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5-Carboxyfluorescein tagged *N*-phenylanthranilamide as a tracer reagent for fluorescence polarization: a robust method to screen MAPK pathway allosteric inhibitors

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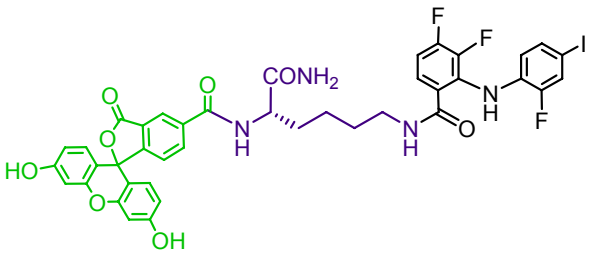
Experimental procedures

N^{α}-(Fluorescein-5-carbonyl)-N^{ε}-(3,4-difluoro-2-(2-fluoro-4-iodophenylamino)benzoyl)-L-lysyl amide; 4



A suspension of the NovaPEG Rink Amide resin (100 mg, 0.064 mmol; 0.64 mmol g^{-1}, Merck Chemicals Ltd. # 01-64-0473) in DMF (2 mL) in a reaction column was washed with DMF (2.5 mL min^{-1}, 10 min), 20% v/v piperidine in DMF (2.5 mL min^{-1}, 5 min) and finally with DMF (2.5 mL min^{-1}, 15 min). The excess DMF was removed, and the resin in the reaction column was treated with a pre-prepared mixture of Dde-L-Lys(Fmoc)-OH [(1)] (133 mg, 0.25 mmol, Merck Chemicals Ltd.), *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) (95 mg, 0.25 mmol) and *N*,*N*-diisopropylethylamine (0.087 mL, 0.5 mmol) in DMF (1 mL). The resultant mixture was gently stirred for 14 h, followed by washing with DMF (2.5 mL min^{-1}, 15 min). The resin product in the reaction column was treated with 20% piperidine (2.5 mL min^{-1}, 10 min), washed with DMF (2.5 mL min^{-1}, 15 min), excess DMF removed and treated with a pre-prepared mixture of 3,4-difluoro-2-(2-fluoro-4-iodophenylamino)benzoic acid[(2,3)] (98 mg, 0.25 mmol), HATU (95 mg, 0.25 mmol) and *N*,*N*-diisopropylethylamine (0.087 mL, 0.5 mmol) in DMF (1 mL) for 10 h. The resin product was washed with DMF (2.5 mL min^{-1}, 10 min), treated with 4% hydrazine monohydrate in DMF (2.5 mL min^{-1}, 30 min), washed with DMF (2.5 mL min^{-1}, 15 min), excess DMF removed and then treated with a pre-prepared mixture of 5-carboxyfluorescein (100 mg, 0.26 mmol), 7-aza-1-hydroxybentriazole (34 mg, 0.25 mmol) and *N*,*N'*-diisopropylcarbodiimide (0.110 mL, 0.25 mmol) in DMF (1 mL) for 14 h. The resin product was then successively washed with DMF (2.5 mL min^{-1}, 15 min), 20% piperidine in DMF (2.5 mL min^{-1}, 15 min), and DMF (2.5 mL min^{-1}, 15 min).

The coloured resin product was collected, rinsed with CH_2Cl_2, dried *in vacuo*, and then treated with the acidolytic mixture trifluoroacetic acid (6 mL) – H_2O (0.6 mL) – iPr_3SiH (0.2 mL) for 2 h at ambient temperature. The suspension was filtered, the filtrate was evaporated to dryness *in vacuo* and the residual material was triturated with diethyl ether (3 x 5 mL) to afford the title compound as a bright yellow solid (48 mg), which was dried *in vacuo* extensively and used directly for FP assays.

Reversed-phase HPLC was carried out using Waters^{TM} 510 pumps, Waters^{TM} 484 detector and Hypersil^{TM} Pep100-C_{18} analytical column (150 x 4.6 mm, 5 μm) at a flow rate of 1.20 mL min^{-1} and the effluent was monitored at 220 nm. Gradient elution was from 50 % to 100 % B over 20 min, and the eluents used were: solvent A (0.06% v/v TFA in Milli-Q water) and solvent B (0.06% v/v TFA in MeCN – Milli-Q water, 9:1 v/v). The solid product was established by HPLC to be >90% pure (**Fig. S1** and **S2**); R_t 8.48 min. RP-HPLC purified sample: ^{1}H NMR (400 MHz, CD_3OD) δ 1.50 (m, 2H), 1.60 (m, 2H), 1.88 (m, 1H), 1.96 (m, 1H), 3.30 (2H, signal masked by CH_3OD), 4.58 (m, 1H), 6.51-6.60 (m, 4 H), 6.71 (d, J = 2.0 Hz, 2H), 6.99 (m, 1H), 7.29 (m, 2H), 7.41-7.46 (m, 2H), 8.23 (dd, J = 1.6 and 8.0 Hz, 1H), 8.50 (s, 1H), 8.71 (d, J = 7.8 Hz, 1H); HR-ToF MS (ES^+) calcd for $C_{40}H_{31}F_3IN_4O_8$ (MH^+) 879.1133, found 879.1170.

(1) Bycroft, B.W., Chan, W.C., Chhabra, S.R. & Hone, N.D. (1993) *J. Chem. Soc., Chem. Commun.*, 778–779.

(2) S. D. Barrett, A. J. Bridges, D. T. Dudley, A. R. Saltiel, J. H. Fergus, C. M. Flamme, A. M. Delaney, M. Kaufman, S. LePage, W. R. Leopold, S. A. Przybranowski, J. Sebolt-Leopold, K. Van Becelaere, A. M. Doherty, R. M. Kennedy, D. Marston, W. A. Howard, Jr., Y. Smith, J. S. Warmus and H. Tecle, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 6501-6504.

(3) S. D. Barrett, C. Biwersi, M. Kaufman, H. Tecle and J. S. Warmus, *WO 02/06213 A2*, 24 January 2002.

