Supporting Information

A dendrimer conjugate of 4-(tetradecanoylamino)benzyl] phosphonic acid (S32826) as an autotaxin inhibitor.

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Experimental procedures

General Chemistry

Reagents and solvents were obtained from commercial suppliers and were not further purified before use. G3 PAMAM dendrimer refers to a 3rd generation polyamidoamine (PAMAM) dendrimer (20 wt. % solution methanol) containing 32 surface groups, (Sigma Aldrich, cat no.412422). ^{1}H NMR was measured on a Bruker DPX300 Fourier transform spectrometer at 300 MHz, using *d*-DMSO solvent as the reference. Chemical shifts are quoted in ppm downfield from TMS; coupling constants (*J*) are quoted in Hertz (Hz). The following abbreviations refer to the observed signals; singlet (s), doublet (d), triplet (t), quartet (q) and multiplet (m). Infra-red (IR) spectra were recorded neat on a Thermo Scientific GladiATR spectrometer and the vibrational frequencies were recorded in cm^{-1}. Mass Spectra were obtained from the EPSRC National Mass Spectrometry Centre at the University of Wales, Swansea, using electron spray ionisation (ESI). Elemental analysis was performed by MEDAC Ltd, Chobam.

Synthesis of [4-(11-Aectylamino-dodecananoylamino)-benzyl]-phosphonic acid diethyl ester.

To a solution of [4-(11-Amino-undecanoylamino)-benzyl]-phosphonic acid diethyl ester (2.00g, 4.69 mmol) in pyridine (10 mL) acetic anhydride (0.5 mL, 5.8 mmol) was added and the reaction mixture was stirred at rt for 16 h. The reaction mixture was added to an ice/water mixture and the resultant precipitate was collected via filtration to give **[**4-(11-Aectylamino-dodecananoylamino)-benzyl]-phosphonic acid diethyl ester (1.89g, 4.03 mmol, 85%) as a white crystalline powder; δ_H (300 MHz *d*-DMSO); 7.99 (s, 1H, NH), 7.49 (d, J = 6, 2H), 7.21 (d, J = 6, 2H), 5.70 (s, 1H, NH), 5.31 (dd, J = 9, 3, 2H), 4.04-3.99 (m, 4H), 3.22 (q, J = 18, 6, 2H), 3.14 (s, 1H), 3.08 (s, 1H), 2.36 (t, J = 6, 3H), 2.00 (s, 3H), 1.72 (t, J = 9, 2H) 1.48-1.46 (m, 2H) and 1.28-1.24 (m, 15H); ν_{max}/cm $^{-1}$ (solid); 3451, 3317, 2982, 2917, 2849, 1671, 1652, 1609, 1542, 1514, 1478, 1411, 1369, 1219; *m/z* (ES) (Found: MH^+, 483.2973, $C_{25}H_{43}O_5N_2P_1$ required *MH*, 483.2910).

Synthesis of [4-(11-Aectylamino-dodecananoylamino)-benzyl]-phosphonic acid

To a solution of [4-(11-Aectylamino-dodecananoylamino)-benzyl]-phosphonic acid diethyl ester (700mg, 1.4 mmol) in anhydrous dichloromethane (10 mL), under $N_{2,}$ trimethylsilyl iodide (400 µL, 2.8 mmol) was added via syringe. The resulting solution was stirred at rt for 8 h, evaporated to dryness and recrystallized in methanol to give [4-(11-Aectylamino-dodecananoylamino)-benzyl]-phosphonic acid (581 mg, 97%, 1.36 mmol) as a white crystalline powder; δ_H (300 MHz *d*-DMSO); 9.79 (s, 1H, NH), 7.49 (d, J = 6, 2H), 7.14 (d, J = 6, 2H), 4.14 (s, 1H, NH), 3.56 (s) 3.15 3.00 (s, 1H), 2.85 2.27 (t, J = 6, 3H), 1.76 (t, J = 9, 2H) and 1.22-1.16 (m, 15H); ν_{max}/cm $^{-1}$ (solid); 3451, 3302, 2917, 2849, 2160, 1652, 1609, 1541, 1514, 1471, 1412, 1369, 1219; *m/z* (ES) (Found: MH^+, 427.2357, $C_{21}H_{35}O_5N_2P_1$ required *MH*, 427.2356).

Synthesis of [4-(12-Acryloylamino-dodecananoylamino)-benzyl]-phosphonic acid diethyl ester.

