Supplementary information for

Small molecule neuropilin-1 antagonists combine anti-angiogenic and anti-tumour activity with immune modulation through reduction of TGFβ production in regulatory T-cells

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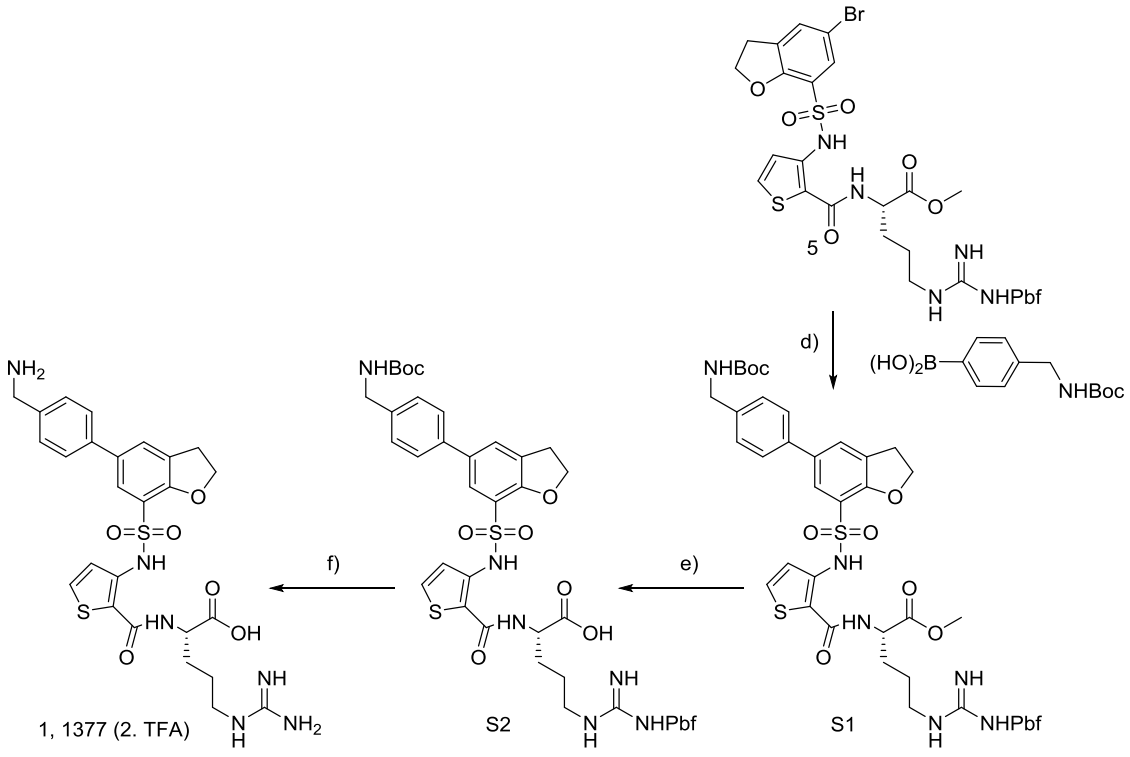
Contents

Large scale synthesis of 1 (1377.2TFA).



Scheme S1. Reagents: (a) (4-(((tert-butoxycarbonyl)amino)methyl)phenyl)boronic acid, $Pd(dppf)Cl_2$.DCM, KOAc, 1,4-dioxane, 90 °C, 18 h, 69% (b) LiOH.H_2O, THF/ /H_2O mixture (2:2), 20˚C, 18h, 82% ; (c) TFA, DCM, 20˚C, 18h, 82%.

Methyl-N^2-(3-((5-(4-(((tert-butoxycarbonyl)amino)methyl)phenyl)-2,3-dihydrobenzofuran)-7-sulfonamido)thiophene-2-carbonyl)-N^w-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)-L-argininate (S1).

