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Borrelidin modulates the alternative splicing of VEGF by targeting spliceosome-associated protein FBP21

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General chemical methods

Reactions were carried out in oven dried glassware under anhydrous conditions and an atmosphere of argon (oxygen free) unless otherwise described. Commercial chemicals were used as received without further drying or purification unless otherwise noted. All solvents were of reagent or HPLC grade. NMR spectra were acquired on a Bruker Avance 500 spectrometer fitted with a 5mm TCI ATM cryoprobe with Z-gradients running at 298 K and operating at 500 MHz and 125 MHz for ^{1}H and ^{13}C respectively. Standard Bruker pulse programs were used to acquire all spectra. Chemical shifts are reported in parts per million and are referenced relative to the solvent resonance. Coupling constants are given in hertz. LCMS was performed on an integrated Agilent HP1100 HPLC system in combination with a Bruker Daltonics Esquire 3000+ spectrometer fitted with an electrospray source. Analytical chromatography was achieved over reverse phase silica (Hypersil C_{18}-BDS, 150 x 4.6 mm column, 3μ particle size) eluted at 1 mL min^{-1} using the following gradient: T=0 min, 25% B; T=15, 100% B. Mobile phase A: Water containing 0.1% v/v formic acid; Mobile phase B: Acetonitrile containing 0.1% v/v formic acid. High-resolution MS were measured on a Bruker BioApex II 4.7e FTICR fitted with an electrospray source and operating in positive ion mode. Compound purity was determined using LCMS and assessed at two concentrations by both MS and UV (at multiple wavelengths); chromatograms were then compared versus a solvent blank. All compounds were additionally assessed by both proton and carbon NMR spectra.

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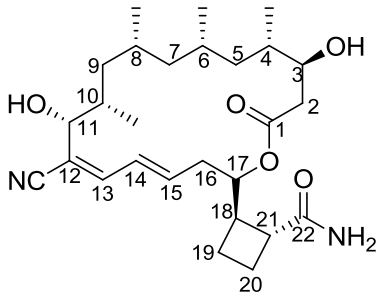
Borrelidin **1**, BC194 **2** and BC153 **3** were produced and isolated as described previously;[1,2] these references include their characterization.

Synthesis of BC235 (6) (achieved using literature method from reference 3)

2 (123.7 mg, 0.26 mmol) was dissolved into tetrahydrofuran (10 mL) with stirring and cooled to -20 °C. To this were added triethylamine (41 μL, 0.29 mmol) and isobutylchloroformate (39 μL, 0.29 mmol). After 30 min at -20 °C the triethylamine hydrochloride salt was removed by filtration and aqueous ammonium hydroxide (25% solution, 100 μL, 1.5 mmol) was added. After stirring for 3 h at -20 °C the solution was adjusted to pH 7 using glacial acetic acid and the solvent removed under reduced pressure. The residue was dissolved into $CHCl_3$ (100 mL) and washed with water (2 x 50 mL), dried over anhydrous $MgSO_4$, filtered and the solvent removed under reduced pressure. The solid residue was chromatographed over flash silica gel eluting with ethyl acetate:$CHCl_3$ (40:60) to yield a colorless oil (90.0 mg, 0.19 mmol, 72% (purity >95%)). HRMS calculated for $C_{27}H_{43}N_2O_5Na$ $[M+Na]^+$ 497.29859, found 497.29692 (Δ 3.35 ppm). NMR data were acquired in $CDCl_3$:



Position	δ_H (ppm)	Multiplicity	Coupling (Hz)	δ_C (ppm)
1	-			172.4
2a	2.45	m		40.4

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2b	2.36	m		
3	3.89	m	12.0	69.9
4	1.58	m		36.0
5a	1.26	m		43.1
5b	0.92	m		
6	1.52	m		27.6
7a	1.12	m		47.5
7b	1.01	m		
8	1.63	m		26.2
9a	1.05	m		37.3
9b	0.73	m		
10	1.88	m		35.2
11	4.11	d	9.5	73.0
12	-			118.2
13	6.80	d	14.5	144.0
14	6.38	t	14.5	126.8
15	6.10	m		138.7
16a	2.51	m		34.3
16b	2.49	m		
17	5.10	m	11.5	76.0
18	2.93	m		39.9
19a	1.75	m		20.9
19b	2.01	m		
20a	2.14	m		21.7
20b	2.14	m		43.6