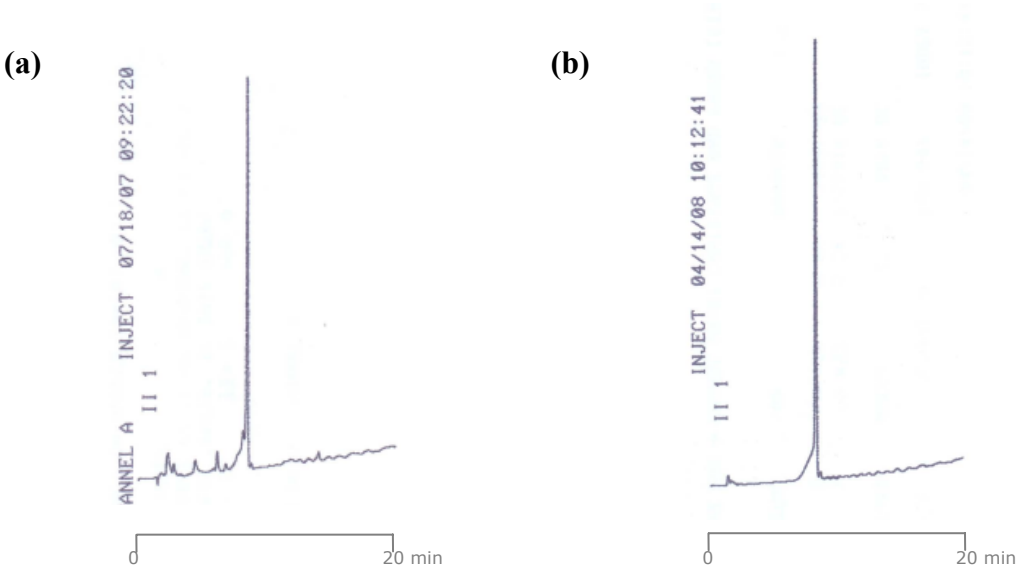


Figure S1 The RP-HPLC chromatograms of the **(a)** semi-purified by ether trituration, and **(b)** HPLC-purified fluorescent tracer **4**. RP-HPLC analyses were carried using Hypersil™ Pep100-C_{18} column (150 x 4.6 mm) at a flow rate of 1.20 mL min^{-1}, the effluent monitored at 220 nm and the elution gradient was 50–100 % B in 20 min; the eluents used were: solvent A (0.06% aq. TFA) and solvent B (0.06% TFA in MeCN – H_2O, 9:1 v/v).

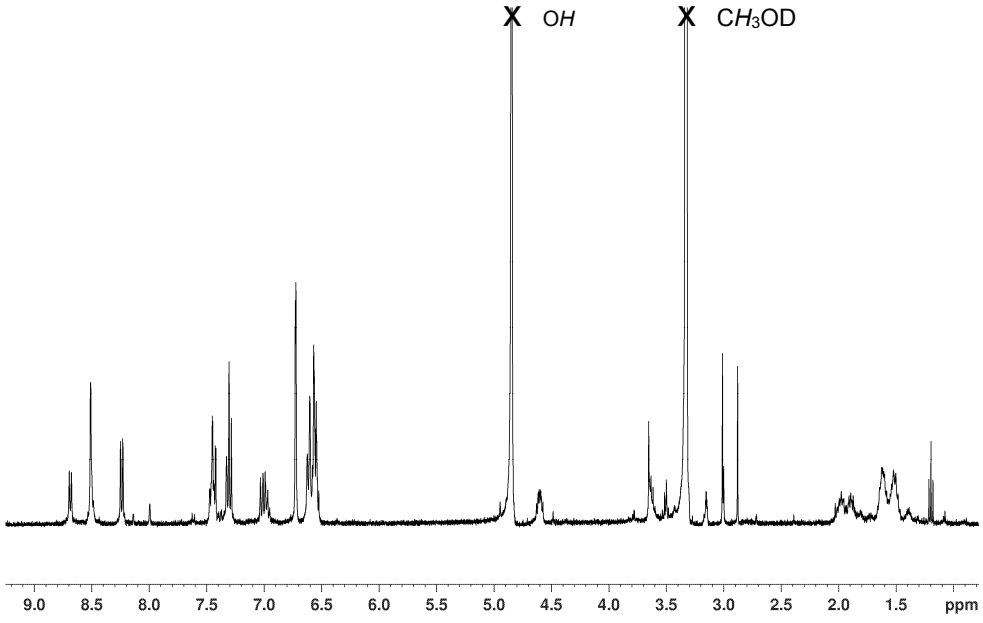


Figure S2 (a) The ^1H NMR (400 MHz) spectrum of the semi-purified fluorescent tracer **4** (6 mg mL^{-1}) in CD_3OD.