To a dry 3 necked 1000ml round bottomed flask, fitted with a stirrer bar, condenser, septum and nitrogen bubbler were added the bromo compound **5** (1 equiv, 21 g, 25.5 mmol), (4-(((tert-butoxycarbonyl)amino)methyl)phenyl)boronic acid (1.5 equiv, 9.6 g, 38.2 mmol), KOAc (3.1 equiv, 7.7 g, 79 mmol) followed by 350 ml of 1,4-dioxane. The contents of the flask were then thoroughly degassed by putting through a vacuum and nitrogen cycle three times. Then Pd(dppf)Cl_2.DCM (0.1 equiv, 21.2 g, 2.5 mmol) was added and the contents degassed again, then the reaction mixture was stirred at 90 ˚C for 18 h. After cooling, the resulting reaction mixture was filtered through Celite, washing through with of methanol (3 x 250ml). The solvents were removed in vacuo and the resulting residue dissolved in EtOAc (200 mL) washed with 1 M hydrochloric acid (3 x 10 mL). The organic layer was washed with NaCl (aqueous sat. solution, 80 mL), dried (Mg_2SO_4) and concentrated in vacuo. The residue was purified by flash column chromatography (silica; EtOAc in DCM 0/100 to 50/50). The desired fractions were collected and concentrated in vacuo to give **6.** Yield: 16.7 g, 17.6 mmol, 69%. Beige foamy solid. ^{1}H NMR (250 MHz, $CDCl_3$) δ 10.61 (s, 1H), 7.87 (m, 2H), 7.52 (m, 4H), 7.34 (m,4H), 5.01 (m,2H), 4.73 (m,4H), 4.32 (m, 4H), 3.71 (s,3H), 3.25 (m,2H), 2.95 (m,2H), 2.63 (s, 3H), 2.52 (s,3H), 2.11 (s, 3H), 1.53 (m,4H), 1.47 (s,12H), 1.43 (s, 6H).

N^2-(3-((5-(4-(((tert-butoxycarbonyl)amino)methyl)phenyl)-2,3-dihydrobenzofuran)-7-sulfonamido)thiophene-2-carbonyl)-N^w-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)-L-arginine (S2).

The ester (**S1**) (1 equiv, 16.7 g, 17.6 mmol) was stirred with lithium hydroxide monohydrate (5 equiv, 3.7 g, 88 mmol) in a tetrahydrofuran/water mixture (2:2; 70 mL) for 18 hours at 20˚C. After this time the organic solvents were removed *in vacuo*, the residue diluted with water (150 mL) and then acidified with 2M hydrochloric acid (100 mL). The resulting very thick slurry was stirred at 20˚C for ~ 1 hour. The solid was collected by filtration, washed with water (3x 50 mL) and dried *in vacuo* to give 7. Yield: 13.6 g, 14.4 mmol, 82%. Off-white solid. ^{1}H NMR (250 MHz, DMSO) δ 8.56 (m,1H), 7.78 (s, 1H), 7.74 (m,2H), 7.58 (m,2H), 7.34 (m,2H), 7.21 (m,2H), 4.64 (m,2H), 4.33 (s,1H), 4.17 (m, 2H), 3.21 (m,2H), 3.01 (m,2H), 2.92 (m,2H), 2.52 (s, 3H), 2.41 (s,3H), 1.96 (s, 3H), 1.62 (m,4H), 1.48 (s,12H), 1.44 (s, 6H).

(S)-1-(4-(3-((5-(4-(ammoniomethyl)phenyl)-2,3-dihydrobenzofuran)-7-sulfonamido)thiophene-2-carboxamido)-4-carboxybutyl)guanidinium 2,2,2-trifluoroacetate (1).

To a solution of the acid (**S2**) (1 equiv, 13.6 g, 14.4 mmol) in dichloromethane (600 mL) was added trifluoroacetic acid (70 mL) and the reaction mixture stirred for 18 hours at 20˚C. After this time some dichloromethane (was added the volatiles were removed *in vacuo*, with DCM (3 x 100ml) added and co evaporated followed by EtOAc (3 x 100ml). To the resulting solid was added an ethyl acetate/methanol mixture (9:1; 200 mL) and the slurry was stirred at ambient for ~ 1.5 h. The solid was collected by filtration, washed with EtOAc (3x 50 mL) and dried *in vacuo* to give 2 as its TFA salt. Yield: 10 g, 12.3 mmol, 82%. Off-white solid.

