Supplementary Material

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Chemistry General Procedures

Commercially available reagents and solvents (HPLC grade) were used without further purification. The following abbreviations have been used: tetrahydrofuran (THF), dichloromethane (DCM), trifluoroacetic acid (TFA), ethyl acetate (EtOAc), carbonyl diimidazole (CDI), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), dimethylaminopyridine (DMAP), 1-methyl-2-pyrrolidinone (NMP), sodium triacetoxyborohydride (STAB), dimethylformamide (DMF). Solvents were removed using a Buchi rotary evaporator. Purification of compounds by flash chromatography column was performed using silica gel, particle size 40–63 µm (230-400 mesh) obtained from Fluorochem. Purification of compounds by preparative HPLC was performed on Gilson systems using reverse phase Axia™ prep Luna C18 columns (10µ, 100 x 21.2 mm), gradient 0-100% B (A = water / 0.05% TFA, B = acetonitrile / 0.05% TFA) over 10min, flow = 25 mL min^{-1}, UV detection at 254 nm.

1H NMR spectra were recorded on a Bruker 300 MHz AV spectrometer in deuterated solvents. Chemical shifts δ □are in parts per million. Thin-layer chromatography (TLC) analysis was performed with Kieselgel 60 F254 (Merck) plates and visualized using UV light.

Analytical HPLC/MS was performed on an Agilent HP1100 LC system using reverse phase Luna C18 columns (3 µm, 50 x 4.6 mm), gradient 5-95% B (A = water / 0.1% Formic acid, B = acetonitrile / 0.1% Formic acid) over 2.25 min, flow = 2.25 mL min^{-1}. UV spectra were recorded at 220 and 254 nm using a G1315B DAD detector. Mass spectra were obtained over the range m/z 150 to 800 on a LC/MSD SL G1956B detector. Data were integrated and reported using ChemStation and ChemStation Data Browser software.

All compounds that were evaluated in biological assays had >95% purity using the HPLC methods described above.

6-Amino-1-(2,6-difluorophenyl)-5-[(2,4-difluorophenyl)carbonyl]pyridin-2(1H)-one (**1**). The synthesis of compound 1 is described in WO03/076405.[14]

tert-Butyl (3, 5-difluoro-4-nitrophenyl)acetate (**7**). A mixture of potassium tert-butoxide (12.3 g, 111.0 mmol) in NMP (100 mL) was cooled to -20°C under nitrogen. A mixture of 2, 6-difluoronitrobenzene (6) (5.0 g, 31.43 mmol) and tert-butylchloroacetate (7.6 mL, 53.11 mmol) in NMP (100 mL) was added slowly at -10 °C to -20 °C over 1.5h. After 1.5h the reaction was quenched by pouring into 2M HCl (120 mL) and ice, then heptane (300 mL) was added. The mixture was stirred for 10 minutes, separated and the aqueous extracted with heptane (2 x 400 mL). The organic layer was washed with brine twice, dried ($MgSO_4$), filtered and washed with heptane. The solution was concentrated *in vacuo* and the residue purified by column chromatography (3-4% EtOAc / Heptane) to provide the title compound as an orange oil (4.34 g, 53% yield). 1H NMR (300 MHz, $CDCl_3$) δ: 7.06 (2H, d, J=8.7Hz), 3.59 (2H, s), 1.48 (9H, s).

(3, 5-Difluoro-4-nitrophenyl) acetic acid (**8**). To a solution of compound **7** (4.34 g, 15.88 mmol) in DCM (10 mL), at 0°C, was added TFA (10 mL). The reaction was warmed to room temperature and stirred for 1.5h. The reaction was concentrated *in vacuo*, slurried in heptane (10 mL), filtered and dried to provide the title compound as an orange solid (2.95 g, 86% yield). 1H NMR (300 MHz, d6-DMSO) δ: 7.45 (2H, d, J=9.6Hz), 3.79 (2H, s).