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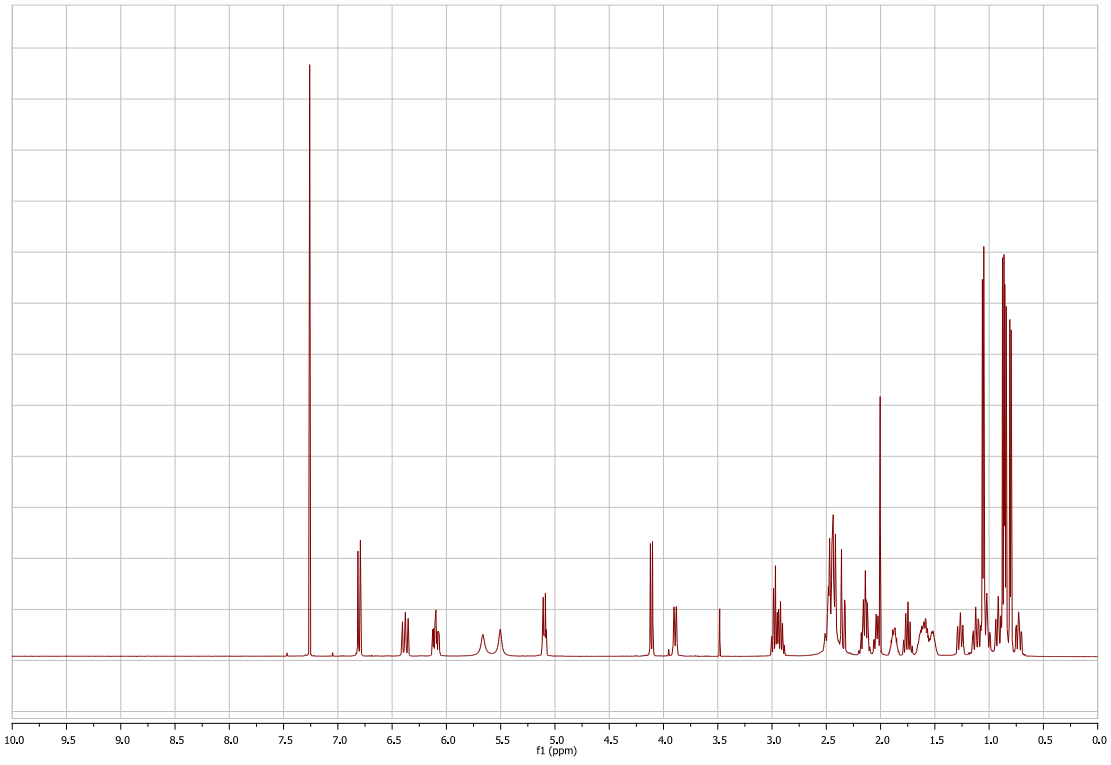
21	2.98	m		
22	-			176.1
4-CH_3	0.87	d	7.0	17.1
6-CH_3	0.80	d	7.0	18.2
8-CH_3	0.85	d	7.0	20.2
10-CH_3	1.05	d	6.0	14.9
12-CN	-			115.9
OH	5.66	bs		
OH	5.50	bs		
NH_2	2.47	b		

^{1}H NMR of BC235 (**6**) in $CDCl_3$



Synthesis of borrelidin biotinamidohexanylhydrazide (4)

