Isoform-selective ATAD2 chemical probe with novel chemical structure and unusual mode of action

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SUPPORTING INFORMATION

METHODS

Chemistry

General Comments.

Chemical names and numbers of the compounds are given in italics and in bold, respectively. Chemical names were generated using ACD/Name Batch or Autonom 2000, following IUPAC nomenclature. Further details can be found in WO 2017/093272 A1.

Analytical Methods.

All NMR spectroscopy was recorded on Bruker Avance III HD spectrometers. $^1$H spectra were obtained at 500 MHz and referenced to residual solvent signals (2.50 ppm for DMSO-d6, ppm for CD$_3$OD and ppm for CDCl$_3$). All spectra were obtained at ambient temperature (22 +/- 1 °C). Peak forms and multiplicities are specified as apparent in the spectra; potential higher-order effects have not been considered. Chemical shifts (δ) are reported in parts per million (ppm), and coupling constants (J) in hertz (Hz). Spin multiplicities are reported as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, bs = broad singlet and mc = centered multiplet.

Chromatographic Method.

Analytical liquid chromatography–mass spectrometry (LC-MS) were performed using UPLC-MS on a MS instrument type: Micromass ZQ; HPLC instrument type: Waters Alliance 2695; UV PDA 2996 Waters detector; column: Waters SunFire C18 2.1x50mm, 3.5µm; mobile phase A: 10 mM formic acid in water, mobile phase B: acetonitrile; gradient: 0.0 min 100% A → 3.1 min
Reagents.

All reagents for which the synthesis is not described below are either commercially available or were synthesized according to literature procedures. All final products were at least 95% pure, as determined by analytical HPLC.

Synthesis of BAY-850

5-Bromo-2-chloro-4-hydroxybenzonitrile 2

To a stirred solution of 2-chloro-4-hydroxybenzonitrile 1, 5.0 g (32.5 mmol) in acetonitrile (100 mL) was added dropwise trifluoromethanesulfonic acid, 3.17 mL (35.8 mmol, 1.1 eq.) at -30 °C. After 10 min at -30 °C, N-bromosuccinimide, 8.11 g (45.6 mmol, 1.4 eq.) was added and the resulting mixture was stirred at r.t. for 18 h. Most of the solvent was removed under reduced pressure and the residue was partitioned between aq. sat. sodium hydrogen carbonate and ethyl acetate. The layers were separated and the aqueous phase was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulfate, filtered and concentrated. The residue was purified by flash column chromatography on silica gel (eluent: cyclohexane/ethyl acetate, 4/1) to give the desired product, 1.26 g (16%) as a white solid, and a mixture fraction containing 50% of the desired product together with the starting material and a regioisomer, 1.13 g (8%) as a white solid. LC-MS: Rt = 3.03 min; MS (ES-): m/z = 230/232 (M-H-). \(^1\)H-NMR (500 MHz, CD\(_3\)OD) δ [ppm] = 7.06 (s, 1H), 7.93 (s, 1H).

5-Bromo-2-chloro-4-hydroxybenzoic acid 3
A stirred solution of 5-bromo-2-chloro-4-hydroxybenzonitrile 2, 1.20 g (5.16 mmol) in ethanol (6 mL) and aq. 30% potassium hydroxide (18 mL) was stirred at 100 °C for 18 h. After cooling to 0 °C, the mixture was acidified with aq. 10% hydrochloric acid and the resulting solid was filtered, washed with water and dried under reduced pressure to give the product, 1.06 g (81%) as a white solid. LC-MS: Rt = 2.62 min; MS (ES-): m/z = 249/251 (M-H)-. 1H-NMR (500 MHz, CD3OD) δ [ppm] = 7.00 (s, 1H), 8.10 (s, 1H).

**Methyl 5-bromo-2-chloro-4-methoxybenzoate 4**

To a stirred solution of 5-bromo-2-chloro-4-hydroxybenzoic acid 3, 700 mg (2.78 mmol) in N,N-dimethylformamide (10 mL) was added potassium carbonate, 1.15 g (8.35 mmol, 3 eq.) followed by methyl iodide, 0.38 mL (6.12 mmol, 2.2 eq.) at r.t. and the resulting mixture was stirred at r.t. for 4 h. The mixture was poured into water, the resulting solid was filtered, washed with water and dried under reduced pressure to give the product, 715 mg (92%) as a white solid. LC-MS: Rt = 3.51 min; no ionisation. 1H-NMR (500 MHz, CDCl3) δ [ppm] = 3.91 (s, 3H), 3.95 (s, 3H), 6.94 (s, 1H), 8.14 (s, 1H).

**Methyl 2-chloro-5-(5-formyl-2-furyl)-4-methoxybenzoate 5**

To a stirred solution of methyl 5-bromo-2-chloro-4-methoxybenzoate 4, 250 mg (0.894 mmol), 5-formylfuran-2-ylboronic acid, 188 mg (1.34 mmol, 1.5 eq.) and [1,1'-bis(diphenylphosphino)ferrocene] dichloropalladium(II), complex with dichloromethane, 110 mg (0.134 mmol, 15 mol%) in degassed tetrahydrofuran (6 mL) was added a solution of sodium carbonate, 190 mg (1.79 mmol, 2.0 eq.) in degassed water (3 mL) and the resulting mixture was stirred at 50 °C for 18 h. After cooling to r.t., the solvent was removed under reduced pressure, water and ethyl acetate were added and the mixture was filtered through Celite. The phases were
separated and the aqueous layer was extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over sodium sulfate, filtered and concentrated to give the crude product 5, 225 mg (84%) as a brown solid. LC-MS: Rt = 3.34 min; MS (ES+): m/z = 295/297 (M+H)+. 1H-NMR (500 MHz, CDCl₃) δ [ppm] = 3.94 (s, 3H), 3.95 (s, 3H), 7.07 (s, 1H), 7.12 (d, 1H), 7.32 (d, 1H), 8.56 (s, 1H), 9.69 (s, 1H).

Methyl 2-chloro-4-methoxy-5-[[1R]-1-(4-methylphenyl)ethyl]amino] methyl)-2-furyl]benzoate 6

To a stirred solution of methyl 2-chloro-5-(5-formyl-2-furyl)-4-methoxybenzoate 5, 223 mg (0.757 mmol) in dichloromethane (8 mL) were added (R)-1-(4-Methylphenyl)ethylamine, 0.12 mL (0.832 mmol, 1.1 eq.) followed by acetic acid, 0.22 mL (3.78 mmol, 5 eq.) and the mixture was stirred at r.t. for 30 min. Sodium triacetoxyborohydride, 240 mg (1.13 mmol, 1.5 eq.) was added in one portion and the resulting mixture was stirred at r.t. for 4 h. The mixture was poured into aq. sat. sodium hydrogen carbonate and extracted with dichloromethane (3x). The combined organic extracts were washed with brine, dried over sodium sulfate, filtered and concentrated. The residue was purified by flash column chromatography on silica gel (eluent: dichloromethane/methanol, 98:2) to give the product 6, 110 mg (35%) as a brown oil. LC-MS: Rt = 2.55 min; MS (ES+): m/z = 414/416 (M+H)+. 1H-NMR (500 MHz, CDCl₃) δ [ppm] = 1.36 (d, 3H), 2.35 (s, 3H), 3.64 (d, 1H), 3.74 (d, 1H), 3.83 (q, 1H), 3.95 (s, 3H), 3.98 (s, 3H), 6.21 (d, 1H), 6.85 (d, 1H), 6.99 (s, 1H), 7.16 (d, 2H), 7.25 (d, 2H), 8.35 (s, 1H).

2-Chloro-4-methoxy-5-[[1R]-1-(4-methylphenyl)ethyl]amino]methyl)-2-furyl]benzoic acid 7
To a stirred solution of methyl 2-chloro-4-methoxy-5-[5-({(1R)-1-(4-methylphenyl)-ethyl}amino)methyl]-2-furyl]benzoate 6, 110 mg (0.266 mmol) in a mixture tetrahydrofuran/methanol/water (3 mL, 1/1/1) was added lithium hydroxide monohydrate, 22 mg (0.532 mmol, 2 eq.) at 0 °C and the resulting mixture was stirred at r.t. for 18 h. The volatiles were removed under reduced pressure, water was added and the mixture was acidified to pH ~ 7 by dropwise addition of aq. 1N hydrochloric acid. The resulting solid was filtered, washed with water and dried to give the crude product 7, 73 mg (69%) as a beige solid. LC-MS : Rt = 2.40 min; MS (ES+): m/z = 400/402 (M+H)+. 1H-NMR (500 MHz, DMSO-d6) δ [ppm] = 1.24 (d, 3H), 2.28 (s, 3H), 3.51 (d, 1H), 3.59 (d, 1H), 3.74 (q, 1H), 3.99 (s, 3H), 6.32 (d, 1H), 6.87 (d, 1H), 7.14 (d, 2H), 7.23 (d, 2H), 7.26 (s, 1H), 8.20 (s, 1H).

4-[(2R)-2-Amino-3-(1,3-dioxo-1,3-dihydro-2H-isooindol-2-yl)propyl]benzonitrile hydrochloride (1:1) 12

A yellow solution of di-tert-butyl azodicarboxylate, 2.7 g (11.7 mmol, 1.2 eq.) in THF (20 mL) was added dropwise at 0 °C into a solution of triphenylphosphine, 3.08 g (11.7 mmol, 1.2 eq.) in THF (90 mL). The resulting solution was stirred for 2 min at 0 °C, then phthalimide, 1.72 g (11.7 mmol, 1.2 eq.) was added in 3 portions, followed by a solution of tert-butyl [(2R)-1-(4-cyanophenyl)-3-hydroxypropan-2-yl]carbamate 11, 2.7 g (9.8 mmol, 1.0 eq.) in THF (20 mL). The resulting yellow solution was stirred at r.t. for 17 h. The reaction medium was concentrated in vacuum and the residue was dry loaded into a silica gel column (12 cm diameter x 20 cm height) using DCM and eluted (cyclohexane / ethyl acetate 9/1 > 6/4). The pure fractions were combined to provide tert-butyl [(2R)-1-(4-cyanophenyl)-3-(1,3-dioxo-1,3-dihydro-2H-isooindol-2-yl)propan-2-yl]carbamate, 3.4 g (86%). LC-MS : Rt = 3.46 min; MS (ES+): m/z = 406 (M+H)+, 350 ([M-tBu]+H)+, 306 ([M-Boc]+H)+, 1H-NMR (500 MHz, DMSO-d6) δ [ppm] =
1.10 (s, 9H), 2.77 (dd, 1H), 2.93 (dd, 1H), 3.65 (d, 2H), 4.01 (m, 1H), 6.88 (d, 1H), 7.42 (d, 2H), 7.72 (d, 2H), 7.79-7.88 (m, 4H).