To a solution of [4-(11-Amino-undecanoylamino)-benzyl]-phosphonic acid diethyl ester (1.50 g, 3.40 mmol) and triethylamine (0.9 mL, 6.80 mmol) in anhydrous dichloromethane (25 mL) cooled to −20 °C was added dropwise a solution of acryloyl chloride (340 mg, 3.75 mmol) in anhydrous dichloromethane (15 mL). The reaction mixture was warmed to 0 °C and stirred for a further 4 h under N_2. The reaction mixture was then washed with saturated aqueous $NaHCO_3$ (30 mL), water (50 mL) and saturated brine (30 mL), dried ($MgSO_4$) and evaporated. The resultant product was stirred in ethyl acetate: petrol (3:7) for 16 h and collected via filtration to give [4-(12-Acryloylamino-dodecananoylamino)-benzyl]-phosphonic acid diethyl ester **2** (715 mg, 1.44 mmol, 43%) as a white crystalline powder; δ_H (300 MHz *d*-DMSO); 8.31 (s, 1H, NH), 7.42 (d, J = 6, 2H), 7.12 (d, J = 6, 2H), 6.23 (dd, J = 18, 3, 1H), 6.08 (d, J = 18, 1H), 6.02 (d, J = 18, 1H), 5.98 (s, 1H, NH), 5.53 (dd, J = 9, 3, 2H), 3.99-3.88 (m, 4H), 3.23 (q, J = 18, 6, 2H), 3.0.7 (s, 1H), 3.00 (s, 1H), 2.29 (t, J = 6, 3H), 1.63 (t, J = 9, 2H) 1.46-1.42 (m, 2H) and 1.20-1.16 (m, 15H); ν_{max}/cm^{-1} (solid); 3293, 2985, 2918, 2850, 1674, 1656, 1627, 1601, 1538, 1470, 1456, 1330, 1305, 1239; *m/z* (ES) (Found: *MH^+*, 495.2978, $C_{26}H_{44}O_5N_2P_1$ required *MH*, 495.2982).

Synthesis of compound 3.

A solution of G3 PAMAM dendrimer (250 mg, 7.24×10^{-3} mmol) and [4-(12-Acryloylamino-dodecananoylamino)-benzyl] phosphonic acid diethyl ester (458 mg, 0.92 mmol 128 eq.) in ethanol (10 mL) was heated with stirring at 100 °C for 48 h in an ACE™ pressure tube. The solution was then transferred to dialysis tubing (MWCO 8 KDa (Biodesign)) and dialyzed in methanol (500 mL), being changed 3 times per day for 3 days. The methanol was then evaporated to give compound dendrimer **3** (200 mg, 0.009 mmol, 30%) as a yellow solid; ν_{max}/cm^{-1} (solid): 3385, 2970, 1621, 1541, 1448, 1361, 1303, 1239.

Synthesis of compound 4.

To a solution of Compound **3** (200 mg, approx. 0.009 mmol) in anhydrous dichloromethane (5 mL), under N_2, trimethylsilyl iodide (0.28 mL, 2 mmol) was added via syringe. The resulting solution was stirred at rt for 8 h. The reaction mixture was then directly transferred into dialysis tubing (MWCO 8 KDa (biodesign)) and dialyzed in methanol (500 mL), being changed 3 times per day for 3 days. The methanol was then evaporated to give compound **4** (16.2 mg, 7.73×10^{-4}, 9%) as an orange oil; ν_{max}/cm^{-1} (solid): 3242, 2952, 2927, 2860, 1709, 1671, 1585, 1508, 1438, 1414, 1388, 1341, 1276. Elemental analysis: $C_{718}H_{1310}N_{154}O_{148}P_{16}$: Theory; C 58.13%, H 8.90%, N 14.54%, P 3.34%; Found; C 45.65%, H 6.14%, N 8.45%, P 3.38%.

Biology

Cell culture.

Human ovarian cancer cells were maintained in RPMI supplemented with 10% FCS, Penicillin/Streptomycin (50 U/mL) and glutamine (2 mM), at 37 °C and 5% CO_2. 3E3 ovarian cancer cells have previously been engineered to express autotaxin[1] whereas 3V3 cells were previously transfected with the empty vector.

Purification of autotaxin.