2-(3,5-Difluoro-4-nitrophenyl)ethanol (**9**). A solution of compound **8** (2.95 g, 13.59 mmol) in THF (30 mL), under nitrogen, was cooled to 0°C and a solution of BH_3Me_2S in THF (10.2 mL, 20.38 mmol) was added dropwise over 5 minutes. The mixture was warmed to room temperature and stirred for 4.5h. The reaction was cooled to 0°C and quenched with methanol (10 mL). The mixture was concentrated *in vacuo* and the residue purified by column chromatography (30-60% EtOAc/Heptane) to provide the title compound as an oil (2.45 g, 89% yield). 1H NMR (300 MHz, $CDCl_3$) δ: 7.03 (2H, d, J=9.3 Hz), 3.97-3.91 (2H, q, J=5.4, 5.7 Hz), 2.93 (2H, t, J=6.2 Hz), 1.52 (1H, t, J=5.0 Hz).

2-(4-Amino-3,5-difluorophenyl)ethanol (**10**). To a solution of compound **9** (2.45 g, 12.06 mmol) in EtOAc (50 mL) was added Pd/C (0.8g). The mixture was stirred under an atmosphere of H_2 for 19h, filtered and concentrated *in vacuo* to provide the title compound as a pale brown solid (2.15 g, 100% yield). 1H NMR (300 MHz, $CDCl_3$) δ: 6.70-6.67 (2H, m), 3.82 (2H, t, J=6.5 Hz), 2.76 (2H, t, J=6.5 Hz).

2-(4-{[1-Amino-3-(2,4-difluorophenyl)-3-oxoprop-1-en-1-yl]amino}-3,5-difluorophenyl)ethyl acetate (**12**). To a mixture of 3-amino-3-[(4-chlorophenyl)thio]-1-(2,4-difluorophenyl)prop-2-en-1-one hydrochloride (**11**, prepared using methods described in WO 2003/076405) (3.99 g, 11.1 mmol) in acetic acid (20 mL) was added 2-(4-amino-3, 5-difluorophenyl) ethanol (compound **7**) (2.00 g, 11.6 mmol) and the mixture heated at 80°C for 20h. The mixture was cooled, concentrated *in vacuo* and the residue triturated in diethyl ether to provide a solid. The solid was partitioned between EtOAc and sat $NaHCO_3$, washed with brine, dried ($MgSO_4$) and concentrated *in vacuo* to provide the title compound as a solid (2.91 g, 67% yield). LC/MS: m/z 397 $[M+H]^+$.

2-{4-[6-Amino-5-(2,4-difluorobenzoyl)-2-oxopyridin-1(2H)-yl]-3,5-difluorophenyl}ethyl acetate (**13**). To a solution of CDI (1.78 g, 10.98 mmol) in THF (36 mL), under nitrogen at 0°C, was added dropwise propiolic acid (675 µl, 10.98 mmol). The mixture was warmed to room temperature and stirred for 1.5h. A solution of compound **12** (2.9 g, 7.32 mmol) in THF (18 mL) was added dropwise and the mixture heated at 80°C for 5h. The mixture was cooled, concentrated *in vacuo* and the residue purified twice by column chromatography (0.7-1% methanol / DCM) to provide the title compound as a solid (1.20 g, 37% yield). 1H NMR (300 MHz, $CDCl_3$) δ: 7.49-7.39 (2H, m), 7.09-6.90 (4H, m), 5.93 (1H, d, J=9.9 Hz), 4.37 (2H, t, J=6.4 Hz), 3.06 (2H, t, J=6.6 Hz), 2.10 (3H, s).

6-Amino-5-(2,4-difluorobenzoyl)-1-[2,6-difluoro-4-(2-hydroxyethyl)phenyl]pyridin-2(1H)-one (**14**). To a mixture of compound **13** (1.1 g, 2.45 mmol) in 6N aq HCl (50 mL) was heated at reflux for 24h. The mixture was cooled, filtered and

washed with water. The precipitate was partitioned between EtOAc and sat. aq $NaHCO_3$, the organic layer further washed with brine, dried ($MgSO_4$) and concentrated *in vacuo* to provide the title compound as a solid (993 mg, 100% yield). 1H NMR (300 MHz, $CDCl_3$) δ: 7.49-7.39 (2H, m), 7.15-6.90 (4H, m), 5.92 (1H, d, J=9.6 Hz), 4.00-3.85 (2H, m), 2.95 (2H, t, J=6.0 Hz).