To a solution of tert-butyl [(2R)-1-(4-cyanophenyl)-3-(1,3-dioxo-1,3-dihydro-2H isoindol-2-yl)propan-2-yl]carbamate, 3.4 g (8.4 mmol) in dichloromethane (110 mL) was added drop wise hydrochloric acid, 14.7 mL (4M in 1,4-dioxane, 59 mmol, 7 eq.) at 0 °C and the resulting mixture was stirred at r.t. for 20 h. LCMS analysis showed complete conversion. The volatiles were removed under reduced pressure, then the residue was co-evaporated two times with dichloromethane, triturated in MTBE, evaporated and dried under high vacuum at 45 °C to give 4-[(2R)-2-amino-3-(1,3-dioxo-1,3-dihydro-2H isoindol-2-yl)propyl]benzonitrile hydrochloride (1:1), 3.0 g (quant. yield) as a white solid. ¹H-NMR (500 MHz, DMSO-d6) δ [ppm] = 2.99 (dd, 1H), 3.24 (dd, 1H), 3.68 (dd, 1H), 3.76 (dd, 1H), 7.53 (d, 2H), 7.76 (d, 2H), 7.84 (s, 4H), 8.43 (br s, 3H).

2-Chloro-N-[(2R)-1-(4-cyanophenyl)-3-(1,3-dioxo-1,3-dihydro-2H isoindol-2-yl)propan-2-yl]-4-methoxy-5-[5-([(1R)-1-(4-methylphenyl)ethyl]amino)methyl]-2-furyl]benzamide 8

To a stirred solution of 2-chloro-4-methoxy-5-[(1R)-1-(4-methylphenyl)ethyl]amino)methyl]-2-furyl]benzoic acid, 230 mg (0.575 mmol) in tetrahydrofuran (5.8 mL) were added TBTU, 194 mg (0.604 mmol, 1.05 eq.) and triethylamine, 0.24 mL (1.73 mmol, 3 eq.) at r.t. After 20 min, 4-[(2R)-2-amino-3-(1,3-dioxo-1,3-dihydro-2H isoindol-2-yl)propyl]benzonitrile hydrochloride 12, 206 mg (0.604 mmol, 1.05 eq.) was added and the resulting mixture was stirred at r.t. for 2 h. Most of the solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate (60 mL). The organic layer was washed successively with aq. sat. sodium hydrogen carbonate, water (2x), brine, dried over
sodium sulfate and concentrated to give 2-chloro-N-[(2R)-1-(4-cyanophenyl)-3-(1,3-dioxo-1,3-
dihydro-2H-isoindol-2-yl)propan-2-yl]-4-methoxy-5-[5-[(1R)-1-(4-methylphenyl)ethyl]amino]
methyl]-2-furyl]benzamide 8, 364 mg (89% yield) as a brown solid. LC-MS: Rt = 2.65 min; MS
(ES+): m/z = 687/689 (M+H)+. 1H-NMR (500 MHz, DMSO-d6) δ [ppm] = 1.28 (d, 3H), 2.24
(s, 3H), 2.87 (dd, 1H), 3.12 (dd, 1H), 3.62 (d, 1H), 3.66 (d, 1H), 3.76-3.85 (m, 3H), 3.90 (s, 3H),
4.57 (m, 1H), 6.33 (d, 1H), 6.84 (d, 1H), 7.06 (s, 1H), 7.11 (d, 2H), 7.27 (d, 2H), 7.46 (s, 1H),
7.50 (d, 2H), 7.73 (d, 2H), 7.76-7.78 (m, 2H), 7.85-7.86 (m, 2H), 8.39 (d, 1H).

N-[(2R)-1-Amino-3-(4-cyanophenyl)propan-2-yl]-2-chloro-4-methoxy-5-[5-[(1R)-1-(4-
methylphenyl)ethyl] amino]methyl]-2-furyl]benzamide 9

To a stirred solution of 2-chloro-N-[(2R)-1-(4-cyanophenyl)-3-(1,3-dioxo-1,3-dihydro-2H-
isoindol-2-yl)propan-2-yl]-4-methoxy-5-[5-[(1R)-1-(4-methylphenyl)ethyl]amino]methyl]-2-
furyl]benzamide, 362 mg (0.527 mmol) in absolute ethanol (8 mL) was added hydrazine, 2.6 mL
(1M in tetrahydrofuran, 2.63 mmol, 5 eq.) and the mixture was stirred at 70 °C for 4 h. The
solvent was then removed in vacuum, the residue was triturated in dichloromethane and filtered.
The filtrate was concentrated and the residue (306 mg, orange oil) was purified by flash column
chromatography on silica gel (eluent: dichloromethane/7N ammonia in methanol, 98/2) to give
the product 9, 254 mg (79%) as an orange solid. LC-MS: Rt = 2.17 min; MS (ES+): m/z =
557/559 (M+H)+. 1H-NMR (500 MHz, CD3OD) δ [ppm] = 1.37 (d, 3H), 2.29 (s, 3H), 2.80 (dd,
1H), 2.85-2.90 (m, 2H), 3.10 (dd, 1H), 3.66 (m, 2H), 3.80 (q, 1H), 3.97 (s, 3H), 4.36 (m, 1H),
6.30 (d, 1H), 6.88 (d, 1H), 7.11 (s, 1H), 7.14 (d, 2H), 7.24 (d, 2H), 7.49 (d, 2H), 7.62 (d, 2H),
7.68 (s, 1H).
Tert-Butyl-(cis-4-{{(2R)-2-[(2-chloro-4-methoxy-5-[[1R]-1-(4-methylphenyl)-
ethyl]amino]methyl}-2-furyl]benzoyl]amino}-3-(4-cyanophenyl)propyl]amino)cyclo-
hexyl)carbamate 10

To a stirred solution of N-[(2R)-1-aminom-3-(4-cyanophenyl)propan-2-yl]-2-chloro-4-
methoxy-5-{{[(1R)-1-(4-methylphenyl)ethyl]amino}methyl}-2-furyl]benzamide 9, 100 mg
(0.18 mmol) in dichloromethane (2 mL) was added 4-N-Boc-aminocyclohexanone, 50 mg (0.233
mmol, 1.3 eq.) followed by acetic acid, 51 µL (0.90 mmol, 5 eq.) and the mixture was stirred at
r.t. for 30 min. Sodium triacetoxyborohydride, 57 mg (0.27 mmol, 1.5 eq.) was added in one
portion and the resulting mixture was stirred at r.t. for 18h. The mixture was poured into aq. sat.
sodium hydrogen carbonate and extracted with dichloromethane (3x). The combined organic
extracts were washed with brine, dried over sodium sulfate, filtered and concentrated. The
residue (257 mg, orange oil) was purified by preparative TLC (eluent: dichloromethane/7N
ammonia in methanol, 97:3) to give the two disatereomers:

Dia #1 product 10, 45 mg (32%) as an orange solid; LC-MS : Rt = 2.37 min; MS (ES+): m/z =
754/756 (M+H)+. $^1$H-NMR (500 MHz, DMSO-d6) δ [ppm] = 1.25 (d, 3H), 1.37 (s, 9H), 1.40-
1.46 (m, 4H), 1.51-1.58 (m, 4H), 2.26 (s, 3H), 2.60-2.64 (m, 3H), 2.77 (dd, 1H), 3.11 (dd, 1H),
3.29 (m, 1H), 3.53 (d, 1H), 3.59 (d, 1H), 3.72 (q, 1H), 3.94 (s, 3H), 4.22 (m, 1H), 6.31 (d, 1H),
6.63 (br d, 1H), 6.86 (d, 1H), 7.11 (d, 2H), 7.16 (s, 1H), 7.24 (d, 2H), 7.48 (d, 2H), 7.51 (s, 1H),
7.74 (d, 2H), 8.25 (d, 1H).

Dia #2, 48 mg (35%) as an orange solid; LC-MS : Rt = 2.31 min; MS (ES+): m/z = 754/756
(M+H)+. $^1$H-NMR (500 MHz, DMSO-d6) δ [ppm] = 0.97-1.06 (m, 2H), 1.11-1.18 (m, 2H), 1.25
(d, 3H), 1.37 (s, 9H), 1.72-1.75 (m, 2H), 1.81-1.86 (m, 2H), 2.26 (s, 3H), 2.31 (m, 1H), 2.63-2.67
N-[(2R)-1-[(4-aminocyclohexyl)amino]-3-(4-cyanophenyl)propan-2-yl]-2-chloro-4-methoxy-5-[5-[(1R)-1-(4-methylphenyl)ethyl]amino{methyl}-2-furyl] benzamide BAY-850

To a stirred suspension of tert-butyl-(4-{[(2R)-2-({2-chloro-4-methoxy-5-[5-((1R)-1-(4-methylphenyl)ethyl]amino{methyl}-2-furyl}benzoyl]amino}-3-(4-cyanophenyl)propyl]amino}cyclohexyl)carbamate, dia #1 10, 44 mg (58 µmol) in dichloromethane (0.5 mL) was added slowly hydrochloric acid, 0.15 mL (4M in 1,4-dioxane, 0.58 mmol, 10 eq.) at 0 °C and the resulting mixture was stirred at r.t. for 48h. The volatiles were removed under reduced pressure and the residue was triturated in methyl tert-butyl ether, filtered, washed with methyl tert-butyl ether and dried under reduced pressure. The impure product obtained was treated with aq. sat. sodium hydrogen carbonate and ethyl acetate. The aqueous phase was extracted using ethyl acetate. The combined organics were dried over sodium sulfate and concentrated. The residue was purified by preparative TLC (eluent: dichloromethane/methanol, 96:4) to give the product, 16 mg (41%) as a beige solid. LC-MS: Rt = 2.03 min; MS (ES+): m/z = 654/656 (M+H)+. 1H-NMR (500 MHz, DMSO-d6) δ [ppm] = 1.25 (d, 3H), 1.38-1.47 (m, 5H), 1.50-1.60 (m, 3H), 2.26 (s, 3H), 2.57 (m, 1H), 2.63-2.66 (m, 2H), 2.71 (m, 1H), 2.78 (dd, 1H), 3.09 (dd, 1H), 3.53 (d, 1H), 3.60 (d, 1H), 3.73 (q, 1H), 3.94 (s, 3H), 4.23 (m, 1H), 6.31 (d, 1H), 6.86 (d, 1H), 7.11 (d, 2H), 7.17 (s, 1H), 7.24 (d, 2H), 7.48 (d, 2H), 7.51 (s, 1H), 7.74 (d, 2H), 8.25 (d, 1H).