Crystal structure views of compound 1 with NRP1B` (EG01377)

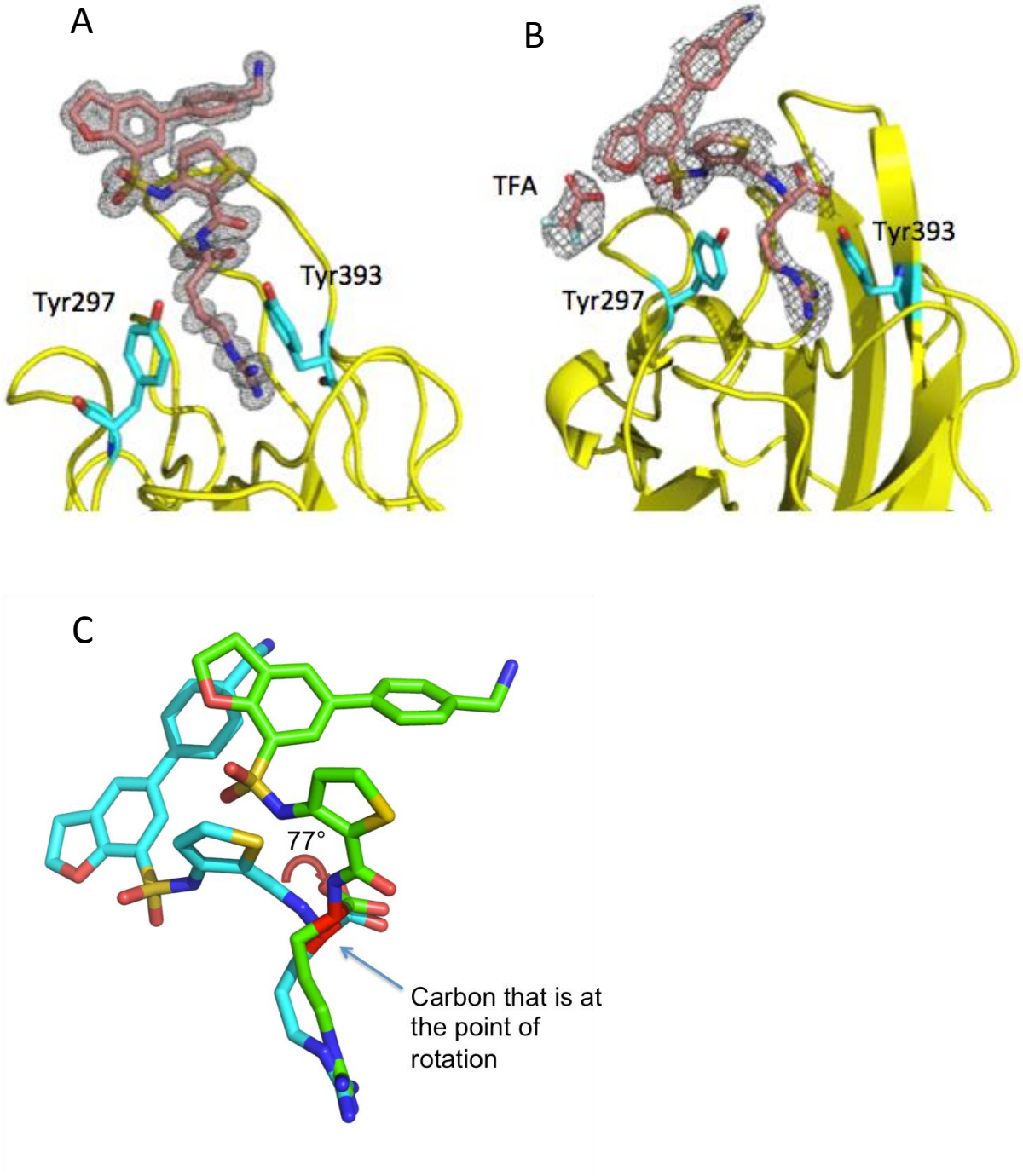
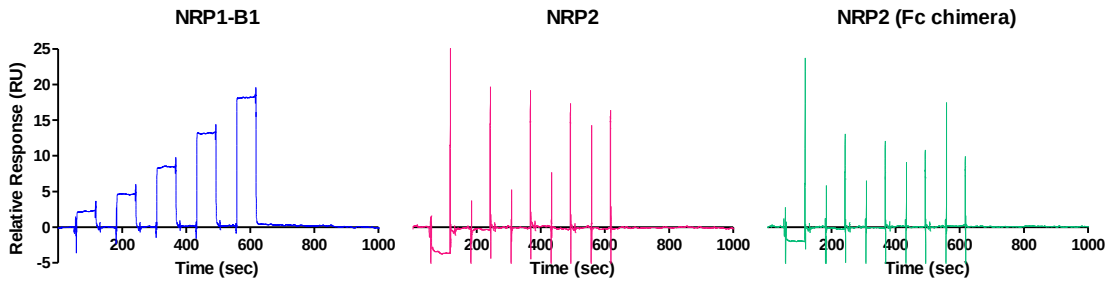