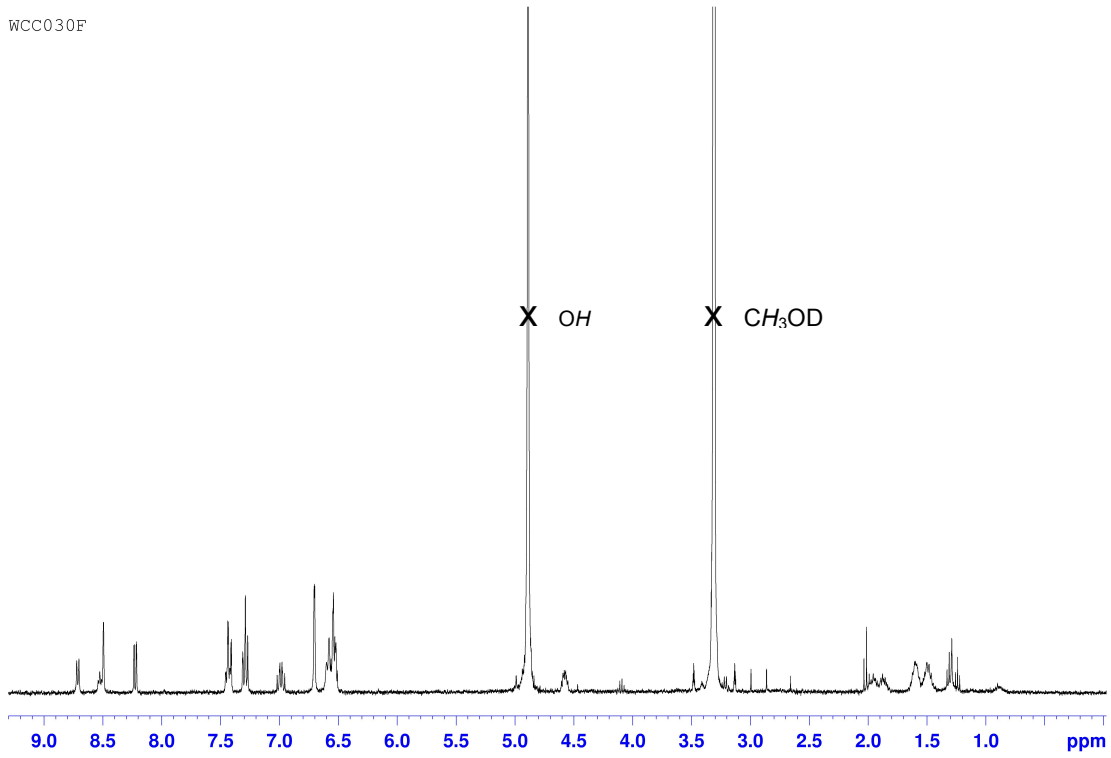


Figure S2 (b) The ^{1}H NMR (400 MHz, CD_3OD) spectrum of the RP-HPLC purified **4**.

RNA isolation and first strand DNA synthesis
Total RNA was purified from HeLa cells using Qiagen RNeasy extraction kit (Cat # 74104). The quality of the RNA was checked by native agarose gel electrophoresis. First strand cDNA was synthesized using the SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturer's protocol.

Cloning human MEK1
The human MEK1 (Gene Bank Accession number: L11284.1) spanning 2,167 bps and contains 11 exons, was cloned by PCR utilizing cDNA derived from HeLa cells as the template. The primers utilized for full length MEK1 amplification were: MEK1 forward :5' CGC GGA TCC ATG CCC AAG AAG AAG CCG 3'; MEK1 reverse:5' GCAA GCT TCG TTA GAC GCC AGC AGC ATG 3'. Direct ligation of the full length MEK1 cDNA into the pRSET-A plasmid (Invitrogen) was performed using *BamHI* and *HindIII* restriction sites introduced into the forward and reverse primers respectively. By employing pRSET-A plasmid, the MEK1 recombinant protein is *N*-terminally tagged with 6 histidine residues for further purification. The final construct was verified by DNA sequence analysis. MEK1 cDNA was inserted into different plasmids, pET-41a, pET-28a (Novagen) and pGEX-4T-1 (Amersham Biosciences) to produce the MEK1 recombinant protein with different tags. MEK1-pRSET-A was transformed into DH5α *Escherichia coli* chemically competent cells for maintenance and propagation.

Expression of human MEK1
BL21(DE3)pLyse *E. coli* cells (Novagen) were utilized as a bacterial host for expression of the recombinant MEK1 protein. For optimization of the MEK1 expression three different expression temperatures were chosen (16, 23 and 37 °C) and also 3 different amounts of IPTG (250, 500 and 1000 μM) and different incubation times (3 and 6 hours) were tried. Optimization was completed and the best conditions were chosen for the MEK1 purification.
The pRSET-A plasmid gave the best expression of MEK1 recombinant protein. Amongst different growth temperatures which were also tried; cell growth at 37 °C and 6 hours of IPTG induction produced the best result. The BL21(DE3)pLyse cells were grown in the LB medium at 37 °C and induced with 500 μM isopropyl-β-D-thiogalactopyranoside (IPTG) for 6 hours to express the MEK1 protein in high yields. Immunoblotting with the MEK1 antibody was performed using the polyclonal MEK1-antibody (Abcam # 32091) which confirms the expression of the MEK1 recombinant protein.

Purification of recombinant His-tagged MEK1
To purify sufficient amount of purified recombinant MEK1 protein for binding assay, 1 litre LB culture was utilized. After 6 h of IPTG induction, the cell pellet was harvested (3000 g, 10 minutes at room temperature) and bacterial cells lysed in lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 1% NP-40 and 0.1% SDS) and then sonicated on ice for 5 x at high output setting. The lysate was centrifuged at high speed (10000 g, 10 min at room temperature), insoluble fraction was spun down and supernatant was collected.

To purify the His-tagged protein under native conditions, nickel-charged resin was employed. Imidazole was added to the supernatant to a final concentration of 10 mM and applied to the prepared 700 μl nickel-charged resin. The column was incubated with gentle shaking overnight at 4 °C. The resin was then washed 3x with TBS, TBS–20 mM Tris and TBS–40 mM Tris (pH 7.5) respectively. The recombinant MEK1 protein was eluted using 1.00 mL of 500 mM imidazole in TBS pH 7.5. Imidazole was removed from the eluted recombinant

protein by dialysis using 20 mM TBS for 16 hours at 4 °C. The purified protein was analysed on 5–20% SDS-PAGE electrophoresis (Fig. S3).