1 (24.2 mg, 0.049 mmol) was dissolved into tetrahydrofuran (THF) (2 mL) and cooled to -15 °C in an ice/salt bath. Isobutyl chloroformate (0.13 mL, 1.0 mmol) and triethylamine (0.13 mL, 1.0 mmol) were dissolved in THF (10 mL) and an aliquot of this mixture (0.54 mL) was added to the **1** solution. After stirring for 40 min the solution was filtered to remove triethylamine hydrochloride. Biotinamidohexanoic acid hydrazide (Sigma, 100 mg, 0.269 mmol) was dissolved in THF (7.5 mL) and water (7.5 mL) and this solution (4.92 mL) added to the freshly filtered activated **1** solution. After 2 h stirring at room temperature the mixture was stored at -20°C overnight, before the solvent was removed under reduced pressure (lyophilisation to remove the water). The residue was dissolved in methanol (4 mL) and diluted with water (15 mL) and passed through a C_{18} Bond Elute cartridge (5 g). This was washed with water (10 mL) and then eluted with methanol (30 mL). The methanol solution was evaporated to dryness to yield an amorphous solid (44.4 mg). The solid was dissolved in methanol (0.75 mL) and purified by preparative LC in a single shot. Column: C_{18} LUNA Phenomenex, 22.5 mm x 250 mm. Solvents: A was water (890 mL), acetonitrile (100 mL) and 1M ammonium acetate (10 mL); B was acetonitrile (900 mL), water (90 mL) and 1M ammonium acetate (10 mL); LC programme: 21 mL min^{-1}, 40% B to 50% B over 30 min. Fractions were assessed by analytical LC and active fractions combined, taken to dryness under reduced pressure and then desalted over a C_{18} SPE cartridge (1g) to yield **4** as a white amorphous solid (19.9 mg, 0.024 mmol, 48 % isolated yield, purity > 95 %). LCMS: negative mode *m/z* = 841.2 $[M-H]^-$; positive mode *m/z* = 843.4 $[M+H]^+$ and 865.3 $[M+Na]^+$. HRMS calculated for $C_{44}H_{70}N_6O_8SNa$ $[M+Na]^+$ 865.48681, found 865.48691 (Δ 0.13 ppm). ^{13}C NMR ($CDCl_3$), δ_C/ppm: 174.7, 173.0, 172.0, 171.7, 164.1, 144.2,

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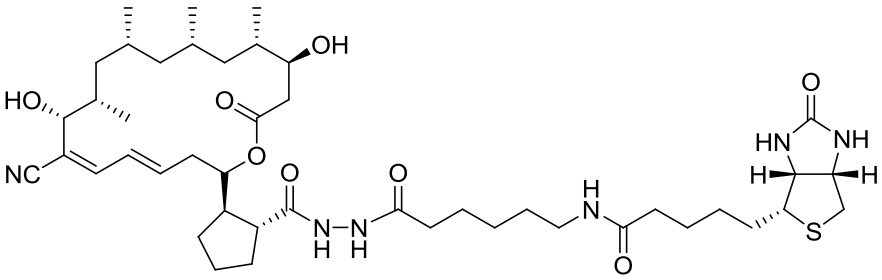
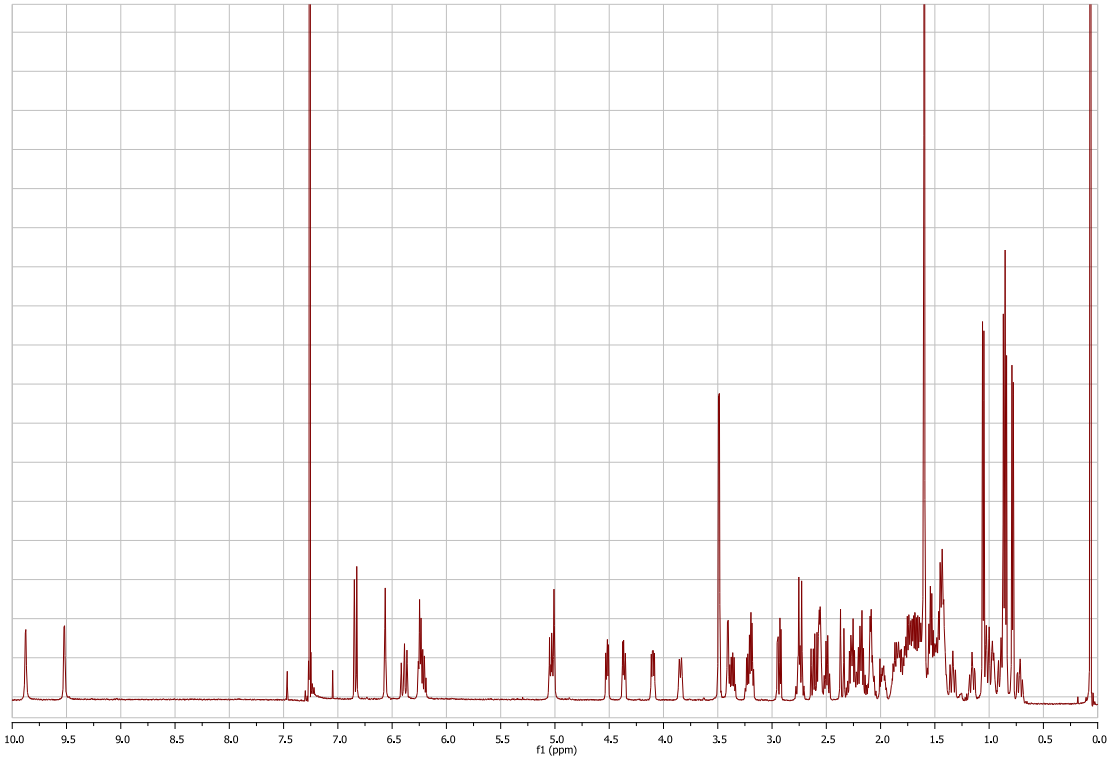
139.2, 126.5, 118.2, 115.7, 76.4, 73.1, 68.0, 62.0, 59.8, 55.4, 50.8, 47.6, 47.4, 46.0, 42.8, 41.4, 41.0, 38.4, 37.2, 36.3, 35.4, 35.0, 33.5, 31.5, 29.9, 28.0, 27.7, 27.3, 27.1, 26.1, 25.4, 25.2, 24.9, 24.3, 20.0, 18.2, 15.6, 14.9.