Synthesis of BAY-460
**Ethyl 5-amino-2-chlorobenzoate 14**

To a stirred solution of 5-amino-2-chlorobenzoic acid 13, 20 g (85% purity, 99 mmol) in dry ethanol (400 mL) was added thionyl chloride, 14.5 mL (198 mmol, 2.0 eq.) over 30 min at 0 °C. The resulting suspension was then allowed to warm to r.t., stirred for 1 h and then heated at 90 °C for 3 h. After cooling to r.t., the mixture was concentrated in vacuum and the resulting purple slurry was partitioned between ethyl acetate and water. The layers were separated and the aqueous phase was washed with ethyl acetate. The aqueous acidic phase was basified with solid sodium hydrogen carbonate and extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over sodium sulfate and concentrated under reduced pressure to yield the product, 18.0 g (91%) as a purple oil. LC-MS: Rt = 2.95 min; MS (ES+): m/z = 200 (M+H)+, 241 ([M+MeCN]+H)+. 1H-NMR (500 MHz,CDCl3) δ [ppm] = 1.39 (t, 3H), 4.37 (q, 2H), 6.71 (dd, 1H), 7.10 (d, 1H), 7.19 (d, 1H).

**Ethyl 2-chloro-5-(5-formylfuran-2-yl)benzoate 15**

A mixture of ethyl 5-amino-2-chlorobenzoate, 18.0 g (90 mmol) in aq. hydrochloric acid, 150 mL (1.5N, 225 mmol, 2.5 eq.) was heated to 80 °C for 1 h. The solution was then cooled to 0 °C and sodium nitrite, 6.53 g (95 mmol, 1.05 eq.) in water (20 mL) was added slowly. After 2 h at 0 °C, the mixture was filtered and the filtrate was treated with 2-furfuraldehyde, 7.5 mL (42.1 mmol). An aqueous solution of cupric chloride, 2.42 g (18 mmol, 0.2 eq.) in water (10 mL) was then added drop wise at r.t. and the resulting mixture was stirred at r.t. for 18 h. The mixture was then extracted with ethyl acetate and the combined organic phase was washed with brine, dried over sodium sulfate and concentrated under reduced pressure to give the product 15, 18.9 g (74%) as a dark brown oily solid. LC-MS: Rt = 3.46 min; MS (ES+): m/z = 279/281 (M+H)+.
\[^1\text{H-NMR}\ (500 \text{ MHz, CDCl}_3) = 1.44 \ (t, \ 3\text{H}), 4.45 \ (q, \ 2\text{H}), 6.89 \ (d, \ 1\text{H}), 7.33 \ (d, \ 1\text{H}), 7.53 \ (d, \ 1\text{H}), 7.84 \ (dd, \ 1\text{H}), 8.21 \ (d, \ 1\text{H}), 9.68 \ (s, \ 1\text{H}).\]

**Ethyl 2-chloro-5-\{[\{1R\}-1-(4-methylphenyl)ethyl\}amino\}methyl\}furan-2-yl\}benzoate 16**

To a stirred solution of ethyl 2-chloro-5-(5-formylfuran-2-yl)benzoate 15, 2.50 g (8.97 mmol) in dichloromethane (83 mL) at r.t. was added \((R)-(+)\)-1-(4-methylphenyl)ethylamine, 1.58 mL (10.8 mmol, 1.2 eq.). The mixture was stirred at r.t. 1 h, and then sodium triacetoxyborohydride, 2.85 g (13.46 mmol, 1.5 eq.) was added in three portions over a 4 h period. The mixture was stirred at r.t. overnight. The mixture was poured into aq. sat. sodium hydrogen carbonate and the layers were separated. The aqueous phase was extracted with DCM (2x) and the combined organic extracts were dried over sodium sulfate, filtered and the solvent was removed under reduced pressure. The residue was purified by flash column chromatography on silica gel (Companion, 80 g column, cyclohexane / ethyl acetate 1/0 to 1/1) to give the product 16, 2.42 g (64%) as brown oil. LC-MS: Rt = 2.57 min; MS (ES+): m/z = 398/400 (M+H). \[^1\text{H-NMR}\ (500 \text{ MHz, CDCl}_3) \delta [ppm] = 1.38 \ (d, \ 3\text{H}), 1.43 \ (t, \ 3\text{H}), 1.75 \ (br \ s, \ 1\text{H}), 2.35 \ (s, \ 3\text{H}), 3.64 & 3.72 \ (2 \ d, \ AB, \ 2\text{H}), 3.82 \ (q, \ 1\text{H}), 4.44 \ (q, \ 2\text{H}), 6.21 \ (d, \ 1\text{H}), 6.61 \ (d, \ 1\text{H}), 7.16 \ (d, \ 2\text{H}), 7.25 \ (d, \ 2\text{H}), 7.43 \ (d, \ 1\text{H}), 7.64 \ (dd, \ 1\text{H}), 8.03 \ (d, \ 1\text{H}).\]

**2-Chloro-5-\{[\{1R\}-1-(4-methylphenyl)ethyl\}amino\}methyl\}2-furyl\}benzoic acid 17**

To a stirred solution of ethyl 2-chloro-5-[5-(\{1R\}-1-(4-methylphenyl)ethyl\}amino\}methyl\}furan-2-yl\}benzoate, 1.0 g (2.5 mmol) in a mixture tetrahydrofuran/methanol/water (24 mL, 1/1/1) was added lithium hydroxide monohydrate, 211 mg (5.0 mmol, 2 eq.) at 0 °C and the resulting mixture was stirred at r.t. for 2.5 h. The volatiles were removed under reduced pressure, water (20 mL) was added and the mixture was acidified
to pH ~ 7 by dropwise addition of aq. 1N hydrochloric acid. The resulting solid was filtered, washed with water, THF, MTBE and dried under reduced pressure to give the product, 0.77 g (79%) as a light brown solid. LC-MS: Rt = 2.41 min; MS (ES+): m/z = 370/372 (M+H)+. 1H-NMR (500 MHz, CD3OD): 1.68 (d, 3H), 2.37 (s, 3H), 4.04 & 4.18 (2 d, AB, 2H), 4.37 (q, 1H), 6.59 (d, 1H), 6.80 (d, 1H), 7.31 (d, 2H), 7.37 (d, 2H), 7.38 (d, 1H), 7.58 (dd, 1H), 7.82 (d, 1H).

(3S)-3-Amino-4-(4-cyanophenyl)-N-methylbutanamide 19

To a stirred solution of (3S)-3-(tert-butoxycarbonylamino)-4-(4-cyanophenyl)butanoic acid 18, 3.0 g (9.86 mmol) in tetrahydrofuran (100 mL) at r.t. was added TBTU, 3.17 g (9.86 mmol) followed by triethylamine, 2.75 mL (19.7 mmol, 2 eq.). After 30 min, methylamine, 5.91 mL (2N in tetrahydrofuran, 11.8 mmol, 1.2 eq.) was added, and the resulting solution was stirred at r.t. for 16 h. More TBTU, 633 mg (0.2 eq) was added, and after 20 min of stirring at r.t., methylamine, 2.0 mL (2N in tetrahydrofuran, 3.9 mmol, 0.4 eq.) was added. The mixture was stirred at r.t. for 4 h. Most of the solvent was removed under reduced pressure and the residue was triturated in ethyl acetate (75 mL), filtered, washed with ethyl acetate (10 mL) and then with pentane. The solid was dried under high vacuum to give the tert-butyl [(2S)-1-(4-cyanophenyl)-4-(methylamino)-4-oxobutan-2-yl]carbamate, 2.9 g (93%) as a white solid. LC-MS: Rt = 2.77 min; MS (ES+): m/z = 318 (M+H)+, 262 ([M-tBu]+H)+, 218 ([M-Boc]+H)+. 1H-NMR (500 MHz, DMSO-d6) δ [ppm] = 1.28 (s, 9H), 2.23 (m, 2H), 2.55 (d, 3H), 2.69 (dd, 1H), 2.81 (dd, 1H), 3.97 (m, 1H), 6.73 (d, 1H), 7.35 (d, 2H), 7.73 (m, 3H).