{4-[6-Amino-5-(2,4-difluorobenzoyl)-2-oxopyridin-1(2H)-yl]-3,5-difluorophenyl}acetaldehyde (**15**). To a mixture of compound **14** (500 mg, 1.23 mmol) in DCM (20 mL) was added Dess-Martin periodinane (783 mg, 1.85 mmol). The mixture was stirred for 3.5h, sat. aq. $Na_2S_2O_3$ (20 mL) and sat. $NaHCO_3$ (20 mL) were added and the mixture stirred vigorously for 30 minutes. The organic layer was separated and the aqueous extracted with DCM. The organic layer was washed with brine, dried ($MgSO_4$) and concentrated to provide the title compound as a solid (497 mg, 100% yield). 1H NMR (300 MHz, $CDCl_3$) δ: 9.88 (1H, s), 7.49-7.40 (2H, m), 7.12-6.91 (4H, m), 5.93 (1H, d, J=9.9 Hz), 3.89 (2H, s).

Cyclopentyl N-(2-{4-[6-amino-5-(2,4-difluorobenzoyl)-2-oxopyridin-1(2H)-yl]-3,5-difluorophenyl}ethyl)-L-leucinate (**2**). To a solution of compound **15** (46 mg, 0.114 mmol) in THF (2 mL) was added cyclopentyl L-leucinate (40 mg, 0.201 mmol, prepared by methods described in WO 2009/060160),[15] stirred for 30 minutes, before the addition of sodium triacetoxyborohydride (80 mg, 0.377 mmol). The reaction was stirred for 24hr, diluted with EtOAc and the organic washed with sat $NaHCO_3$, brine, dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by column chromatography (0.75-1.25% MeOH / DCM), and then purified by preparative HPLC to provide the title compound (29 mg, 31% yield). LC/MS: m/z 588 $[M+H]^+$. 1H NMR (300 MHz, CD_3OD) δ: 7.57-7.48 (2H, m), 7.32-7.10 (4H, m), 5.84 (1H, d, J=9.6 Hz), 5.41-5.30 (1H, m), 4.10-4.03 (1H, m), 3.45-3.30 (2H, m), 3.20-3.14 (2H, m), 2.05-1.60 (11H, m), 1.10-0.95 (6H, m).

(4S)-4-[(tert-Butoxycarbonyl)amino]-5-(cyclopentyloxy)-5-oxopentanoic acid (**17**). To a solution of Boc-L-Glu(OBzl)-OH (compound **16**) (15 g, 44.5 mmol) in dichloromethane (220 mL) in an ice bath, was added cyclopentanol (4.8 mL, 53.3 mmol), EDC (9.4 g, 48.9 mmol) and DMAP (543 mg, 4.4 mmol). The reaction mixture was allowed to warm to room temperature and stirred for 12 hours for complete reaction. The reaction mixture was diluted with DCM (200 mL) and washed with 1M HCl, 1M Na_2CO_3 and brine. The organic layer was then dried over magnesium sulphate and evaporated under reduced pressure. The product was purified by column chromatography using ethyl acetate/heptane (1:4) to give 12.4 g, 69% yield of title compound as a white solid. 1H NMR (300 MHz, $CDCl_3$), δ: 7.38 (5H, m), 5.70 (1H, m), 5.10 (2H, s), 5.05 (1H, m), 4.25 (1H, m), 2.47 (2H, m), 2.15 (1H, m), 1.95-1.55 (9H, bm), 1.47 (9H, s).

