treated with 10 µM of 2-PMPA (2-(Phosphonomethyl)-pentandioic acid, Sigma-Aldrich) and allowed to incubate for 1 hour. Subsequently DMSO (vehicle control), compounds (dissolved in DMSO, 100 µM, 10 µM, 1 µM, 0.1 µM, 0.01 µM, 0.001 µM, 0.0001 µM, 0.00001 µM), or doxorubicin hydrochloride (positive control) were added, in triplicate, and incubated for 48 hr at 37 °C with 5 % CO_2. The final DMSO concentration in wells was 1%. Following this, MTS assay reagent (Promega) was added for 3 hrs and the absorbance measured at 490 nm using the Polarstar Optima microplate reader (BMG Labtech). Doxorubicin demonstrated toxicity to all cell lines and this was used as the 100% inhibition of cell proliferation value. Experiments were performed in triplicate, with different cell passages and freshly prepared compound dilutions.

Quantification of Cathepsin Activities

1 x 10^6 cells were washed in PBS and lysed for 30 minutes at 4°C in 100 µL of 50 mM sodium acetate (pH 5.5), 0.1 M NaCl, 1 mM EDTA, and 0.2% Triton X-100. Lysates were clarified by centrifugation

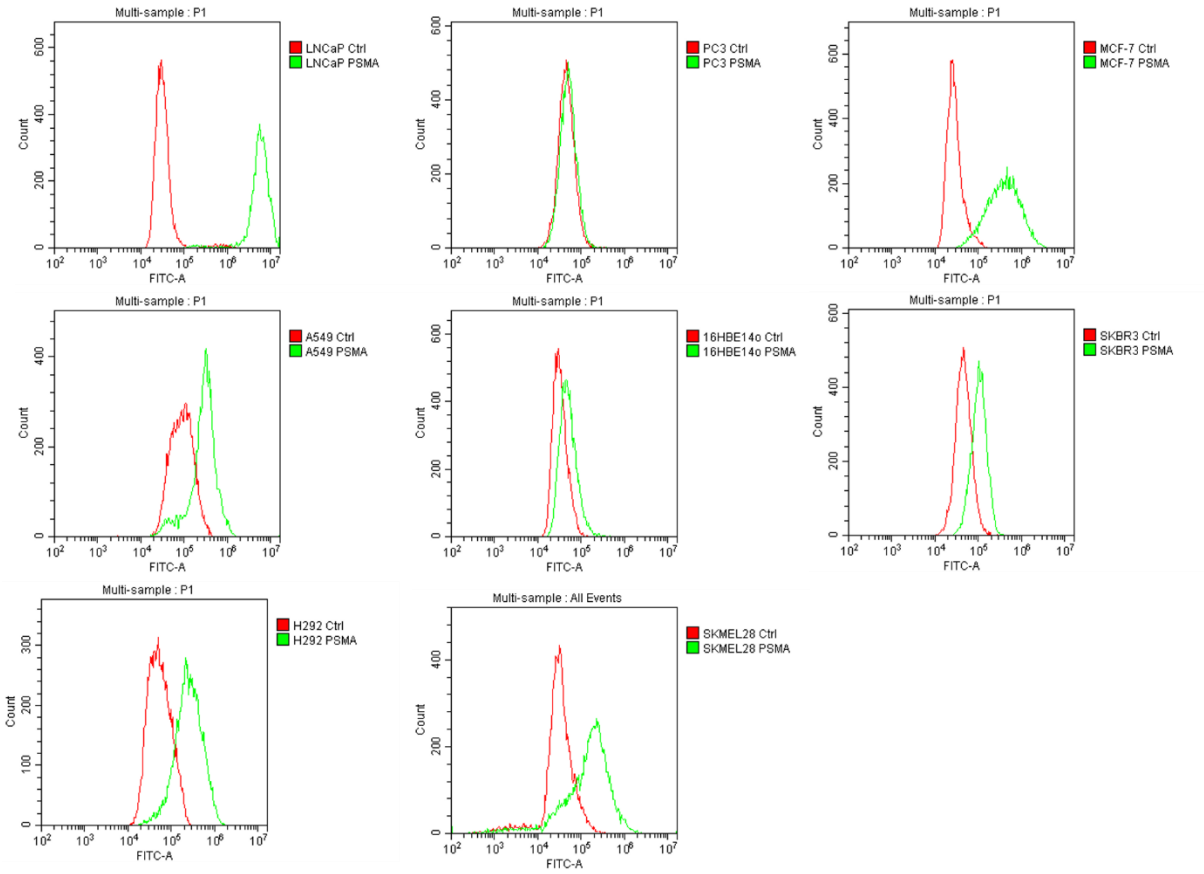
and immediately used for determination of proteolytic activity. For this, 1 µL of cell lysates was incubated at 37°C for 30 minutes in lysis buffer (100 µL) in the presence of z-RR-AMC (Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride, 20 µM). The AMC (amino-4-methylcoumarin) released as a result of proteolytic activity was quantified with a Clariostar plate reader (excitaion: 380 nm; emission: 440 nm), and normalized by total protein content, determined by absorbance at 280 nm with a NanoDrop microvolume spectrophotometer. Relative fluorescence was compared to the non-cancerous 16HBE14o cell line (normalised to 100), which showed no difference in fluorescence compared to the buffer in all experiments. Experiments were performed in triplicate, with different cell passages.