To a stirred suspension of tert-butyl [(2R)-1-(4-cyanophenyl)-4-(methylamino)-4-oxobutan-2-yl]carbamate, 4.11 g (12.9 mmol) in dichloromethane (170 mL) was added slowly hydrochloric acid, 32 mL (4N in dioxane, 129 mmol, 10 eq.) at r.t. and the resulting mixture was stirred at r.t.
for 16 h. The resulting suspension was cooled at 0 °C, 80 mL of aq. sat. sodium hydrogen carbonate was slowly added, followed by solid potassium carbonate until basic pH was obtained. The aqueous phase was saturated with sodium chloride, then extracted using a DCM / MeOH mixture (95:5, 5 x 100 mL). The combined organics were dried over sodium sulfate, concentrated and dried to provide the product 19 as an off white solid, 2.60 g (92%). LC-MS: Rt = 0.44 min; MS (ES+): m/z = 218 (M+H)+. 1H-NMR (500 MHz, CDCl3) δ [ppm] = 2.20 (dd, 1H), 2.39 (dd, 1H), 2.74 (dd, 1H), 2.80 (d, 3H), 2.88 (dd, 1H), 3.48 (m, 1H), 6.68 (br s, 1H), 7.32 (d, 2H), 7.61 (d, 2H).

2-Chloro-N-[(2S)-1-(4-cyanophenyl)-4-(methylamino)-4-oxobutan-2-yl]-5-[5-([(1R)-1-(4-methylphenyl)ethyl] amino)methyl]furan-2-yl]benzamide BAY-460

To a stirred suspension of 2-chloro-5-[5-([(1R)-1-(4-methylphenyl)ethyl]amino)methyl]-2-furyl]benzoic acid 17, 100 mg (0.27 mmol) in tetrahydrofuran (2.7 mL) were added triethylamine, 75 µL (0.54 mmol, 2 eq.) followed by TBTU, 87 mg (0.27 mmol) at r.t. After 15 min (light trouble solution), (3S)-3-amino-4-(4-cyanophenyl)-N-methylbutanamide 19, 71 mg (0.32 mmol, 1.2 eq.) was added in one portion and the resulting mixture was stirred at r.t. for 2 h (light trouble solution). Most of the solvent was removed under reduced pressure and the residue was partitioned between dichloromethane and aq. sat. sodium hydrogen carbonate. The layers were separated and the aqueous phase was extracted with dichloromethane. The combined organic extracts were dried over sodium sulfate, filtered and the solvent was removed under reduced pressure. The solid obtained was triturated in diethyl ether, filtered, washed with diethyl ether and dried under reduced pressure to give the product BAY-460, 90 mg (56%) as a yellow solid. LC-MS: Rt = 2.39 min; MS (ES+): m/z = 569/571 (M+H)+. 1H-NMR (500 MHz, CD3OD) δ [ppm] = 1.37 (d, 3H), 2.31 (s, 3H), 2.55 (d, 2H), 2.73 (s 3H), 2.97 (dd, 1H), 3.10 (dd, 1H), 3.65
(2 d, AB, 2H), 3.78 (q, 1H), 4.70 (m, 1H), 6.30 (d, 1H), 6.72 (d, 1H), 7.16 (d, 2H), 7.22 (d, 2H), 7.40 (d, 1H), 7.44 (d, 1H), 7.50 (d, 2H), 7.65-7.68 (m, 3H).
Cloning, expression and purification of human ATAD2 bromodomain

The human ATAD2 BD (Uniprot Q6PL18, residues 981-1108) was cloned into a pNIC28-Bsa4 and pGTVL2 vector containing cleavable N-terminal hexahistidine and GST tags, respectively, following the previously described protocol (1). Expression vectors were transformed into BL21(DE3)-Rosetta-pRARE2 cells. Pre-cultures were incubated at 37 °C overnight (O/N) in the presence of 50 μg/ml of kanamycin and 34 μg/ml of chloramphenicol in Terrific Broth (TB) media. Then, fresh TB media was inoculated with the precultures and grown at 37 °C in 2 L flasks. Expression was induced by adding IPTG at a final concentration of 200 μM and the temperature was lowered to 18°C. Following overnight incubation, cells were harvested by centrifugation (4000 g at 4°C for 40 min), resuspended in buffer A (500 mM NaCl, 50 mM HEPES pH 7.5, 10 mM imidazole, 0.5 mM TCEP, 5% glycerol) with 1:200 (v/v) protease Inhibitor Cocktail III (Calbiochem), and cell disruption was performed using the EmulsiFlex-C5 high pressure homogenizer (Avestin-Mannheim). Lysates were clarified by centrifugation (15000 g for one hour) and soluble fractions incubated with nickel affinity resin (NiNTA, GE Healthcare), previously equilibrated with buffer A, for one hour at 4°C in a rotatory mixer. Samples were placed into a gravity column and purified in four elution steps; 1) buffer A + 50 mM imidazole, 2) buffer A + 100 mM imidazole, 3) buffer A + 150 mM imidazole, and 4) buffer A + 250 mM imidazole. The eluted protein was treated with TEV protease and dialysed in buffer A for the His6-tagged protein or without TEV for the GST-tagged version (GST-ATAD2). To discriminate between the His6-tagged and non-tagged (ATAD2) protein, a second affinity chromatography purification was carried out in the same manner. This time, the flow-through was kept for further experiments. Both samples, ATAD2 and GST-ATAD2, were then subjected
to size exclusion chromatography (SEC) column (16/600 HiLoad Superdex200 prep grade, GE Healthcare) using buffer B (500 mM NaCl, 20 mM HEPES pH 7.5, 0.5 mM TCEP). Protein purity was assessed with SDS-PAGE and MALDI-TOF MS, and proteins concentrated up to 7 mg/mL with a 3.5 kDa Amicon Ultra concentrator (Millipore).

Alternatively, the protocol reported in (2) was used to generate GST-ATAD2 BD. A synthetic cDNA encoding the ATAD2 BD (residues 981 to 1108) with an N-terminal TEV cleavage site was codon-optimized by Eurofins (Ebersberg, Germany) for expression in Escherichia coli, subcloned into pDONR221 and subsequently recombined with a modified pDEST vector containing a 5 prime GST tag sequence by Gateway cloning (Invitrogen, USA). The protein was overexpressed in E. coli BL21 (DE3) induced overnight by 0.1 mM IPTG (isopropyl β-D-thiogalactoside) at 17 °C. The cell pellet from a 20 L fermenter was re-suspended in 400 mL buffer containing 50 mM HEPES (pH 7.5), 500 mM NaCl, 5% glycerol, 0.5 mM TCEP and protease inhibitor cocktail (ETDA-free). Cells were lysed by using a high pressure homogenizer (Microfluidics, USA). The lysate was centrifuged at 30,000g at 4 °C for 30 min. The GST-fusion protein was purified by GST affinity chromatography using GSH-sepharose (GE Healthcare Life Science). For TSA, native MS and ITC experiments the GST-tag was removed by TEV cleavage overnight at 4 °C. Both tagged and untagged proteins were further purified by size exclusion chromatography (SEC) using a Superdex 200 26/60 high load column (GE Healthcare Life Science) and a buffer containing 20 mM sodium phosphate (pH 7.5), 50 mM NaCl, 5% glycerol, 0.5 mM TCEP.

For the ATAD2 construct bearing the ATPase domain, cDNA coding for residues 342 to 1390 was codon-optimized by Eurofins for expression in insect cells with an N-terminal TEV cleavage site. The construct was provided with an N-terminal FLAG tag by Gateway cloning and purified
from baculovirus-infected Sf9 cells using anti-FLAG M2 beads (Sigma-Aldrich), and a binding buffer containing 50 mM Tris (pH 8.5), 250 mM NaCl 5% glycerol, 0.5 mM TCEP, complete+EDTA, 0.1% NP40. Proteins were eluted with 150 µg/mL FLAG peptide and further purified by size exclusion chromatography using a Superdex 200 26/60 high load column (GE Healthcare Life Science) and a buffer containing 50 mM Tris (pH 8.5), 250 mM NaCl, 5% glycerol, 0.5 mM TCEP.


**Compound Screening**

Library Design

The 11 DNA-encoded chemical libraries (DELs) (3) amounting to 65 billion compounds tested were generated using a “split-and-pool” strategy (4) in three synthetic cycles. The single sub-library of 110-million formyl acid derivatives leading to BAY-850 was prepared as follows: First, a headpiece comprised of a short DNA oligonucleotide with a primary aliphatic amine was split into 300 wells and in each well a unique Fmoc amino acid was acylated onto the primary amine and a unique encoding oligonucleotide tag was ligated onto the headpiece (cycle B). This step was followed by pooling of the 300 conjugates and the subsequent splitting of this mixture into 150 wells in which unique individual formyl acids were installed by acylation and encoded followed by pooling and splitting into 2300 wells in which unique amines were installed by reductive amination and encoded.

Affinity-Mediated Selection Protocol

The DNA-encoded chemical library and GST-ATAD2 were combined in solution and affinity-mediated selection for ATAD2-binders was initiated by incubation in 60 μl of a model cytosolic incubation buffer containing HEPES (20 mM), potassium acetate (134 mM), sodium acetate (8 mM), sodium chloride (4 mM), magnesium acetate (0.8 mM), sheared salmon sperm DNA (1mg/ml, Invitrogen), Tween 20 (0.02%) at pH 7.2. Multiple individual DNA-encoded chemical libraries were combined with the concentration of each individual library at 1 μM. After 1 hour of incubation the mixture was flowed over a 5 μl bed of glutathione affinity matrix with twenty passages followed by eight washes with 200 μl incubation buffer aliquots. Retained library members were eluted by incubation with 60 μl of incubation buffer at 85°C for 5 minutes.
followed by a further incubation with a second 5 µl matrix bed to remove any eluted protein. This entire selection protocol was repeated with fresh addition of GST-ATAD2 to half of the round-one eluate. Encoding oligonucleotides present in the output of the second selection round were amplified using Platinum PCR SupermixTM (Invitrogen) with denaturation at 94°C, annealing at 55°C and extension at 72°C for 24 cycles using 5’- and 3’- primer oligonucleotides (each at 0.5 µM) that each incorporate sequences complementary to the library DNA tailpiece or headpiece along with Illumina READ1 or READ2 sequences required to support clustering and subsequent single-read 100 base-pair sequencing on an Illumina HiSeq 2500. Sequencing was also performed for PCR-amplified samples of the naïve (unselected) library and the output of a no-target selection performed in the absence of ATAD2 in parallel. Sequence data were converted back into encoded chemical information computationally and demographic and statistical information were calculated for individual building block combinations with normalization to the naïve library sequence data.
Biochemical Assays