^{1}H NMR of **4** in d_4-methanol



Synthesis of BC153 biotinamidohexanylhydrazide (5)

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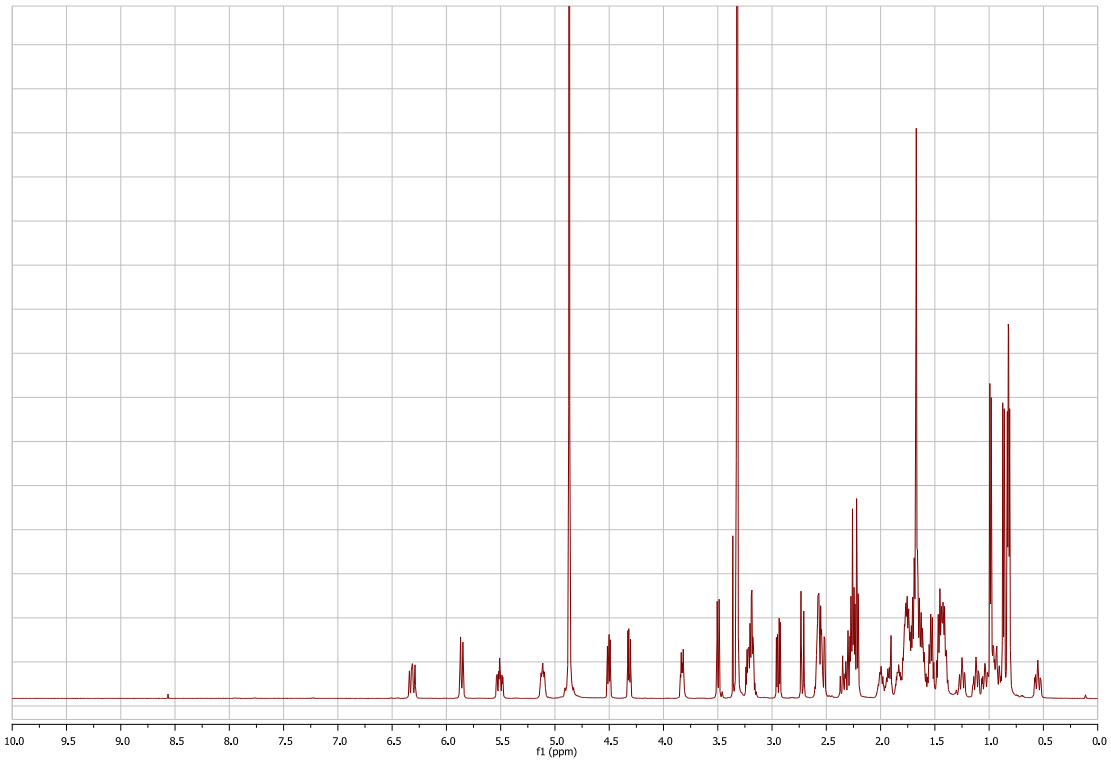
This was made in a similar method to that above starting with **3** (19.4 mg, 0.041 mmol) to yield **5** as a white amorphous solid (18.3 mg, 0.022 mmol, 54 % isolated yield, purity > 95 %). LCMS: negative mode *m/z* = 830.2 $[M-H]^-$; positive mode *m/z* = 854.4 $[M+Na]^+$. HRMS calculated for $C_{44}H_{73}N_5O_8SNa$ $[M+Na]^+$ 854.50721, found 854.50900 (Δ 2.10 ppm). ^{13}C NMR (d_4-methanol), δ_C/ppm: 177.9, 176.1, 174.7, 174.5, 166.3, 138.6, 131.2, 130.0, 128.5, 86.1, 77.1, 73.0, 63.5, 61.8, 57.2, 50.1, 49.8, 47.3, 44.7, 41.2, 40.3, 39.5, 39.1, 36.9, 34.8, 34.6, 33.5, 31.3, 30.1, 29.9, 29.6, 28.4, 27.6, 27.3, 27.0, 27.0, 26.3, 20.6, 18.9, 18.7, 16.6, 11.3.