Relative fluorescence intensity of cell lines compared to non-cancerous cell line 16HBE14o normalised by protein content.

Determination of GCPII (PSMA) Expression

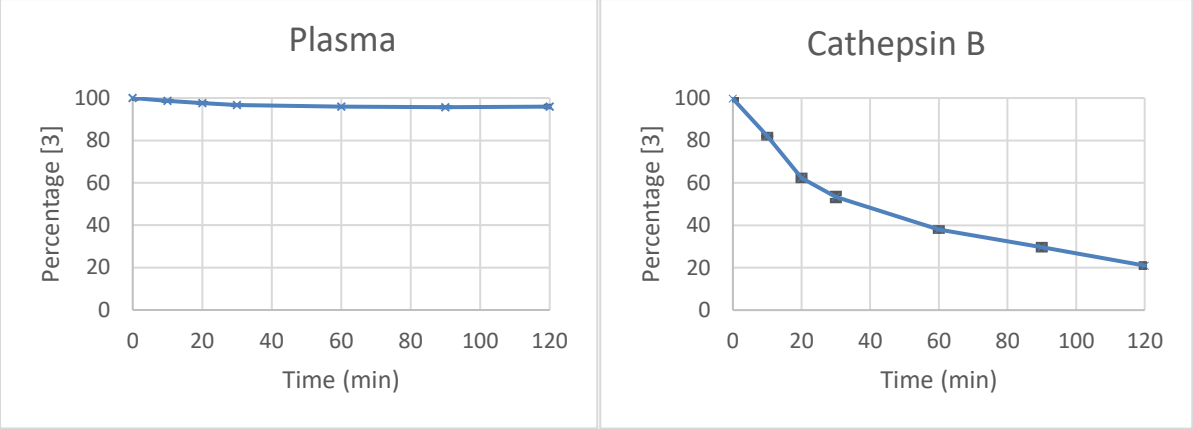
1 x 10^6 cells were washed in PBS and fixed in 100 µL of 4% paraformaldehyde in PBS for 15 mins at room temperature. After washing with 0.05% BSA in PBS cells were incubated in 100 µL of 0.05% BSA in PBS with (PSMA) or without (Ctrl) 1 µL of primary antibody (YPSMA-1, Abcam, 1 µg/mL) for 1 hour. After washing with 0.05% BSA in PBS (1 mL) cells were incubated with 1 µL secondary antibody (Goat pAb to Mouse IgG (FITC), ab6785) in 100 µL of 0.05% BSA in PBS. After washing with 0.05% BSA in PBS cells were suspended in 300 µL of 0.05% BSA in PBS and analysed using flow cytometry on a Beckman Coulter Cytoflex. Experiments were performed in triplicate, with different cell passages.



Comparative histograms of cell lines comparing cell count to fluorescence intensity of the FITC-A channel. Ctrl was treated with only secondary antibody, PSMA was treated with YPSMA antibody. Representative examples of three different experiments.

Stability to human plasma and Cathepsin B.

Method taken from a published procedure with minor modifications. (Bioconjugate Chem. 2016, 27, 1040−1049). Human plasma was purchased from VWR (Biowest Human plasma). Cathepsin B extracted from human liver was obtained frozen at 27.7 µM in 20 mM sodium acetate and 1 mM EDTA at pH 5.0. The enzyme was activated by incubation at ambient temperature for 15 min with 30 mM dithiothreitol and 15 mM ethylenediaminetetraacetic acid (EDTA) at pH 5.5. In the free drug release assay, the activated cathepsin B at a final concentration of 125 nM (enzyme to substrate ratio 1:1000) in the reaction buffer (25 mM sodium acetate and 1 mM EDTA at pH 5.5), or human plasma, was mixed with SMDC **3** at a final concentration of 10 µM. The reaction mixtures were incubated at 37 °C for 120 min, with sample aliquots (10 µL) taken at multiple time points. Each aliquot of sample was immediately quenched by adding methanol (1 mL), and the sample centrifuged to separate precipitated protein. A reaction without enzyme was also included as buffer only blank. The samples from this study were analysed by RP-HPLC reading at 254 nm to determine the amount of SMDC **3**. 7-amino-4-methylcoumarin was used as an internal standard.



The stability of SMDC **3** in plasma or in the presence of cathepsin B. The average of triplicate data is shown ± standard deviation.