Time-resolved fluorescence resonance energy transfer (TR-FRET) binding competition assay

Assays were performed at room temperature (RT) in 384-well low volume black microtiter plates (Greiner) in a final volume of 5 µL. Test compounds were serially diluted in DMSO (3.5-fold, 12-points, 0-20 µM) using a Precision liquid handling robot (Biotek) and 50 nl were dispensed onto the plates at 100X the test concentration with a Hummingbird capillary dispenser (Cartesian Technologies). Next, 2 µl of 10 nM GST-ATAD2 BD [in assay buffer 50 mM Hepes pH 7.5, 100 mM NaCl, 50 mM KF, 0.25 mM CHAPS, 0.05% bovine serum albumin (BSA) and 1 mM dithiotreitol (DTT)] were added with a Multidrop (Thermo Fisher Scientific) and the plates were incubated for 15 min. Finally, 3 µl of 50 nM of C-terminal biotinylated synthetic acetylated peptides derived from histone H4 a.a.1-25 (K12 mono-acetylated or K5,8,12,16 tetra-acetylated) and detection reagents (10 nM anti-6His-XL665 (Cisbio), 2.5 nM streptavidin Eu (Perkin Elmer), both in assay buffer) were dispensed and further incubated for at least 1 h. TR-FRET signals corresponding to the number of protein-peptide complexes in equilibrium were acquired either with Viewlux (Perkin Elmer) or Pherastar (BMG Lab Technologies) microtiter plate readers. The normalized ratios of the fluorescence emission at 665 nm and at 620-622 nm after excitation at 330-350 nm, at increasing compound concentrations were used to calculate IC50 values with the Screener Software (Genedata) by regression analysis based on a four-parameter equation [minimum, maximum, IC50, Hill; Y = Max + (Min - Max) / (1 + (X/IC50)^Hill).

AlphaScreen® binding competition assays
Compound binding was determined by the displacement of a tetra-acetylated biotinylated peptide from a Hexa His tagged ATAD2 BD or a FLAG tagged ATAD2 (342 - 1390) protein using AlphaScreen® Histidin (Nickel Chelate) or FLAG (M2) Detection Kits. Compound was dispensed from DMSO stocks into assay plates (ProxiPlate-384 Plus, Perkin Elmer) using an Echo 525 Liquid Handler (Labcyte). Assays were performed at 25°C in a volume of 20 µl in a buffer that was 25 mM HEPES pH7.4, 100mM NaCl, 0.1% BSA, 0.05% CHAPS with 25 nM peptide (SGRGK(ac)GGK(ac)GLGK(ac)GGAK(ac)RHRK(biotin)-acid), 100 nM ATAD2, 0.2 % DMSO. Peptide and protein were pre-mixed, added to the compound plate and incubated for 30 minutes after which AlphaScreen® beads were added to a final concentration of 6 µg/ml. Assay plates were incubated for 60 minutes then luminescence was measured using a Pherastar FS (BMG).

ADP Glo® ATPase activity assay

ATPase assays were conducted using the ADP-Glo® kit (Promega) according to manufacturer’s instructions. Compound and ATP was dispensed into assay plates (ProxiPlate-384 Plus, Perkin Elmer) using an Echo 525 Liquid Handler (Labcyte). Assays were performed at 25°C in a volume of 4 µl in a buffer that was 20 mM HEPES pH7.4, 50 mM NaCl, 0.5 mM TCEP, 1 mM MgCl2 and 0.1% BSA. Reactions were initiated by the addition of 100 nM ATAD2 protein (342 - 1390) and incubated for three hours. Reactions were then terminated by the addition of 4 µl of the ADP Glo® reagent, to deplete unhydrolysed ATP, which was then incubated for one hour at 25 °C. Luciferase/luciferin reactions were then initiated by the addition of 4 µl ADP-Glo® detection reagent which then added incubated at 25 °C for one hour after which assay plates were read on BMG Pherastar FSX.
Biophysical Assays

Microscale Thermophoresis (MST)

For experiments with fluorescence labeled protein, GST-ATAD2 BD was labeled using the RED-NHS kit (Nano Temper Technologies) according to the manufacturer’s instructions. Alternatively, Alexa Fluor 647 was covalently attached by NHS coupling to the N-terminal primary amine groups of the protein. Experiments were performed on the Monolith NT.115 Instrument using the RED detector. When Nano Temper proprietary labeling was chosen, a protein concentration of 20 nM and a buffer containing 25 mM HEPES pH7.4, 150 mM NaCl, 0.05% Tween 20 and 0.5 mM TCEP were used. For experiments using Alexa Fluor 647 labeling, the concentration of GST-ATAD2 BD was 50 nM, and the buffer 50 mM Hepes pH 7.5, supplemented with 150 mM NaCl and 0.05% Pluronic. Compounds were screened in a twelve point threefold serial dilution beginning at 100 μM compound concentration down to 0.5 nM either manually or using an Echo 525 Liquid Handler (Labcyte). After 30 minutes incubation, the protein and compound samples were centrifuged for 5 min at 14000 rpm and then loaded into Monolith™ NT.115 Series MST Premium Coated Capillaries. The MST experiment was performed using LED Power of 20% or 40 %, and MST Power of 40% or 80% (depending on whether Nanotemper proprietary- or Alexa Fluor 647 labeling were used) with thermophoresis occurring over 30 seconds. K_D values were determined with the MO.Affinity Analysis Software v2.1 from Nanotemper.

Experiments with unmodified protein were performed on the Monolith NT.Automated Instrument using the Label free detector with a GST-ATAD2 BD concentration of 500 nM. Compounds were screened in a twelve point two-fold serial dilution beginning at 500 μM
compound concentration down to 244 nM. The assay was performed in buffer containing 150 mM NaCl, 50 mM HEPES 7.5, 0.05% Pluronic. After 30 minutes incubation, the samples were loaded into Monolith™ NT.Automated Zero Background MST Premium Coated Capillary Chips and the MST experiment was performed using the Monolith NT.Automated (LED Power 50 %, MST Power 80%). $K_D$ values were determined with the MO.Affinity Analysis Software v2.1 from Nanotemper.

Isothermal titration calorimetry (ITC)

Experiments were carried out on a NanoITC microcalorimeter (TA Instruments) at 37 °C in 20 mM HEPES pH 7.5, 50 mM NaCl, 0.5 mM TCEP, 5% glycerol. ATAD2 protein solution was buffer exchanged by dialysis into the ITC buffer. The experiment was performed in reverse titration mode. Protein concentration in the syringe was 200 μM and the BAY-850 inhibitor concentration in the cell was 40 μM. Thermodynamic parameters were calculated using $\Delta G = \Delta H - T \Delta S = -RT \ln K_B$, where $\Delta G$, $\Delta H$ and $\Delta S$ are the changes in free energy, enthalpy and entropy of binding respectively using a single binding site model.

Thermal Shift Assay (TSA) / Differential Scanning Fluorimetry (DSF)

Thermal melting experiments with GST-tagged and untagged ATAD2 BD and were carried out in 384-well plates using a ViiATM Real-Time PCR machine (Thermo Fisher Scientific). Melting curves were obtained at a protein concentration of 5.3 μM in presence of environment sensitive fluorescence probe SYPRO Orange (Invitrogen) at a dilution of 1:625 from the supplied stock, using buffer containing 20 mM HEPES pH7.5; 200 mM NaCl; 10% Glycerol; 0.5 mM TCEP. For binding experiments, ligands in serial dilution (0.049 μM to 100 μM, 5-fold) were added to the mixture, as control 1% DMSO was used. Excitation and emission filters for the SYPRO
Orange dye were set to 465 and 590 nm, respectively and scans were measured from 25°C to 95 °C at a rate of 4 °C/min.

To assess the bromodomain selectivity of BAY-850, thermal melting experiments were carried out using an Mx3005p machine (Agilent Technologies). Proteins were buffered in 10 mM HEPES, pH 7.5, 500 mM NaCl and assayed in a 96-well plate at a final concentration of 2 μM in 20 μL volume. BAY-850 was added to obtain a final concentration of 10 μM and SYPRO Orange was added at a dilution of 1:1000 from the supplied stock. Excitation and emission filters for the SYPRO Orange dye were set to 465 and 590 nm, respectively. The temperature was raised with a step of 3°C per minute from 25 to 96°C, and fluorescence readings were taken at each time interval.

All TSA data were analyzed either with the midpoint or the Boltzmann methods using the Genedata Assay Analyzer Software or Excel Macros (5).

Native Mass Spectrometry

ATAD2 samples were buffer exchanged by dialysis against 100 mM ammonium acetate (pH 6.8) and 1 mM DTT using Slide-A-Lyzer dialysis cassettes with a molecular weight cut-off of 10 kDa (Thermo Fisher Scientific). The protein concentration after dialysis was determined by absorbance at 280 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). For the titration experiments ATAD2 protein samples were diluted to 10 μM with 100 mM ammonium acetate and incubated with ligand concentrations ranging from 1.25 to 50 μM with a final DMSO concentration of 1 %. The peptide competition experiments were performed by incubating 10 μM ATAD2 protein with 10 μM peptide and subsequently with increasing concentration of ligand (1.25 – 50 μM) at a final DMSO concentration of 1.2 %.
Nano-electrospray MS data were collected on a Waters SYNAPT G2-S quadrupole time-of-flight mass spectrometer connected to a Triversa NanoMate chip-based nano-ESI-source (Advion) operated at a chip nozzle voltage of 1.7 kV in the positive ion mode and a pressure of 2.7 bar at the back of the conductive pipet tip. The source temperature was kept at room temperature. All measurements were performed at three different cone voltages (50, 75 and 100 V) in order to find optimal conditions to obtain a narrow peak shape without disrupting the non-covalent protein-ligand interactions. Calibration of the instrument was performed using sodium iodide clusters (2 µg/µL in 2-propanol:water; 1:1) up to m/z 3500. In order to improve the signal-to-noise ratio mass spectra were accumulated for 1-2 min. The instrument was controlled and data were evaluated using MassLynx software version 4.1.