Figure S1: Two crystallised forms of **1** with NRP1b1: A low resolution 2.8Å structure (Cyan), B high resolution 0.9Å (green). C rotation of the C-Carboxyl bond results in interconversion of the conformations.

Compound 1 shows no detectable NRP2 binding by SPR.



Supplementary Figure S2. Selective binding of EG01377 for NRP1 over NRP2. SPR binding of EG01377 (62.5 – 1000 nM) to immobilised NRP1-B1, NRP2 (Life Technologies), and NRP2 (Fc Chimera, R&D systems) using the single-cycle kinetics method. Consecutive injections of 60 sec show binding only to NRP1-B1, with injection spikes only being observed to both NRP2 surfaces.

Materials: Surface Plasmon Resonance experiments were performed using a Biacore T200 instrument at a constant temperature of 25 °C. Sensor chips, buffer stock solutions, and immobilisation reagents were purchased from GE Healthcare. Recombinant human NRP1-B1 was produced in-house. Recombinant human NRP2-His tagged was purchased from Life Technologies. Recombinant human NRP2-Fc chimera was purchased from R&D systems.

Immobilisation: The proteins were immobilised on a CM5 chip and PBS containing 0.05 % surfactant P20 was used as the running buffer during immobilisation. Flow cell 1 was treated with EDC/NHS and Ethanolamine and used as a reference flow cell. NRP1-B1 (30 µg/mL in 10 mM sodium acetate pH 5) was immobilised on flow cell two at 1207 RU. NRP2-Fc chimera (50 µg/mL in 10 mM sodium acetate pH 5) was immobilised on flow cell 3 at 5067 RU. NRP2 (50 µg/mL in 10 mM sodium acetate pH 5) was immobilised on flow cell 4 at 5907 RU (and NRP1-B1 was immobilised onto a CM5 chip using random amine coupling.

Single-cycle binding: PBS containing 0.05 % surfactant P20 and 2 % DMSO was used as the running buffer and sample dilution buffer throughout these experiments. EG01377 was injected over the immobilised proteins for 60 sec at increasing concentration (62.5 – 1000 nM) using a two-fold dilution factor.

Data processing: Binding curves were corrected for variations in DMSO concentration using the BIAevaluation software, and the response curve of a blank sample was subtracted. The DMSO corrected blank-subtracted curves were plotted using GraphPad Prism.

Table S1. Data and refinement statistics for the crystal structures of NRP1 b1 domain in complex with **1** at 0.9 Å and 2.8 Å resolution.

	6FMC	6FMF
Data Collection		
Space group	$P4_1$	$P4_1$
Cell axis (Å)	43.44, 43.44, 91.23	39.65, 39.65, 91.78
Resolution range (Å)	43.44-0.90	39.66-2.81
Rmerge	0.057	0.180
I/σ	10.5	10.9
Completeness (%)	94.7	98.1
Number of reflections	117,894	3,271
Refinement statistics		
Rwork/Rfree	0.11/0.13	0.23/0.29
Average B factor ($Å^2$)	19.0	32
R.m.s deviation from ideal		
Bond lengths (Å)	0.020	0.004
Bond angles (°)	2.6	1.3
Ramachangran plot (%)		
Residues in favored region	98	97
Residues in allowed region	2	3