Cyclopentyl N-(tert-butoxycarbonyl)-5-hydroxy-L-norvalinate (**18**). Compound **17** (12.4 g, 30.5 mmol) was dissolved in EtOAc (200 mL) and purged with nitrogen before addition of 20% $Pd(OH)_2$ on carbon catalyst (1.3 g). The reaction flask was then purged with hydrogen gas for a period of 5 minutes before leaving under a balloon of hydrogen for 5 hours for complete reaction. The catalyst was removed by filtration, washing with 50 mL EtOAc and the combined mother liquors were evaporated under reduced pressure. The title compound was isolated as a clear oil (7.73 g, 85%) and required no further purification. 1H NMR (300 MHz, $CDCl_3$), δ: 10.0 (1H, bs), 5.70 (2H, m), 4.28 (1H, m), 2.47 (2H, m), 2.15 (1H, m), 1.95-1.55 (9H, bm), 1.47 (9H, s).

Cyclopentyl (2S)-5-hydroxy-2-[(tert-butoxycarbonyl)amino] pentanoate (**19**).

Ethyl chloroformate (2.45 mL, 25.6 mmol) was added at -20°C to a stirred solution of compound **18** (6.73 g, 21.4 mmol) and N-methyl morpholine (3.05 mL, 27.8 mmol) in THF (50 mL). The reaction mixture became very thick with precipitation of a white solid. The reaction was therefore diluted further with THF (100 mL) to aid mixing and left stirring at -20°C for 2 hours. The precipitated solid was filtered off and the filtrate was added over a period of 20 minutes to a solution of sodium borohydride (2.43 g, 64.1 mmol) in THF (20 mL) and water (5 mL) at 0°C. The reaction mixture was allowed to stir to room temperature and left for 4 hours for complete reaction. The mixture was acidified to pH 5 with 1M HCl and the THF removed under reduced pressure. The aqueous solution was extracted with EtOAc (3 × 100 mL) and dried over magnesium sulphate. The product was purified by column chromatography (DCM-5%MeOH / DCM) and isolated as a clear oil (5.0 g, 78%). 1H NMR (300 MHz, $CDCl_3$), δ: 5.20 (2H, m), 4.25 (1H, m), 3.65 (2H, m), 2.00-1.57 (12H, bm), 1.47 (9H, s).

Cyclopentyl (2S)-5-bromo-2-[(tert-butoxycarbonyl)amino] pentanoate (**20**).

To a slurry of N-bromo succinimide (3.54 g, 19.9 mmol) in DCM (30 mL) was added a solution of triphenyl phosphine (4.87 g, 18.8 mmol) in DCM (15 mL). The solution was stirred for a further 5 minutes before addition of pyridine (644µl, 7.96 mmol) and a solution of compound **19** (2.0 g, 6.64 mmol) in DCM (20 mL). The solution was stirred for 18h, concentrated *in vacuo* and the residual solvent azeotroped with toluene (3 × 30 mL). The residue was triturated with diethyl ether (30 mL) and ethyl acetate:heptane (1:9, 2 × 30 mL). The combined ether and ethyl acetate / heptane solution was concentrated onto silica and purified by column chromatography using ethyl acetate/heptane (1:9 – 2:8) to provide 1.34g (55% yield) of title compound as a clear oil. 1H NMR (300 MHz, $CDCl_3$), δ: 5.25 (1 H, m), 5.05 (1H, bd), 3.45 (2H, m), 2.00-1.55 (12H, bm), 1.45 (9H, s).

Cyclopentyl (S)-5-{4-[6-Amino-5-(2,4-difluorobenzoyl)-2-oxo-2H-pyridin-1-yl]-3,5-difluorophenoxy}-2-tert-butoxycarbonylaminopentanoate (**22**). To a stirred mixture of 6-amino-5-(2,4-difluorobenzoyl)-1-(2,6-difluoro-4-hydroxy-phenyl)-1H-pyridin-2-one (compound **21**) [prepared by methods described in WO 2003/076405] (100 mg, 0.265 mmol) and K_2CO_3 in DMF (1.5 mL) was added compound **16** (96 mg, 0.265 mmol). The reaction mixture was stirred at 60°C for 2h. LCMS shows disappearance of the starting phenol, product (54%) and impurity (17%). The reaction mixture was diluted with EtOAc (15 mL) and washed sequentially with sat. aq. $NaHCO_3$ (3 mL) and water (10 mL). The EtOAc layer was dried over Na_2SO_4, filtered and