Size Exclusion Chromatography (SEC)

To assess ATAD2 bromodomain dimerisation by BAY-850, ATAD2 and GST-ATAD2 preparations were each equally separated into two samples; one of which was incubated with BAY-850 (50 mM DMSO stock solution) in a molar ratio of 1:1, while the other was incubated with the same amount of DMSO instead of compound (apo samples). The samples were then analysed by SEC using 16/600 HiLoad Superdex200 prep grade column and buffer B. Column calibration was performed using commercial gel filtration standards (Biorad).
**Cell-based Assays**

Cell cultures, proliferation inhibition and GI$_{50}$ determination

Cells were cultivated at 37°C and 5% CO2 in different mediums; HMEC in Mammary Epithelial Cell Growth Medium (Promocell, # C-21215), SupplementMix (Promocell, # C-39115), MCF7 in RPMI 1640 (w/o phenolred) (Gibco #11835-063), 10% FCS (heat inactivated, sterile filtrated) (Biochrom; # S 0415), 10 µg/ml human Insulin (Sigma Aldrich #I9278-5ML), 100 pM E2 (estradiol) (Sigma Aldrich # E8875), MDA-MB-231 in DMEM/Ham’sF12 (Biochrom FG 4815 ), 10% FCS (heat inactivated, sterile filtrated) (Biochrom; # S 0415) and NCI-H526 in RPMI 1640 (Biochrom; # FG 1215), 10% FCS (heat inactivated, sterile filtrated) (Biochrom; # S 0415). Compounds were tested in 3 days long proliferation assays in dose-response manner up to 20 µM compound concentration. For GI50 value determination normal and cancer cells were seeded into 96-well microtiter plates in 90 µl cell culture medium and incubated for 24 h at 37 °C and 5% CO2. In dose-response experiments 10 µl compounds were added to reach the final compound concentrations and then cells were incubated for additional 72 h. Cell viability was measured with addition of alamarBlue® (Invitrogen) to the medium in Victor X3 Multilabel Plate Reader (Perkin Elmer). Each experiment was performed in triplicates and GI$_{50}$ values calculated in GraphPad Prism 7.

Gene expression study with quantitative PCR

MCF7 cells were seeded in RPMI 1640 medium w/o phenolred including 10% FCS, 2 mM L-Glutamine, 10µg/ml Insulin and 100 pM E2 (estradiol) with 150 000 cells per well in 6-well plates. Compound treatments started 24 h after seeding and were performed for an additional 24 h in a dose-response manner up to 6.67 µM concentration. Higher compound concentrations
were not tested due to potential off-target-related cytotoxicity. Cells were harvested and after lysis RNA isolation was performed with RNeasy Plus Mini Kit (QIAGEN, # 74136), followed by cDNA synthesis using SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Thermo Fisher Scientific, cat# 11752250). For the assay, TaqMan® Fast Advanced Master Mix (ThermoFisherScientific, #4444557) was used with Taqman probes against ATAD2 (ThermoFisherScientific, Hs00204205_m1), CCNA2 (ThermoFisherScientific, Hs00996788_m1), CDK1 (ThermoFisherScientific, Hs00938777_m1), CDK2 (ThermoFisherScientific, Hs01548894_m1), KIF4A (ThermoFisherScientific, Hs00602211_g1), TOP2A (ThermoFisherScientific, Hs01032137_m1) and Human HPRT1 as housekeeping-gene (ThermoFisherScientific, 4326321E). 7900HT Fast Real-Time PCR System was used with 384-Well Block Module.

Fluorescence recovery after photobleaching (FRAP)

FRAP experiment was performed as previously described with minor modifications (2). MCF7 cells were transfected with TagGFP-tagged wild type and mutant ATAD2 using lipofectamine 3000 (ThermoFisher). Medium was replaced by DMEM without phenol red complemented with 25 mM Hepes 4 hours after transfection to prevent autofluorescence. 24 hours after transfection cells were treated with 1 µM compounds for 1 h at 37 °C. Bleaching and image acquisition were performed on an inverted Zeiss LSM700 confocal microscope using a 488 nm Diode-Pumped Solid State Laser with a 40X, 1.3 N.A. oil-immersion objective at 30 °C. A defined area of interest (2.8 µm x 2.8 µm) was photobleached at full laser power and time lapses were taken every 480 msec at 1 % laser power and at 30 °C. The image series were imported in Fiji and average fluorescence was in the bleached region (F(t)ROI), the total cell nucleus (F(t)total) and a background (F(t)BG) were determined. The relative fluorescence intensity (F(t)norm) was then
calculated following equation shown below where F(i) is the mean intensity of a region in the five prebleach scans.

\[ F(t)_{\text{norm}} = \frac{(F(t)_{\text{ROI}} - F(t)_{\text{BG}})}{(F(t)_{\text{total}} - F(t)_{\text{BG}})} \times \frac{(F(i)_{\text{total}} - F(i)_{\text{BG}})}{(F(i)_{\text{ROI}} - F(i)_{\text{BG}})} \]

The normalized curves were fitted to double exponential curve and half recovery time was calculated using GraphPad Prism 6. For each groups at least 15 images were analyzed and student t-test was used to determine significant differences between the groups.
**Pharmacokinetics.**

Permeability Assay.

Caco2 cells purchased from the DSMZ were seeded at a density of 4.5 x 10^4 cells per well and grown for 15 d in DMEM with typical supplements. Cells were kept at 37 °C in a humidified 5% CO2 atmosphere. Before the permeation assay was run, the culture medium was replaced by a FCS-free Hepes carbonate transport buffer (pH 7.2). For assessment of monolayer integrity the transepithelial electrical resistance was measured. Test compounds were predissolved in DMSO and added either to the apical or basolateral compartment at a final concentration of 2 µM. Before and after 2 h incubation at 37 °C, samples were taken from both compartments. Analysis of compound content was conducted after precipitation with methanol by LC/MS/MS analysis. Permeability (Papp) was calculated in the apical to basolateral (A → B) and basolateral to apical (B → A) directions. The efflux ratio basolateral (B) to apical (A) was calculated by dividing Papp B-A by Papp A-B. Reference compounds were analyzed in parallel as assay control.

Metabolic Stability in Hepatocytes.

A hepatocyte cell suspension of the respective species was filtered through sterile gauze in 50 mL Falcon tubes and centrifuged at 50×g for 3 min at RT. The cell pellet was re-suspended in 30 mL WME and centrifuged through a Percoll® gradient twice at 100×g. The hepatocytes were washed again with WME and resuspended in medium containing 5% FCS. Liver cells were distributed in WME containing 5% FCS to glass vials at a density of 1.0 × 10^6 vital cells/mL. BAY-850 and BAY-460 were added at a final concentration of 1 µM. During incubation, the hepatocyte suspensions were continuously shaken at 580 rpm and aliquots were taken at 2, 8, 16, 30, 45 and 90 min, to which equal volumes of cold methanol were immediately added. Samples
were frozen at -20 °C overnight, then centrifuged for 15 min at 3000 rpm and the supernatant was analyzed by LC/MS/MS. The half-lives of BAY-850 and BAY-460 were determined from the concentration-time plot. The intrinsic clearances were calculated from the half-life.

Metabolic Stability in Liver Microsomes.

The in vitro metabolic stability was determined by incubating compounds at 1 µM concentration in a suspension of liver microsomes in 100 mM phosphate buffer, pH 7.4 (NaH2PO4 x H2O + Na2HPO4 x 2H2O) and at a protein concentration of 0.5 mg/mL at 37 °C. The microsomes were activated by adding a co-factor mix containing 8 mM glucose-6-phosphate, 4 mM MgCl2; 0.5 mM NADP and 1 IU/mL G-6-P-dehydrogenase in phosphate buffer, pH 7.4. The metabolic assay was started shortly afterwards by adding BAY-850 or BAY-460 to the incubation in a final volume of 1 mL. During incubation, the microsomal suspensions were continuously shaken at 580 rpm and aliquots were taken at 2, 8, 16, 30, 45 and 60 min, to which equal volumes of cold methanol were immediately added. Samples were frozen at -20 °C overnight, then centrifuged for 15 min at 3000 rpm and the supernatant was analyzed by LC/MS/MS. The half-lives of BAY-850 and BAY-460 were determined from the concentration-time plot. The in vitro blood clearances were calculated from the half-life.
SUPPLEMENTARY FIGURES AND LEGENDS

Figure S1. Synthesis route of BAY-850 and BAY-460

A

B
A) *Scheme 1. Synthesis of BAY-850.* Reagents and conditions: a) NBS, CF$_3$SO$_3$H, CH$_3$CN, -30°C, 16%; b) KOH, H$_2$O, 100°C, 81%; c) CH$_3$I, K$_2$CO$_3$, DMF, 92%; d) 5-formylfuran-2-ylboronic acid, Pd(dppf)Cl$_2$, K$_2$CO$_3$, THF, H$_2$O, 50°C, 84%; e) (R)-1-(4-Methylphenyl)ethylamine, NaHB(OAc)$_3$, HOAc, CH$_2$Cl$_2$, 35%; f) LiOH, THF, H$_2$O, CH$_3$OH, 69%; g) TBTU, 12, THF, 89%; h) H$_2$NNH$_2$, THF, 70°C, 79%; i) 4-N-Boc-aminocyclohexanone, NaHB(OAc)$_3$,CH$_2$Cl$_2$, 32%; j) HCl, dioxane, CH$_2$Cl$_2$, 41%; k) 1. DBAD, P(Ph)$_3$, phthalimide, THF, 0°C; 2. HCl, dioxane, CH$_2$Cl$_2$, 86%.