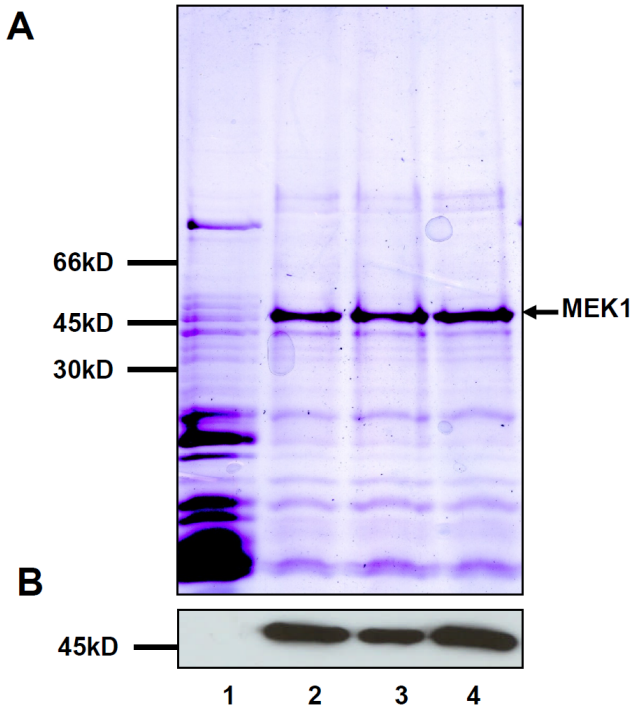
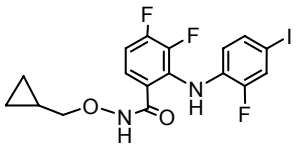


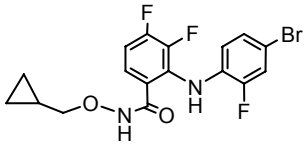
Figure S3 Expression analysis of MEK1, **(A)** BL21(DE3)pLyse *E. coli* cells were lysed by sonication and 50 µl of soluble fraction was analysed on SDS-PAGE and stained with Coomassie blue. The arrow shows recombinant MEK1 protein (MEK1 MW: 43 kD, the overall estimated fusion protein His-MEK1 MW: 46 kD).
Lane 1: BL21(DE3)pLyse *E. coli* cells only, lane 2: 250 µM IPTG, 3 h induction; lane 3: 500 µM IPTG, 3 h induction, lane 4: 500 µM IPTG, 6 h induction. All the cells were grown at 37 °C.
(B) The corresponding lanes were immuno-bloted with the MEK1 antibody. Induction with 500 µM IPTG for 6 h yielded the highest amount of recombinant MEK1.

***N*-(Cyclopropylmethoxy)-3,4-difluoro-2-(2-fluoro-4-iodophenylamino)benzamide;[2,3] 6**



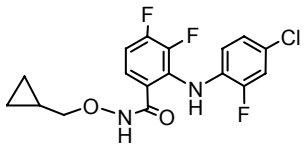
A solution of pentafluorophenyl 3,4-difluoro-2-(2-fluoro-4-iodophenylamino)benzoate (792 mg, 1.41 mmol) in CH_2Cl_2 (10 mL) was added *N*,*N*-diisopropylethylamine (0.50 mL, 2.83 mmol) followed by *O*-cyclopropylmethylhydroxylamine hydrochloride (350 mg, 2.83 mmol). The mixture was stirred at ambient temperature for 72 h, and was then washed with water (3 x 15 mL), brine (15 mL), dried over $MgSO_4$, and the organic extract was evaporated to dryness to give an orange gum. The gum was purified by silica column chromatography, using EtOAc as the eluent to give the title compound as an orange fluffy solid (107 mg, 16%); m.p. 121-123 °C. ^{1}H NMR (300 MHz, D_6-DMSO) δ 0.18 (m, 2H), 0.46 (m, 2H), 1.03 (m, 1H), 3.57 (d, J = 6.8 Hz, 2H), 6.65 (m, 1H), 7.19 (m, 1H), 7.33 (m, 2H), 7.55 (d, J = 10.9 Hz, 1H), 8.67 (s, 1H), 11.74 (s, 1H); MS (ES^+) calcd for $C_{17}H_{15}F_3IN_2O_2$ (MH^+) 463.01, found 462.84.

2-(4-Bromo-2-fluorophenylamino)-*N*-(cyclopropylmethoxy)-3,4-difluorobenzamide;[3] 7



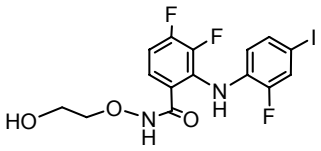
A solution of 2-(4-bromo-2-fluorophenylamino)-3,4-difluorobenzoic acid (370 mg, 1.07 mmol) in THF (3 mL) and DMF (3 mL) was added *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) (446 mg, 1.18 mmol) and *N*,*N*-diisopropylethylamine (0.56 mL, 3.21 mmol). The mixture was stirred at ambient temperature for 1 h, after which *O*-cyclopropylmethylhydroxylamine hydrochloride (200 mg, 1.61 mmol) was added and the mixture was stirred for a further 48 h. The mixture was then concentrated *in vacuo*, diluted with EtOAc (20 mL), washed with water (4 x 15 mL), dried over Na_2SO_4, and the organic extract was evaporated to dryness to give a coloured residual material. The residual material was suspended in CH_2Cl_2 (5 mL), filtered and the filtrate was loaded directly onto a silica chromatography column, which was then eluted with CH_2Cl_2 to give the title compound as a white solid (72 mg, 16%); m.p. 116-118 °C. ^{1}H NMR (300 MHz, D_6-DMSO) δ 0.18 (m, 2H), 0.46 (m, 2H), 1.04 (m, 1H), 3.58 (d, J = 7.2 Hz, 2H), 6.79 (m, 1H), 7.19 (m, 2H), 7.34 (m, 1H), 7.49 (d, J = 8.9 Hz, 1H), 8.73 (s, 1H), 11.74 (s, 1H); MS (ES^+) calcd for $C_{17}H_{15}BrF_3N_2O_2$ (MH^+) 415.03 and 417.02, found 414.85 and 416.88.