3E3 cells were seeded in a T75 flask and when 50% confluent the medium was replaced with serum free medium (10 mL) and incubated overnight. The next day the supernatant was collected and centrifuged (3000 rpm, 4 °C, 15 min). The supernatant was loaded onto the HiTrap Con A sepharose column (GE healthcare) and recirculated for 1 h. 20 mL tris buffered saline (TBS; 20mM tris, 0.5 M NaCl, pH 7.4) was passed through the column and then autotaxin eluted with 2 mL α-methylmannoside (0.5M) in TBS overnight. The next day, a 2ml fraction was collected, and dialyzed against TBS. The retentate was then used directly as the source of autotaxin for the bis *p*NPP assay.

FS-3 assay.

Inhibition of autotaxin activity was evaluated using a commercially available assay to measure they hydrolysis of FS-3, a fluorescence-quenched analogue of lysophosphatidylcholine (Echelon Biosciences, Inc. Salt Lake City, UT). Compounds to be tested were prepared as stock solutions in DMSO, compound 4 (10 μM) and G3 PAMAM dendrimer (1 mM) in Tris buffer (1 M, pH 8.8). 80 μL reaction buffer containing autotaxin and 10 μL drug were pre-incubated for 10 min at 37 °C Subsequently, 10 μL FS-3 substrate was added and the rate of fluorescence was measured at 37 °C every minute for 30 min using a Synergy2 multi-mode microplate reader (BioTek instruments, Inc.) (λ=528 nm). Data was analyzed using GraphPad Prism software.

Bis-pNPP Assay.

Inhibition of autotaxin activity was measured using the lysophosphatidylcholine analogue bis-*para*-nitrophenylphosphate (bis-*p*NPP). In an opaque 96-well plate, 20 μL 5X reaction buffer (250 mM Tris HCl, 25 mM KCl, 5 mM $CaCl_2$, 5 mM $MgCl_2.6H_2O$, NaCl 700 mM, pH 7.8), 10 μL bis-*p*NPP (1 mM), 20 μL inhibitor was added to each well, followed by the addition of 50 μL autotaxin purified from 3E3 cells as described above. After incubation (37°C for 4 h) absorbance was measured using a plate reader (λ=405 nm) A_{405} was determined and data was analyzed using graph pad prism software to fit a 4 parameter Hill equation.

Wound healing assay.

3V5 and 3E3 cells were plated at a of density 100,000 cells/ml in 6 well plates using RPMI supplemented with FCS (10%). When the cells had reached confluence the medium was removed and a plastic pipette tip was drawn across the centre of the plate to produce a clean wound area. The wells were washed with PBS twice to remove any debris and remaining FCS. The medium was then replaced with serum free medium containing lysophosphatidyl choline (0.5 μM), and either no inhibitor, S32826 (30 nM) or the S32826 dendrimer

conjugate (1.3 μM or 2.6 μM). DMSO was added to each well to normalize the amount of DMSO present to 0.5%. The cells were viewed by phase contrast microscopy immediately after wounding. After 16 h incubation at 37°C the cells were stained and fixed with methylene blue (200 μL, Sigma M9140; 0.5% w/v in 50% H_2O, 50% EtOH) and viewed using light microscopy. The wound closure was measured using a calibration image to determine distance (μm) using imageJ software. The area of a section of the wound pre- and post-migration was then measured and the percentage wound closure was calculated as

$$Percentage\ wound\ closure = \left(\frac{(a_b - a_a)}{a_b}\right) \times 100$$

Where a_b represents the area of the wound immediately after wounding and a_a represents the area of the wound after migration.

Caspase 3/7 activity assay.

3E3 and 3V5 cells were plated at a density of 5000 cells per well in a 96-well plate. The plates were incubated with 80 μL serum free medium supplemented with lysophosphatidic choline (0.5 μM). The cells were incubated in the presence of the indicated combinations of carboplatin (300 μM), S32826 (300 nM) or compound 4 (10 μM) for 18 h. The cells were centrifuged (1000 rpm, 2 min), the supernatant was removed and 30 μL PBS and 30 μL of Caspase-Glo 3/7 reagent (Promega) were added to the cells before measuring luminescence. To normalize for cell number, identical samples were prepared at the same time and stained with SRB as described.[2]

References

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2. Witham, J.; Vidot, S.; Agarwal, R.; Kaye, S. B.; Richardson, A., Transient ectopic expression as a method to detect genes conferring drug resistance. *Int. J. Cancer* 2008, *122* (11), 2641-2645.