B) *Scheme 2. Synthesis of BAY-460.* Reagents and conditions: a) SOCl$_2$, C$_2$H$_5$OH, 90°C, 91%; b) HCl, H$_2$O 80°C, then NaNO$_2$, 0°C, 2-furfuraldehyde, CuCl$_2$, 74%; c) (R)-1-(4-methylphenyl)ethylamine$_2$, NaBH(OAc)$_3$, CH$_2$Cl$_2$, 64%; d) LiOH, THF, H$_2$O, 50°C, 79%; e) 19, TBTU, NEt$_3$, THF, 56%; f) 1. TBTU, NEt$_3$, CH$_3$NH$_2$, THF, 93%; 2. HCl, dioxane, CH$_2$Cl$_2$, 92%.
Figure S2. Analytical Data of BAY-850 and BAY-460.
A) $^1$H-NMR (300 MHz, CD$_3$OD) and LC-MS spectra for BAY-460.

B) $^1$H-NMR (400 MHz, CD$_3$OD) and LC-MS spectra for BAY-850
Figure S3. NMR-observed effects upon binding of a BAY-850 congener to ATAD2-BD.
(A) 1H, 15N labelled ATAD2-BD (50 μM) was titrated with the non-labelled BAY-850 congener N-[(2R)-1-amino-3-(4-cyanophenyl)propan-2-yl]-2-chloro-5-[[1R]-1-(4-methylphenyl)ethyl]amino{methyl}furan-2-yl]benzamide at concentrations between 50 and 250 μM. This compound was used for the NMR experiment due to its superior solubility (experimental details on its preparation can be found in WO 2017/093272 A1 as example 53 in p. 176). Concentration-dependent signal perturbations, predominantly line broadening effects are not solely located in the classical histone peptide binding site. Additionally, a few chemical shift perturbations were observed. Reference 2D 1H,15N-HSQC spectrum of ATAD2-BD without compound (red) and of ATAD2-BD at a protein-to-compound ratio of 1:5 (blue).

(B) Concentration-dependent signal perturbations were observed and mapped onto the molecular surface of the ATAD2-BD - Histone H4K12ac peptide structure (PDBcode 4QUT) based on backbone amide resonance assignments. Amino acids displaying resonance perturbations in the presence of the compound are colored green, residues that were not significantly perturbed are colored white and residues for which backbone assignments are not available are shown in light blue. The H4K12ac peptide is shown in stick representation whereby C, N and O atoms are colored orange, blue and red, respectively.
Figure S4. Extended binding data for BAY-860, BAY-460 and related compounds.

A-C) Correlation plots of potencies and affinities of BAY-850 (blue), BAY-460 (red) and compounds of the same chemical series (colorless) in biochemical and biophysical binding assays. A) Comparison of IC₅₀’s in TR-FRET assay with K12 mono-acetylated H4 peptide as ligand (primary assay) vs. assay with K5,8,12,16 tetra-acetylated H4 peptide as ligand. B) Comparison of potencies in primary biochemical assay vs. affinities (Kᵃ) obtained with fluorescence labeled and “label-free” GST-ATAD2 BD in MST binding assays. C) Comparison of potencies in primary biochemical assay vs. TSA Tm shifts obtained with GST-tagged and untagged ATAD2 BD. The line of equivalence is indicated in solid gray, and dashed gray lines indicate 10-fold offsets. Pearson coefficients are indicated in gray, next to the symbol labels.

D) Inhibition [%] vs. log [BAY-850] (M). HillSlope 0.743, IC₅₀ 1.52e-006.

E) Thermodynamic parameters: Kᵃ=753.3 nM, N=1.45, ΔH=-2.16 kcal/mole, ΔS= 21.11 cal/mole °K.
D) AlphaScreen® normalized dose-response curve corresponding to the displacement of a Histone H4 tetraacetylated peptide from an ATAD2 construct bearing the ATPase domain (342-1390) by BAY-850.

E) ITC titration at 37 °C of BAY-850 on tag-free ATAD2 BD. Analysis of the data with a single site binding model (lower panel) delivered the thermodynamic and affinity data shown next to the graph.
Figure S5. Extended selectivity data for BAY-850 and BAY-460.

A) Residual BD binding to their ligands after treatment with 1 and 10 µM BAY-850 or 10 µM BAY-460 (right).

B) BAY-850 ∆Tm values for other Bromodomain family proteins
Figure S6. Extended native MS data.
A) BAY-850-induced dimerization of ATAD2: Nano-ESI raw spectra of ATAD2 BD (10 µM) with increasing concentrations of BAY-850. Mass signals derived from monomeric and dimeric ATAD2 are indicated. The low molecular mass regions show the mass signal of unbound BAY-850. Fraction bound indicates the portion of free dimeric ATAD2 versus ligand-bound dimeric ATAD2. At concentrations above 25 µM unspecific binding of two and more ligand molecules are observed.

B) Binding of histone H4 peptide to ATAD2 in presence of BAY-850: Deconvoluted nano-ESI spectra of ATAD2 with and without BAY-850 in absence of lysine 12 acetylated histone H4, H4AcK12 (left) and in presence of H4AcK12 (right) obtained under native conditions.
Figure S7. Regulation of target gene expressions by BAY-850 and BAY-460.

Effect of increasing concentrations of compounds on the expression of the target genes indicated in the x-axis. Fold expression values were calculated relative to the housekeeping gene HPRT1. Data for BAY-850 is shown in the left hand panel and for BAY-460 on the right hand panel.
SUPPLEMENTARY TABLES
| Parameter | BAY-850 | BAY-460 |
|-----------|---------|---------|
| IC50 (M)  | 1.66E-07| >0.00002|
| KD (M)    | 1.52E+00| <0.00002|
| ΔH (kcal/mol) | -2.16 | -2.16 |
| ΔS (cal/mol*K) | 21.11 | 21.11 |
| ΔTm (°C) @25 µM | 1.45 | 1.45 |
| ΔTm (°C) @250 µM | 11.4 | 11.4 |
| ΔTm (°C) @2500 µM | 4.5 | 4.5 |

**Table S1. Potencies of BAY-850 and BAY-460 in biochemical and biophysical assays**

- **GST-ATAD2 / Histone H4 AcK12 Interaction (HTRF)**
- **GST-ATAD2 / Histone H4 AcK 5,8,12,16 Interaction (HTRF)**
- **His-ATAD2 / Histone H4 AcK 5,8,12,16 Interaction** (AlphaScreen)
- **Bromocresol TM MST (GST-ATAD2)**
- **TSA (GST-ATAD2)**
- **TSA (untagged-ATAD2)**
- **ITC (Hist-ATAD2)**

**Assay Parameters**
- **IC50 (M)**: Inhibitory concentration 50%.
- **KD (M)**: Dissociation constant.
- **ΔH (kcal/mol)**: Enthalpy change.
- **ΔS (cal/mol*K)**: Entropy change.
- **ΔTm (°C) @25 µM, 250 µM, 2500 µM**: Temperature change at various concentrations.
Table S2. Effects of 1 µM BAY-850 and BAY-460 on kinase activities. Measurements were performed at the Cerep/Eurofins KinaseProfiler panel (www.eurofinspanlabs.com)