^1H NMR of **5** in d_4-methanol



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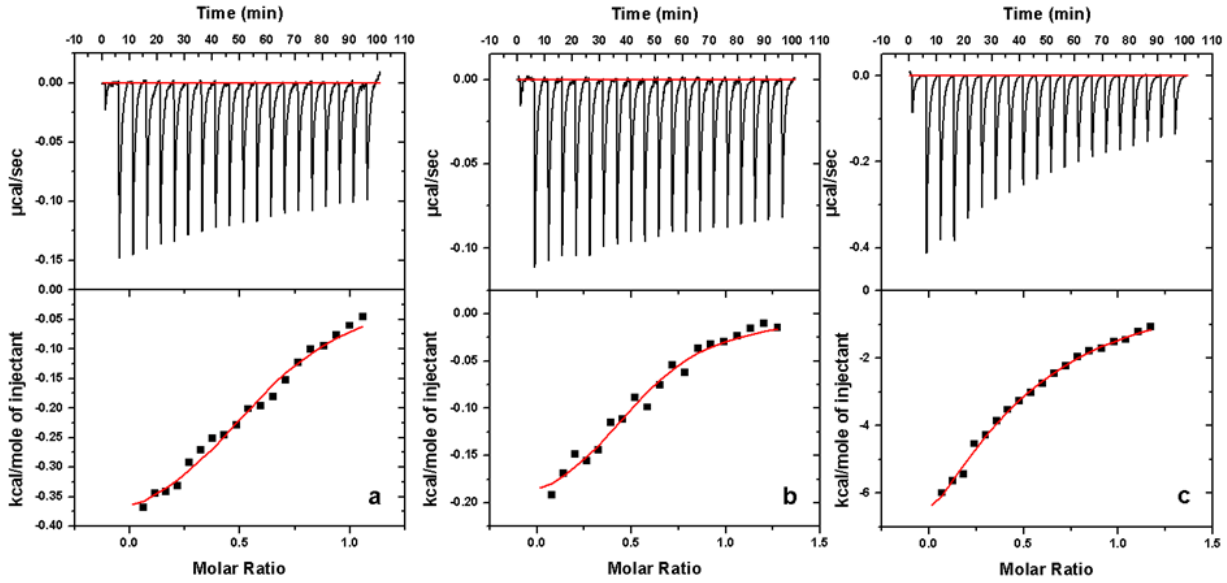
Isothermal titration calorimetry data

Supplementary Figure S1. FBP21 WW domains bind **1** & **2**. The isothermal titration calorimetry traces are shown for (a) **1**; (b) **3**; (c) the control peptide SMB; the thermodynamic data are summarized below.



Ligand	K_d (µM)	ΔG kcal/mol	ΔH kcal/mol	TΔS kcal/mol	N
1	11.8 ± 2.7	-6.5	-0.43 ± 0.0	6.1	0.62 ± 0.0
2	9.7 ± 3.1	-6.6	-0.22 ±0.0	6.4	0.55 ± 0.0
SMB	16.4 ± 1.9	-6.3	-12.7 ± 1.4	-6.4	0.44 ±0.4

In vitro HUVEC anti-angiogenesis assays

Angiogenesis assays were carried out at the angiogenesis resource center of the NCI and details of the assay protocols are given below in Supplementary Table S1. **1** and **3** were screened, along with TNP-470 and paclitaxel (Taxol®) which were used as reference compounds.