concentrated to dryness. Purification by flash chromatography (20% EtOAc / heptane) yielded the desired product as a white solid (50mg, 29%). LCMS purity 100%, m/z 662 $[M+H]^+$, 1H NMR (300 MHz, CD_3OD), δ: 1.45 (9H, s),1.60-2.10 (12H, m), 4.05-4.15 (3H, m), 5.15-5.25 (1H, m), 5.75 (1H, d), 6.85-6.95 (2H, m), 7.10-7.20 (2H, m), 7.40-7.60 (2H, m).

Cyclopentyl-(S)-2-Amino-5-{4-[6-amino-5-(2,4-difluorobenzoyl)-2-oxo-2H-pyridin-1-yl]-3,5-difluorophenoxy}pentanoate (**3**). A mixture of compound **22** (10 mg) and 20% TFA/ DCM (0.5 mL) was allowed to stand at ambient temperature for 3 h. The reaction mixture was concentrated to dryness under reduced pressure. The residue was triturated with Et_2O (2 mL) to give a white precipitate (9.3mg, 91%). LCMS purity 98%, m/z 562 $[M+H]^+$, 1H NMR (300 MHz, CD_3OD), δ: 1.65-2.25 (12H, m), 4.15-4.25 (3H, m), 5.35-5.45 (1H, m), 5.85 (1H, d,), 6.90-7.00 (2H, m), 7.15-7.25 (2H, m), 7.50-7.65 (2H, m).

N-[2-(4-{6-Amino-5-[(2,4-difluorophenyl)carbonyl]-2-oxopyridin-1(2H)-yl}-3,5-difluorophenyl)ethyl]-L-leucine (**4**). Compound **4** was synthesised as described in WO 2009/060160 (Example 67). LCMS purity 99% m/z 520 [M+H]+, 1H NMR (400 MHz, CD_3OD), δ: 7.57-7.48 (2H, m), 7.32-7.14 (4H, m), 5.84 (1H, d, J=9.6Hz), 3.95-3.85 (1H, m), 3.45-3.32 (2H, m), 3.21-3.15 (2H, m), 1.95-1.65 (3H, m), 1.05 (6H, t, J=6.2Hz)

5-(4-{6-Amino-5-[(2,4-difluorophenyl)carbonyl]-2-oxopyridin-1(2H)-yl}-3,5-difluorophenoxy)-L-norvaline (**5**). Compound **5** was synthesised as described in WO 2007/129036 (Example 12). LCMS purity 97%, m/z 494 [M+H]+, 1H NMR (400 MHz, CD_3OD), δ: 1.80-2.10 (4H, m), 3.90-4.00 (1H, m), 4.00-4.10 (2H, m), 5.65 (1H, d), 6.75-6.80 (2H, m), 6.95-7.05 (2H, m), 7.30-7.45 (2H, m).

Abbreviations

hCE1/2, human carboxylesterase 1/2; TNF-α, tumor necrosis factor α; AA, amino acid; BT MOPS, Bis-Tris (Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAP, Mitogen-activated protein; MAPKAPK2, MAP kinase-activated protein kinase 2; h, hours.