2-(4-Chloro-2-fluorophenylamino)-*N*-(cyclopropylmethoxy)-3,4-difluorobenzamide;[3] **8**



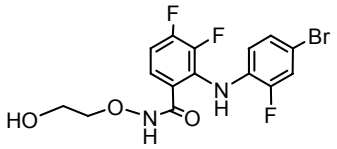
A solution of 2-(4-chloro-2-fluorophenylamino)-3,4-difluorobenzoic acid (325 mg, 1.07 mmol) in THF (3 mL) and DMF (3 mL) was added HBTU (446 mg, 1.18 mmol) and *N*,*N*-diisopropylethylamine (0.56 mL, 3.21 mmol). The mixture was stirred at ambient temperature for 1 h, after which *O*-cyclopropylmethylhydroxylamine hydrochloride (200 mg, 1.61 mmol) was added and the mixture was stirred for a further 48 h. The mixture was then concentrated *in vacuo*, diluted with EtOAc (20 mL), washed with water (4 x 15 mL), dried over Na_2SO_4, and the organic extract was evaporated to dryness to give a coloured residual material. The material was suspended in CH_2Cl_2 (5 mL), filtered and the filtrate was loaded directly onto a silica chromatography column, which was then eluted with CH_2Cl_2 to give the title compound as a pale yellow solid (90 mg, 23%); m.p. 101-103 °C. ^{1}H NMR (300 MHz, D_6-DMSO) δ 0.18 (m, 2H), 0.47 (m, 2H), 1.04 (m, 1H), 3.58 (d, J = 6.9 Hz, 2H), 6.85 (m, 1H), 7.10 (m, 1H), 7.16 (m, 1H), 7.37 (m, 2H), 8.75 (s, 1H), 11.76 (s, 1H); MS (ES^+) calcd for $C_{17}H_{15}ClF_3N_2O_2$ (MH^+) 371.08, found 370.94.

3,4-Difluoro-2-(2-fluoro-4-iodophenylamino)-*N*-(2-hydroxyethoxy)benzamide;[2,3] **9**



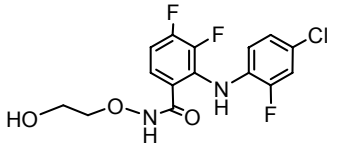
A solution of 3,4-difluoro-2-(2-fluoro-4-iodophenylamino)benzoic acid (500 mg, 1.27 mmol) in THF (3 mL) and DMF (3 mL) was added HBTU (529 mg, 1.40 mmol) and *N*,*N*-diisopropylethylamine (0.22 mL, 1.27 mmol). The mixture was stirred at ambient temperature for 1 h, after which *O*-hydroxyethylhydroxylamine (195 mg, 2.54 mmol) and *N*,*N*-diisopropylethylamine (0.45 mL, 2.54 mmol) were added and the mixture was stirred for a further 48 h. The mixture was then concentrated *in vacuo* and loaded directly onto a silica chromatography column, which was then sequentially eluted with CH_2Cl_2 and 10% v/v MeOH in CH_2Cl_2 to give a buff solid (180 mg). The sample was recrystallised from CH_2Cl_2–hexane to afford the title compound as an off-white solid (100 mg, 28%); m.p. 126-128 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 3.69 (t, J = 4.8 Hz, 2H), 3.92 (t, J = 4.8 Hz, 2H), 6.74 (m, 1H), 7.00 (m, 1H), 7.16 (m, 1H), 7.31 (dd, J = 2.2 and 10.8 Hz, 1H), 7.38 (m, 1H); MS (ES^+) calcd for $C_{15}H_{13}ClF_3N_2O_3$ (MH^+) 452.99, found 452.77.

2-(4-Bromo-2-fluorophenylamino)-3,4-difluoro-*N*-(2-hydroxyethoxy)benzamide;[(2)] 10



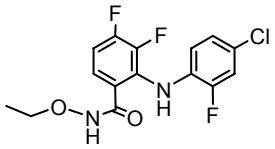
A solution of 2-(4-bromo-2-fluorophenylamino)-3,4-difluorobenzoic acid (500 mg, 1.23 mmol) in THF (3 mL) and DMF (3 mL) was added HBTU (514 mg, 1.23 mmol) and *N*,*N*-diisopropylethylamine (0.21 mL, 1.23 mmol). The mixture was stirred at ambient temperature for 1 h, after which *O*-hydroxyethylhydroxylamine (189 mg, 2.46 mmol) and *N*,*N*-diisopropylethylamine (0.43 mL, 2.46 mmol) were added and the mixture was stirred for a further 48 h. The mixture was then concentrated *in vacuo* and loaded directly onto a silica chromatography column, which was then sequentially eluted with CH_2Cl_2 and 10% v/v MeOH in CH_2Cl_2 to give a solid. The solid material was recrystallised from CH_2Cl_2–hexane to afford the title compound as a pale pink solid (140 mg, 28%); m.p. 138-140 °C. ^{1}H NMR (300 MHz, D_6-DMSO) δ 3.53 (br s, 2H), 3.80 (br s, 2H), 6.78 (m, 1H), 7.18 (m, 2H), 7.36 (m, 1H), 7.48 (dd, J = 2.1 and 10.9 Hz, 1H), 8.68 (s, 1H), 11.83 (s, 1H); MS (ES^+) calcd for $C_{15}H_{13}BrF_3N_2O_3$ (MH^+) 405.01 and 407.00, found 404.83 and 406.82.

2-(4-Chloro-2-fluorophenylamino)-3,4-difluoro-*N*-(2-hydroxyethoxy)benzamide;[(3)] 11



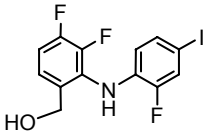
A solution of 2-(4-chloro-2-fluorophenylamino)-3,4-difluorobenzoic acid (500 mg, 1.66 mmol) in THF (5 mL) was added 1,1'-carbonyl diimidazole (538 mg, 3.32 mmol). The mixture was stirred at ambient temperature for 1 h, after which *O*-hydroxyethylhydroxylamine (511 mg, 6.64 mmol) was added and the mixture was stirred for a further 18 h. The mixture was evaporated to dryness. The residual material was dissolved in EtOAc (20 mL), washed with 2 M aqueous HCl (10 mL), water (4 x 15 mL), dried over Na_2SO_4, and the organic extract was evaporated to dryness to give a coloured oil, which was purified by silica chromatography column, using EtOAc as eluent, to afford the title compound as an off-white solid (306 mg, 51%); m.p. 144-146 °C. ^{1}H NMR (300 MHz, D_6-DMSO) δ 3.53 (br s, 2H), 3.81 (br s, 2H), 4.68 (br s, 1H), 6.85 (m, 1H), 7.06-7.19 (m, 2H), 7.40 (m, 2H), 8.70 (s, 1H), 11.83 (s, 1H); MS (ES^+) calcd for $C_{15}H_{13}ClF_3N_2O_3$ (MH^+) 361.06, found 360.95.