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Supplementary Table S1

Compound	HUVEC growth inhibition assay, IC_{50} (nM)	HUVEC cord formation assay, IC_{50} (nM)
1	3.25	46.5
3	2380	>30,000
TNP-470	3.16±0.82	1000
Paclitaxel	1.65±0.12	50

Growth inhibition assay

HUVEC ($1.5x10^3$) were plated in a 96-well plate in EBM-2 (100 µL) (Clonetic # CC3162). After 24 h (day 0), the test compound (100 µL) was added to each well at 2 times the desired concentration (5-7 concentration levels) in EBM-2 medium. On day 0, one plate was stained with 0.5% crystal violet in 20% methanol for 10 min, rinsed with water, and air-dried. The remaining plates were incubated for 72 h at 37 ºC. After 72 h, plates were stained with 0.5% crystal violet in 20% methanol, rinsed with water and air-dried. The stain was eluted with 1:1 solution of ethanol :0.1M sodium citrate (including day 0 plate), and absorbance was measured at 540 nm with an ELISA reader (Dynatech Laboratories). Day 0 absorbance was subtracted from the 72 h plates and data was plotted as percentage of control proliferation (vehicle treated cells). The IC_{50} (drug concentration causing 50% inhibition) was calculated from the plotted data.

Cord formation assay

Matrigel (60 µL of 10 mg/mL; Collaborative Lab # 35423) was placed in each well of an ice-cold 96-well plate. The plate was allowed to sit at room temperature for 15 min then it was incubated at 37 ºC for 30 minutes to permit the matrigel to polymerize. In the mean time,

HUVEC were prepared in EGM-2 (Clonetic # CC3162) at a concentration of $2x10^5$ cells mL^{-1}. The test compound was prepared at 2 times the desired concentration (5 concentration levels) in the same medium. The cells (500 µL) and the drug at 2 times concentration (500 µL) were mixed and a portion of this suspension (200 µL) was placed in duplicate on the polymerized matrigel. After 24 h incubation, triplicate pictures were taken for each concentration using a Bioquant Image Analysis system. Drug effect (IC_{50}) is assessed compared to untreated controls by measuring the length of cords formed and number of junctions.

Retinal pigmented endothelial cell culture

Human retinal pigmented epithelial (RPE) cells (obtained from ATCC, Teddington, UK), are well-characterised and constitutively express both $VEGF_{165}$ and $VEGF_{165}b$.[4] Cells were routinely subcultured in DMEM:F12 (1:1) media supplemented with 10% foetal bovine serum (FBS) and trypsinised with a trypsin/EDTA solution (Sigma) between passages 20-30. Once a confluency of 70-80% was achieved, RPE cells were plated in 6 well plates. To determine the effects of borrelidin or its analogs, cells were cultured in fresh medium in the absence of FBS for 24 h prior to stimulation. Both borrelidin and analogs were prepared in 2 mL of serum-free medium, at the concentrations indicated in the text. Cell number was assessed following stimulation. No changes in cell morphology were apparent upon visualisation of the cells following stimulation with borrelidin or its analogues. Following treatment with borrelidins, conditioned media was collected and stored at -20 °C until analysis by ELISA or Western Blotting. Subsequently, cells were washed with ice-cold 1XPBS and lysed in 50 µL RIPA buffer containing a protease inhibitor cocktail (Sigma,

UK).

Conditioned media and cell lysate VEGF measurements

Total pan VEGF concentrations of neat RPE conditioned medium and cell lysates were measured according to the manufacturer's instructions using a standard DuoSet VEGF ELISA (R&D Systems Cat No.DY293BE). $VEGF_{xxx}b$ was determined with a similar sandwich ELISA (R&D Systems Cat. No. MAB3045). For the $VEGF_{xxx}b$ ELISA, the capture antibody was a monoclonal biotinylated mouse anti-human antibody raised against the terminal nine amino acids of $VEGF_{165}b$ (R&D Systems); a standard curve for this assay was built with recombinant human $VEGF_{165}b$ (R&D Systems). This antibody has been characterised previously[4,5] and specifically detects the $VEGF_{xxx}b$ family of isoforms but not the conventional $VEGF_{xxx}$ isoforms.