References

14 C. Alonso-Alija, M. Michels, H. Schirok, K-H Schlemmer, J. Bell, M.F. Fitzgerald, S. Dodd, A. Gill, WO/2003/076405

15 D.F.C. Moffat, S. Pintat, S. Davies,. WO/2009/060160

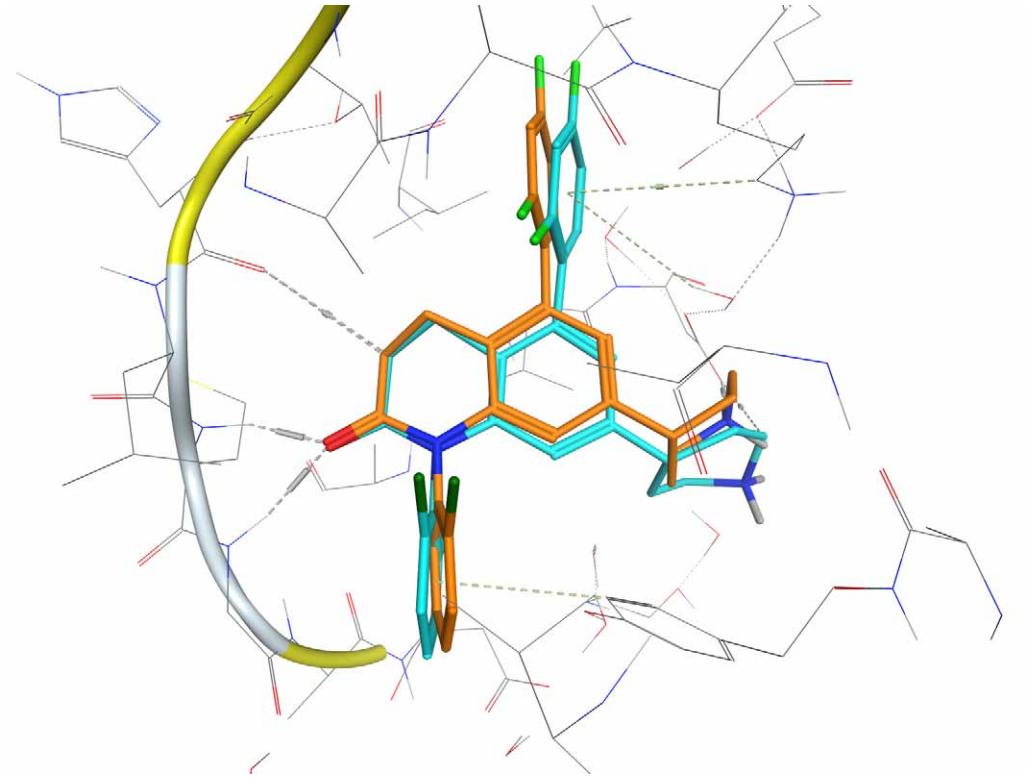

Fig. S1 Overlay of experimental (cyan) and predicted (orange) binding modes of the ligand from crystal structure 1OVE