2-(4-Chloro-2-fluorophenylamino)-*N*-ethoxy-3,4-difluorobenzamide;[3] 13



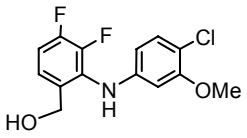
A solution of 2-(4-chloro-2-fluorophenylamino)-3,4-difluorobenzoic acid (500 mg, 1.66 mmol) in THF (2.5 mL) and DMF (2.5 mL) was added HBTU (691 mg, 1.82 mmol) and *N*,*N*-diisopropylethylamine (0.28 mL, 1.66 mmol). The mixture was stirred at ambient temperature for 1 h, after which *O*-ethylhydroxylamine hydrochloride (322 mg, 3.32 mmol) and *N*,*N*-diisopropylethylamine (0.57 mL, 3.32 mmol) were added and the mixture was stirred for a further 48 h. The mixture was concentrated *in vacuo* to give a thick oil, which was dissolved in CH_2Cl_2 (5 mL) and then purified by silica chromatography, using CH_2Cl_2 as eluent, to give a pale yellow solid. The solid material was recrystallised from CH_2Cl_2–hexane to afford the title compound as a pale yellow solid (152 mg, 27%); m.p. 104-106 °C. ^{1}H NMR (300 MHz, D_6-DMSO) δ 1.12 (t, J = 7.0 Hz, 3H), 3.80 (q, J = 7.2 Hz, 2H), 6.85 (m, 1H), 7.06-7.20 (m, 2H), 7.39 (m, 2H), 8.72 (s, 1H), 11.74 (s, 1H); MS (ES^+) calcd for $C_{15}H_{13}ClF_3N_2O_2$ (MH^+) 345.06, found 345.01.

(3,4-Difluoro-2-(2-fluoro-4-iodophenylamino)phenyl)methanol; 14



A solution of 3,4-difluoro-2-(2-fluoro-4-iodophenylamino)benzoic acid (1.00 g, 2.54 mmol) in THF (10 mL) and DMF (10 mL) was added HBTU (1.06 g, 2.79 mmol) and *N*,*N*-diisopropylethylamine (0.44 mL, 2.54 mmol). The mixture was stirred at ambient temperature for 1 h, after which *N*-hydroxysuccinimide (586 mg, 5.09 mmol) and *N*,*N*-diisopropylethylamine (0.88 mL, 5.08 mmol) were added and the mixture was stirred for a further 48 h. The mixture was concentrated *in vacuo* to give a thick oil, which was dissolved in CH_2Cl_2 (5 mL) and then purified by silica chromatography, using CH_2Cl_2 as eluent, to give succinimidyl 3,4-difluoro-2-(2-fluoro-4-iodophenylamino)benzoate as an off-white solid (440 mg, 35%). A sample of the isolated solid (424 mg, 0.86 mmol) was dissolved in a mixture of THF (10 mL) and water (2.5 mL). To the solution was added portionwise sodium borohydride (163 mg, 4.3 mmol) and the resultant mixture was refluxed for 6 h, cooled to ambient temperature, and allowed to stand overnight. Water (20 mL) and EtOAc (20 mL) were added followed by 2 M aqueous HCl (20 mL). The organic phase was extracted, washed with brine (30 mL) and concentrated *in vacuo* to give a pink oily material, which was purified by silica column chromatography, using EtOAc–hexane as eluent, to afford the title compound as a pale pink solid (181 mg, 56%); m.p. 78-79 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.95 (br s, 1H), 4.65 (s, 2H), 6.39 (m, 1H), 6.50 (s, 1H), 6.95 (m, 1H), 7.07 (m, 1H), 7.26 (m, 1H), 7.39 (dd, J = 2.1 and 10.6 Hz, 1H); HR-ToF MS (ES^+) calcd for $C_{13}H_{10}F_3INO$ (MH^+) 379.9754, found 379.9833.

(2-(4-Chloro-3-methoxyphenylamino)-3,4-difluorophenyl)methanol; 15



A solution of 2-(4-chloro-3-methoxyphenylamino)-3,4-difluorobenzoic acid (400 mg, 1.28 mmol) in THF (10 mL) was added 1,1'-carbonyl diimidazole (415 mg, 2.56 mmol). The mixture was stirred at ambient temperature for 1 h, after which *N*-hydroxysuccinimide (369 mg, 3.20 mmol) was added and the mixture was stirred for a further 16 h. The mixture was evaporated to dryness to give a thick coloured oil, which was purified by silica column chromatography, using hexane–diethyl ether as eluent, to give succinimidyl 2-(4-chloro-3-methoxyphenylamino)-3,4-difluorobenzoate as a brown solid (330 mg, 63%). A sample of the isolated solid (300 mg, 0.73 mmol) was dissolved in a mixture of THF (7 mL) and water (2 mL). To the solution was added sodium borohydride (139 mg, 3.65 mmol) and the resultant mixture was stirred for 6 h. To the mixture was then added water (20 mL) and EtOAc (20 mL), and the organic phase was extracted with brine (30 mL) and evaporated to dryness to give a coloured solid, which was purified by silica column chromatography to yield the title compound as a purple solid (98 mg, 45%); m.p. 96-97 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.82 (br s, 1H), 3.83 (s, 3H), 4.64 (s, 2H), 6.27 (d, J = 8.5 Hz, 1H), 6.38 (s, 1H), 6.92 (m, 1H), 7.05 (m, 1H), 7.17 (d, J = 8.2 Hz, 1H); HR-ToF MS (ES^+) calcd for $C_{14}H_{13}ClF_2NO_2$ (MH^+) 300.0597, found 300.0583.