Western blotting

Cell lysate samples were quantified using the Bradford Assay for protein quantification (Biorad, UK). Samples were dissolved in loading buffer containing Tris 1M (pH 6.8), 10% SDS, 30% Glycerol, 10% Bromophenol Blue and 5% β-mercaptoethanol, and boiled for 5 min. 30 μg protein per lane were separated by SDS/PAGE (12%) and transferred to a 0.2 μm Polyvinylidene Fluoride (PVDF) membrane. Subsequently, membranes were blocked for 60 min with 5% skimmed milk / 1XPBS - 0.05% Tween, and probed overnight at room temperature with antibodies against $VEGF_{xxx}b$ (in house clone 56/1; 1:250) and β-tubulin

(Sigma; 1:2000) in a solution containing 2.5% skimmed milk / 1xPBS - 0.05% Tween. Membranes were washed in 0.05% 1xPBS - 0.05% Tween and then incubated with a secondary HRP-conjugated antibody (Pierce). Immunoreactive bands were visualised using ECL reagent (Pierce) and subsequently quantified using ImageJ analysis; the resulting densities were normalized to those of β-tubulin (n=3).

RNA analysis

RNA from RPE cells treated with compounds as described above was extracted, reverse transcribed and amplified by PCR as decribed previously.[6]

IGF-1 treatment experiments

RPE cells were split to six-well plates ($1x10^5$ cells per well) and grown until 95% confluent cells. Twenty-four hours before treatment cultured medium was replaced with serum free RPMI-1654 medium (Sigma) containing 1% ITS (Sigma) and 0.5% PSS (Sigma). Subsequently, the medium was replaced with fresh serum free RPMI-1610 medium (Sigma) containing 1% ITS (Sigma), 0.5% PSS (Sigma) and increasing concentrations of IGF-1 (Sigma). The conditioned media were collected 24 h or 48 h after stimulation.

RPE transfection with FBP21 and analysis

RPE cells were grown in six-well plates, each seeded with 3×10^5 cells and subsequently

transfected with 1 µg of pWV327 or control (empty) vector using Lipofectamine (Invitrogen). Following incubation at 37 °C for 48 h, and treatment with IGF-1 as above where stated, conditioned medium was collected and cells were lysed in buffer containing: 20 mM Tris, pH7.4, 1.5%w/v Triton X-100, 150 mM NaCl, 10% w/v glycerol and protease inhibitors cocktail (Sigma). VEGF isoforms were measured in cell lysate using ELISA. Correction of the FBP21 cDNA clone is described below.

Correction of the FBP21 cDNA expression plasmid pVW327

pOriGene-FBP21 is a pCMV6 based expression vector containing the human formin binding protein 21 (FBP21) complementary DNA (cDNA) and was obtained from OriGene Technologies Inc. (USA). However, upon restriction digest and sequencing the entire insert it became apparent that an additional 2299 bp of DNA was inserted into the 5' region of *fbp21* at base 75 when compared to the published *fbp21* sequence in GenBank (NM_007187). This was corrected as follows: pOrigene-FBP21 was digested with *Eco*RI, removing most of the cDNA insert, including all of the unwanted sequence. To repair the *fbp21* coding sequence, two PCR products were then inserted (PCR 325 generated by amplifying the 5' region of *fbp21* using oligos BV170 and BV171; and PCR 326 generated by amplifying the 3' region of *fbp21* using oligos BV172 and BV173) using a 3-part ligation. This introduced a silent *Eag*I site at the junction to aid analysis. The resulting plasmid (pWV327) was verified by restriction digest and sequencing, and represents a corrected vector for the expression of *fbp21* to produce FBP21 protein as described in the above mentioned GenBank sequence.

Oligo	Sequence
BV170	5'-GGTGGGAGGTCTATATAAGCAGAGCTCG
BV171	5'-ATATCGGCCGATTGTCTGCTATCCAGCACTTGCAGTAA
BV172	5'-ATATCGGCCGAGTGTTGAATTTCATGAAAGAGGAAAGAATCATAAG
BV173	5'-CTCCATATGGGTTTGATTTCTTAAGAGTTTTCGA

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