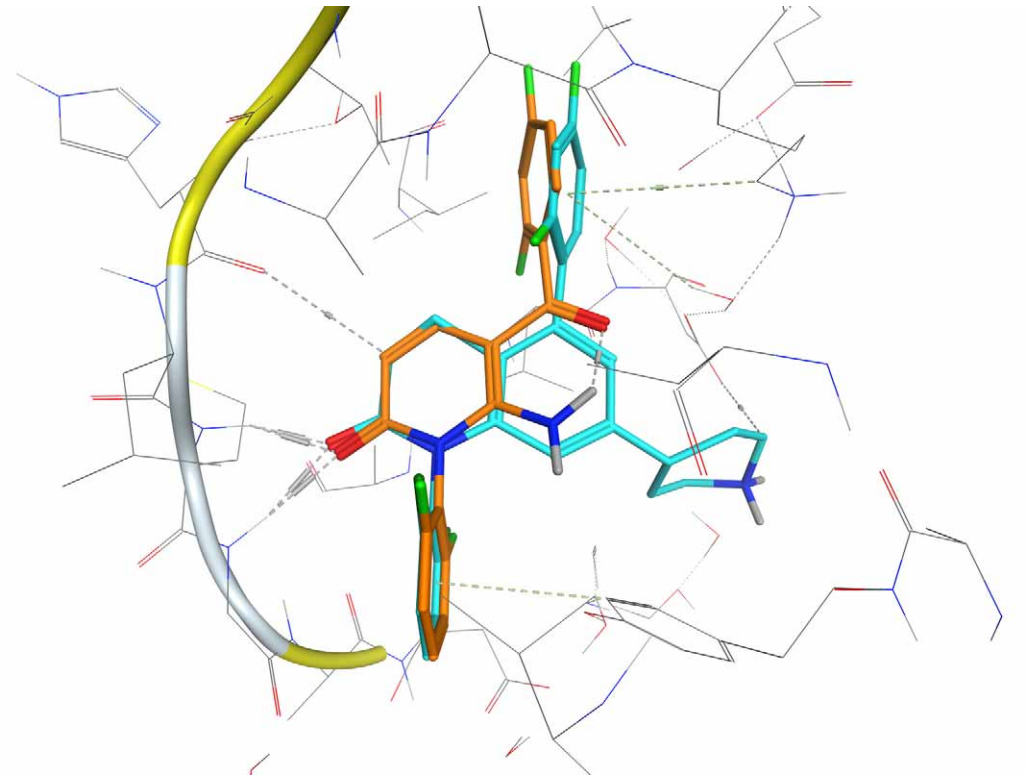

Fig. S2 Overlay of the predicted binding mode of 1 (orange) on crystal structure 1OVE (cyan ligand)

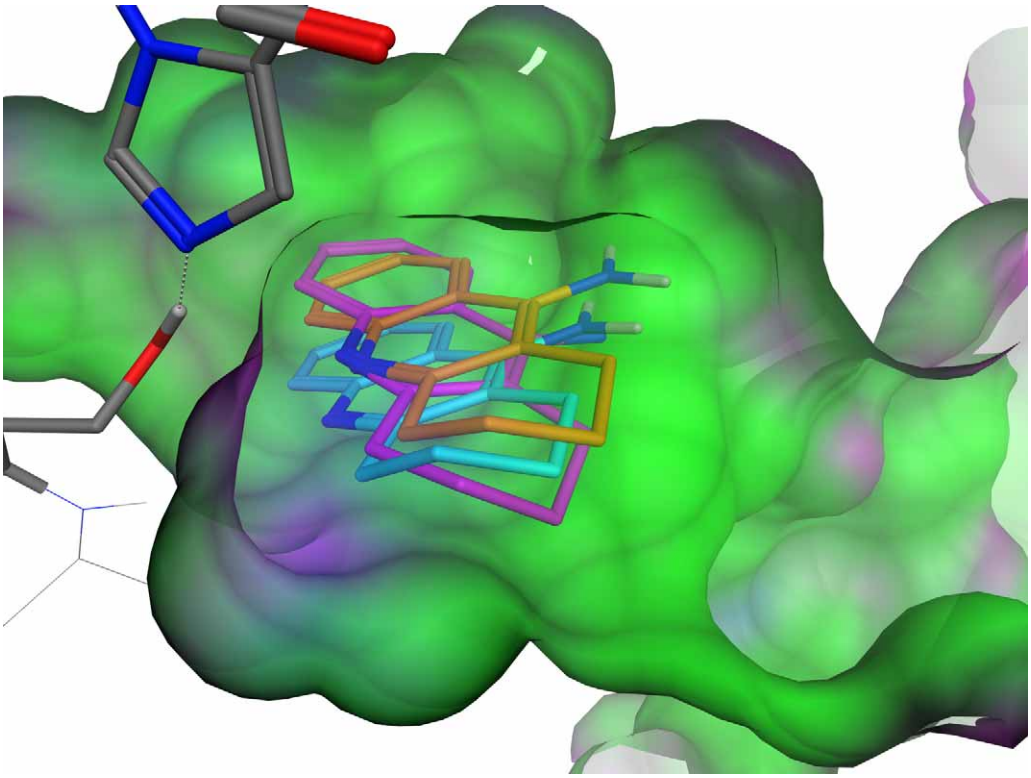

Fig. S3 Observed (magenta) and predicted binding of tacrine in hCE1. Docking with water 19 present : orange, without water 19 : cyan