Fluorescence polarization assay for MEK1

To determine the binding of chemical reagents to the human MEK1 recombinant protein, a fluorescence polarization assay was employed. The assay is based on competition between the fluorescent tracer [N^{α}-(fluorescein-5-carbonyl)-N^{ϵ}-(3,4-difluoro-2-(2-fluoro-4-iodophenyl amino)benzoyl)-L-lysyl amide **4**] and increasing amounts of competing compound, determined as fluorescence polarization intensity (mP). Binding of different concentrations of the fluorescein-tagged reagent **4** to increasing amounts of MEK1 recombinant protein was first analysed. The fluorescent tracer **4** binding to MEK1 showed a K_d 485 nM. Negative controls were also performed using ERK1 recombinant protein, albumin and 5-carboxyfluorescein (5-FAM; Merck Chemicals Ltd.).

Test compounds **6–15** were initially dissolved in DMSO to obtain 10 mM stock solutions and stored at –20 °C. All the compounds were soluble in DMSO at 10 mM concentration. Dilutions of the test compounds were then freshly prepared, prior to the assay, as a 10–100x stocks of their final assay concentrations in aqueous Tris buffered saline (TBS) pH 7.5. The competition assays for a panel of chemical reagents were carried out with the 2 nM fluorescent tracer **4**, 500 nM MEK1 with increasing concentration of the competing ligands **6–15** in TBS.

Each assay sample was performed in a total volume of 150.0 µl, by adding 7.5 µl MEK1 (10 µM), 2.0 µl fluorescent tracer **4** (150 nM), 2.0–75.0 µl challenge compound and finally an appropriate volume of TBS. The mixture was then incubated in the dark at the room temperature for 45 min. Samples of each reaction (35 µl) were pippetted in triplicate into wells of black opaque 384 ShallowWell plates. Fluorescence polarization was measured using a Wallace Envision instrument (PerkinElmer), using the inbuilt FITC FP mirrors and filters (λ_{ex} = 480 nm; λ_{em} = 535 nm). One background well was included containing assay buffer (TBS) alone and the background correction for mP values was automatically calculated by the Envision data analysis software. Polarization was calculated according to the standard equation: $P = (V - H)/(V + H)$, in which P denotes polarization, V denotes vertical emission intensity and H represents horizontal emission intensity. The inhibitory concentration 50% (IC_{50}) was calculated using Prism™ statistical software (version 5).

The inhibitor dissociation constant (K_i) was calculated as described by the Cheng-Prusoff equation(4): $IC_{50} = K_i(1 + [\text{tracer ligand}]/K_{d(\text{tracer ligand})})$. Thus, under our experimental conditions, $K_i = IC_{50}/(1 + (0.002/0.485)) = IC_{50}/1.00412$ µM.

(4) Cheng, Y-C & Prusoff, W.H. (1973) *Biochem. Pharmacol.*, **22**, 3099–3108.

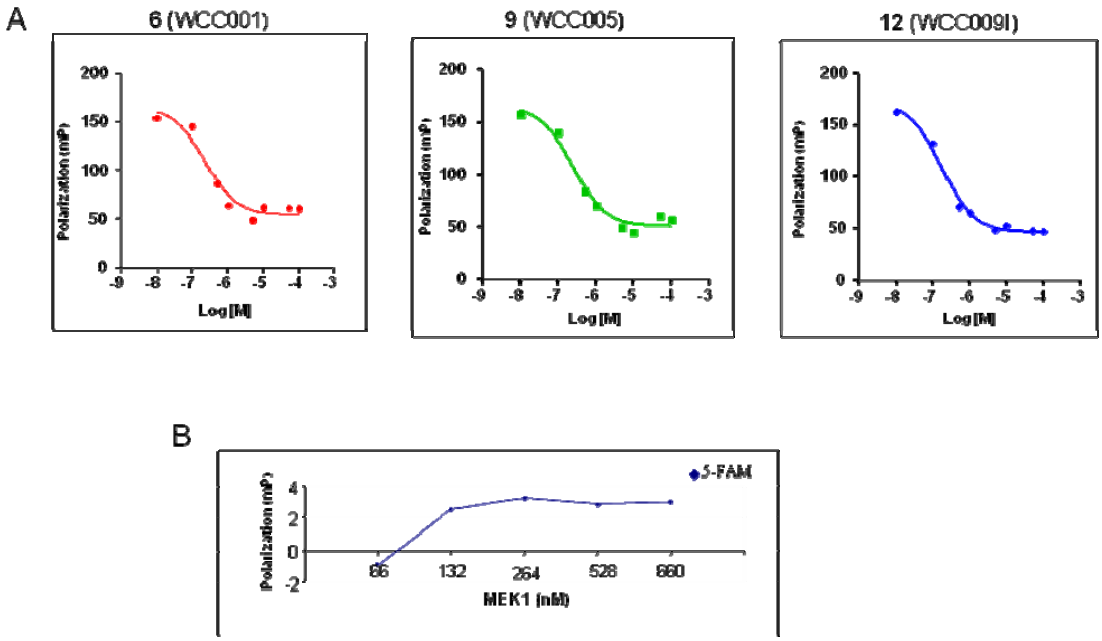


Figure S4 (**A**) Examples of competitive inhibition of the fluorescent tracer **4**–MEK1 interaction determined using fluorescent polarization. (**B**) Control experiment showing that the FP of 5-FAM (2 nM) was unaffected by increasing concentrations of MEK1.

Inhibition of ERK1 phosphorylation in TPA-stimulated HEK293T cells by the MEK1 inhibitors

To assess the effect of PD184352 analogues on ERK1 phosphorylation, the HEK293 were plated at 2 x 10^5 cells per well in a 12 well plate and serum starved for 16 h. The cells were pre-treated with 0.5, 1 and 2 µM of each inhibitor for 1.5 h at 37 °C incubation, followed by stimulation with 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 200 nM) for 10 or 30 min at 37 °C incubation. In order to assess the basal level of phospo-ERK1 and the possible effect of DMSO untreated cells, TPA treated and DMSO+TPA treated cells were also prepared as controls. Following stimulation, the cells were immediately lysed by pippeting up & down in ice-cold buffer containing protease and phosphatase inhibitors (20 mM Tris, 1 mM EGTA, 0.1% TritonX-100, 1 mM NaF, 10 mM β-glycerophosphate, protease inhibitor cocktail, Roche) and the plate was left on gentle shaking for 1 h at 4 °C. Cell lysate from each well (150 µM) was loaded on SDS-PAGE gel (5–20%), and followed by immunoblotting with mouse monoclonal ERK1/2 (p44/42); Cell Signalling#9107) and rabbit monoclonal phospho-p44/42 antibody (Cell signalling#4377). Infrared-dye labelled secondary anti-mouse (LI-COR#926-32220) and anti-rabbit (LI-COR#926-32211) were used. Membranes were imaged with the LI-COR Odyssey Infrared Imaging System.