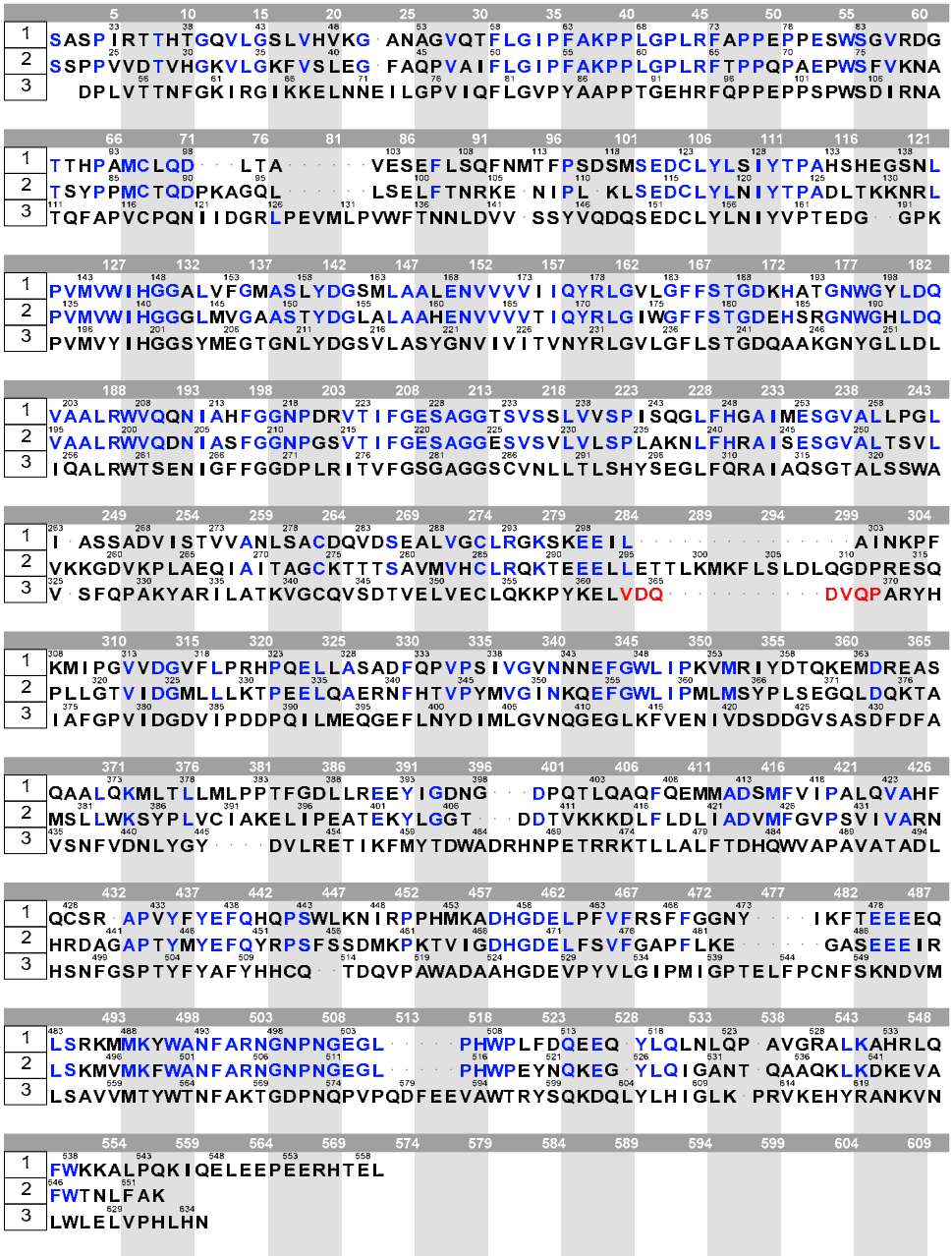

Fig. S4 Sequence alignment of hCE2 (chain 1), 1MX1 (chain 2) and 3B3Q (chain 3)

Blue residues show sequence identity between hCE1 and hCE2. Red residues indicate the region in which crystal structure 3B3Q was used as an alternative template.