To determine the effect of MEK1 inhibitors on SAPK/JNK, the HEK293 cell were plated out at 2 x 10^5 cells per well in a 12 well plate and serum starved for 16 h. Cells were pre-treated with 2 µM of MEK1 inhibitors for 1.5 h and stimulated with 500 ng mL^{-1} anisomycin for 10 minutes at 37 °C incubation. The cells were then lysed and analyzed on SDS-PAGE as described above. Mouse monoclonal phospho- SAPK/JNK antibody (Cell signaling#9255)

and rabbit polyclonal SAPK/JNK antibody (Cell signalling#9252) were utilized. Infrared-dye labelled secondary antibody and Infrared Imaging System was used as described before.

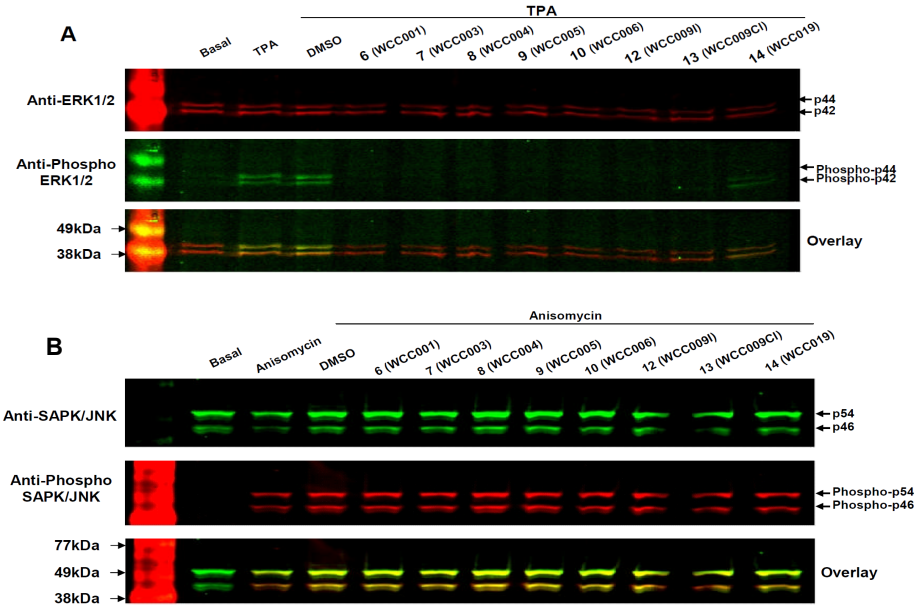


Figure S5 (**A**) Inhibition of ERK1/2 phosphorylation by eight MEK1/2 inhibitors (2 μM) in TPA stimulated (200 nM) HEK293 cells, ERK1 (p44) and ERK2 (p42). (**B**) The lysate from anisomysin stimulated (500 ng mL^{-1}) HEK293 cells treated with eight MEK1 inhibitors (2 μM) and immunoblotted using total and phospho-JNK antibodies. The inhibitors showed no effect on JNK phosphorylation. Phospho-SAPK/JNK mouse mAb detects endogenous levels of p46 and p54 SAPK/JNK dually phosphorylated at Thr183 and Tyr185.

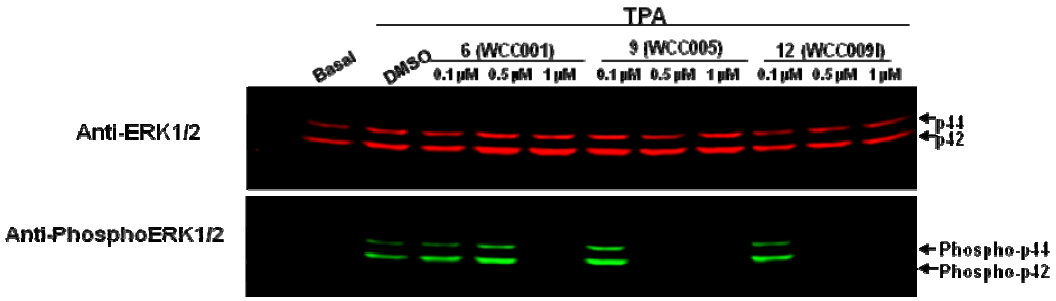


Figure S6 Inhibition of cellular ERK1/2 phosphorylation using lower concentrations (0.1, 0.5 and 1.0 μM) of MEK1/2 inhibitors **6**, **9** and **12**.

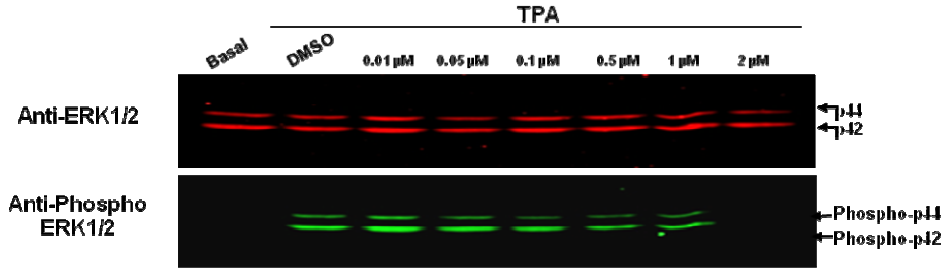


Figure S7 The fluorescent tracer **4**-mediated cellular inhibition of MEK1/2, resulting in a reduced phosphorylation of ERK1/2. The HEK293 cells were stimulated by TPA for 30 min.