| Kinase     | Residual Activity (%) | Kinase     | Residual Activity (%) |
|------------|-----------------------|------------|-----------------------|
| BAY-850 @ 1 µM | BAY-460 @ 1 µM | BAY-850 @ 1 µM | BAY-460 @ 1 µM |
| Abl(h)     | 96 95                 | EphA1(h)   | 105 106               |
| ACK1(h)    | 107 106               | EphA2(h)   | 93 114                |
| ACTR2(h)   | 94 107                | EphA3(h)   | 69 107                |
| ALK(h)     | 96 135                | EphA4(h)   | 99 109                |
| ALK1(h)    | 123 122               | EpA5(h)    | 90 100                |
| ALK2(h)    | 89 96                 | EpA6(h)    | 105 112               |
| ALK4(h)    | 92 92                 | EpA7(h)    | 107 106               |
| ALK5(h)    | 101 104               | EpB1(h)    | 123 108               |
| Arg(h)     | 87 86                 | EpB2(h)    | 122 123               |
| AMPKα1(h)  | 94 89                 | EpB3(h)    | 97 98                 |
| AMPKα2(h)  | 89 93                 | EpB4(h)    | 91 90                 |
| A-Raf(h)   | 101 99                | ErbB2(h)   | 91 91                 |
| ARK5(h)    | 94 101                | ErbB3(h)   | 99 99                 |
| ASK1(h)    | 94 101                | FAK(h)     | 100 105               |
| Aurora-A(h)| 98 100                | Faz(h)     | 96 98                 |
| Aurora-B(h)| 96 122                | Fes(h)     | 94 99                 |
| Aurora-C(h)| 94 103                | FGFR1(h)   | 94 81                 |
| Axl(h)     | 94 96                 | FGFR2(h)   | 102 102               |
| Bmx(h)     | 90 91                 | FGFR3(h)   | 85 91                 |
| BMPR2(h)   | 99 98                 | FGFR4(h)   | 96 108               |
| Brx(h)     | 72 92                 | Fgr(h)     | 87 94                 |
| BRK(h)     | 98 105                | Frl(h)     | 102 92                |
| Brk1(h)    | 100 100               | Fr3(h)     | 103 103               |
| Brk2(h)    | 99 97                 | Fr4(h)     | 93 106                |
| Btk(h)     | 114 98                | Fmx(h)     | 111 114               |
| B-Raf(h)   | 97 102                | Fyn(h)     | 104 112               |
| CalM(h)    | 108 126               | GCK(h)     | 94 106                |
| CalM2(h)   | 98 118                | GCN2(h)    | 102 102               |
| CalM3(h)   | 99 119                | GRK1(h)    | 100 100               |
| CalM4(h)   | 99 98                 | GRK2(h)    | 105 108               |
| CalM5(h)   | 94 75                 | GRK3(h)    | 99 99                 |
| CalM6(h)   | 87 93                 | GRK5(h)    | 99 94                 |
| CalM7(h)   | 88 94                 | GRK6(h)    | 100 98                |
| CalM8(h)   | 100 98                | GRK7(h)    | 102 104               |
| CalM9(h)   | 92 112                | GSK3β(h)   | 106 100               |
| CalM10(h)  | 100 106               | GSK3β(h)   | 101 99                |
| CDK1(cyclinB) | 95 105            | Hck(h)     | 99 100                |
| CDK2(cyclinA) | 96 105          | HPK1(h)    | 101 107               |
| CDK2(cyclinE) | 99 99              | HPK2(h)    | 105 106               |
| CDK3(cyclinE) | 99 107            | HPK3(h)    | 96 96                 |
| CDK4(cyclinD1) | 103 101          | HPK4(h)    | 90 109                |
| CDK5pS25(h) | 102 103          | HPK5(h)    | 96 106                |
| CDK5pS35(h)| 88 104               | HRI(h)     | 93 103                |
| Kinase                  | Residual Activity (%) | Kinase                  | Residual Activity (%) |
|------------------------|-----------------------|------------------------|-----------------------|
| CDK9/cyclin T1(h)      | 99/99                 | IGF-1R(h), activated   | 117/111               |
| CDK12/cyclinK(h)       | 98/98                 | IKKα(h)                | 95/96                 |
| CDK13/cyclinK(h)       | 107/110               | IKKβ(h)                | 88/95                 |
| CDK14/cyclinY(h)       | 99/105                | IKKε(h)                | 89/98                 |
| CDK18/cyclinY(h)       | 103/108               | IR(h)                  | 97/106                |
| CDKL1(h)               | 101/103               | IR(h), activated       | 100/102               |
| CDKL3(h)               | 93/99                 | JAK2(h)                | 101/107               |
| CDKL5(h)               | 95/104                | JNK2α2(h)              | 93/94                 |
| CDKL6(h)               | 99/98                 | JNK2α2(h)              | 93/94                 |
| Chk1(h)                | 106/111               | JAK3(h)                | 100/102               |
| CHK2(h)                | 100/112               | Jnk2α2(h)              | 93/94                 |
| CHK2(h)                | 98/110                | JNKα1α1(h)             | 100/102               |
| CHK1(h)                | 104/101               | JNK3(h)                | 93/106                |
| CK2α1(h)               | 102/94                | KDR(h)                 | 82/86                 |
| CK2α1(h)               | 106/107               | Lck(h)                 | 75/95                 |
| CLK1(h)                | 106/111               | Lck(h) activated       | 85/94                 |
| CLK1(h)                | 91/106                | LMPK1(h)               | 100/102               |
| CLK2(h)                | 92/106                | LMPK2(h)               | 101/92                |
| CLK3(h)                | 99/99                 | LKB1(h)                | 101/99                |
| CLK4(h)                | 103/96                | LCK1(h)                | 110/112               |
| cRaf(h)                | 98/121                | Lyn(h)                 | 90/100                |
| CSK(h)                 | 99/119                | LRRK2(h)               | 104/96                |
| c-RAF(h)               | 97/106                | LTK1(h)                | 94/89                 |
| DAPK2(h)               | 123/117               | MAPK2(h)               | 92/97                 |
| DCAMK2(h)              | 96/128                | MAPK3(h)               | 98/102                |
| DCAMK3(h)              | 89/106                | MAPK4(h)               | 96/99                 |
| DDR1(h)                | 112/106               | MAPK5(h)               | 113/115               |
| DDR2(h)                | 104/117               | MAPK4P(K22h)           | 107/111               |
| DPRK(h)                | 100/104               | MAPK3P(K33h)           | 107/108               |
| DRAK1(h)               | 70/104                | MEK1(h)                | 98/109                |
| DRAK2(h)               | 94/96                 | MEK2(h)                | 115/113               |
| Dyrk1A(h)              | 120/110               | MARK1(h)               | 148/115               |
| Dyrk1B(h)              | 101/109               | MARK3(h)               | 98/104                |
| Dyrk2(h)               | 98/103                | MARK4(h)               | 91/88                 |
| Dyrk3(h)               | 99/107                | MBK2(h)                | 110/106               |
| eEF-2K(h)              | 80/104                | MBK3(h)                | 95/98                 |
| EGFR(h)                | 103/104               | MELK(h)                | 98/107                |

RAW TEXT END
| Kinase     | Residual Activity (%) | Kinase     | Residual Activity (%) |
|------------|-----------------------|------------|-----------------------|
|            | BAY-850 @ 1 µM | BAY-460 @ 1 µM | BAY-850 @ 1 µM | BAY-460 @ 1 µM |
| Mer(h)     | 99               | 116          | ROCK-I(h)      | 105          | 107          |
| Mer(h)     | 63               | 99           | ROCK-II(h)     | 96           | 98           |
| MNK(h)     | 94               | 90           | Ron(h)         | 92           | 104          |
| MKK6(h)    | 88               | 92           | Ros(h)         | 137          | 124          |
| MKK7(h)    | 92               | 91           | Rsk1(h)        | 85           | 96           |
| MLK(h)     | 95               | 98           | Rsk2(h)        | 85           | 91           |
| MLK2(h)    | 97               | 103          | Rsk3(h)        | 72           | 102          |
| MLK2(h)    | 97               | 98           | Rsk4(h)        | 97           | 59           |
| MK2(h)     | 102              | 103          | SAPK2a(h)      | 107          | 104          |
| MRCKα(h)   | 99               | 103          | SAPK2b(h)      | 92           | 93           |
| MRCKβ(h)   | 100              | 103          | SAPK3(h)       | 109          | 102          |
| MSK1(h)    | 85               | 92           | SAPK4(h)       | 101          | 104          |
| MSK2(h)    | 77               | 86           | SBK1(h)        | 98           | 94           |
| MSK3(h)    | 96               | 109          | SGK1(h)        | 94           | 104          |
| MST1(h)    | 102              | 93           | SGK2(h)        | 82           | 104          |
| MST2(h)    | 97               | 101          | SGK3(h)        | 83           | 101          |
| MST3(h)    | 96               | 81           | STK1(h)        | 97           | 99           |
| MST4(h)    | 94               | 90           | STK2(h)        | 96           | 93           |
| mTORα(h)   | 98               | 100          | STK3(h)        | 102          | 119          |
| mTORαB(h)  | 108              | 109          | SLK(h)         | 95           | 116          |
| MLK2(h)    | 94               | 87           | Snk(h)         | 96           | 105          |
| MYLK2(h)   | 99               | 108          | SNRK(h)        | 104          | 121          |
| MYO3B(h)   | 116              | 108          | SRPK1(h)       | 99           | 105          |
| NDR2(h)    | 91               | 85           | SRPK2(h)       | 98           | 104          |
| NIK(h)     | 114              | 122          | STK16(h)       | 95           | 108          |
| NIK2(h)    | 106              | 116          | STK25(h)       | 104          | 104          |
| NBK4(h)    | 106              | 116          | STK32A(h)      | 97           | 100          |
| NBK3(h)    | 107              | 117          | STK32B(h)      | 102          | 111          |
| NBK5(h)    | 111              | 110          | STK32C(h)      | 95           | 101          |
| NBK7(h)    | 99               | 96           | STK33(h)       | 99           | 99           |
| NBK9(h)    | 127              | 117          | Syk(h)         | 97           | 114          |
| NMR(h)     | 98               | 97           | TAF1L(h)       | 96           | 104          |
| NBK11(h)   | 107              | 112          | TAX1(h)        | 96           | 103          |
| NLK(h)     | 88               | 100          | TAO1(h)        | 97           | 100          |
| NUAK2(h)   | 93               | 97           | TAO2(h)        | 96           | 105          |
| PAK2(h)    | 98               | 100          | Tec(h)         | 90           | 99           |
| PAK4(h)    | 102              | 98           | TGFRB1(h)      | 103          | 100          |
| PAK3(h)    | 101              | 96           | TGFRB2(h)      | 94           | 96           |
| PAK5(h)    | 103              | 104          | Tie2 (h)       | 79           | 117          |
| PAK15(h)   | 95               | 96           | TLK1(h)        | 94           | 95           |
| PAR-1(Bc)  | 98               | 102          | TLK2(h)        | 94           | 92           |
| PAK5(h)    | 72               | 101          | TNK(h)         | 100          | 100          |
| Kinase  | Residual Activity (%) | Kinase  | Residual Activity (%) |
|---------|-----------------------|---------|-----------------------|
|         | BAY-850 @ 1 µM        |         | BAY-460 @ 1 µM        |
| PDGFRβ(h) | 95 110                | TrkB(h) | 64 106                |
| PDHK4(h)  | 103 99                | TrkC(h) | 96 91                 |
| PKC1(h)  | 73 90                 | TSSK1(h) | 101 102              |
| PKCγ1(h) | 68 81                 | TSSK2(h) | 101 103              |
| PKCγ2(h) | 103 96                | TSSK3(h) | 97 100                |
| PK-1(h)  | 110 100               | TSSK4(h) | 98 97                 |
| PKM-2(h) | 92 83                 | TTBK1(h) | 99 105                |
| PKM-3(h) | 84 74                 | TTBK2(h) | 96 102                |
| PKM-4(h) | 106 107               | TTK(h)  | 95 108                |
| PKA(c)   | 66 89                 | Txxk(h)  | 88 96                 |
| PKB(a)   | 85 69                 | TYK2(h)  | 105 109               |
| PKB(b)   | 65 95                 | UUK1(h)  | 95 92                 |
| PKB(y)   | 97 95                 | UUK2(h)  | 94 105                |
| PKCα    | 97 96                 | UUK3(h)  | 98 99                 |
| PKCβ1   | 94 98                 | VRK1(h)  | 93 97                 |
| PKCβ2   | 99 107                | VRK2(h)  | 91 96                 |
| PKCβ3   | 98 105                | Wee1(h)  | 102 106               |
| PKCβ4   | 100 107               | Wee1B(h) | 89 94                 |
| PKCγ    | 85 102                | WNK1(h)  | 109 94                |
| PKCδ    | 104 120               | WNK2(h)  | 101 110               |
| PKCε    | 96 96                 | WNK3(h)  | 99 109                |
| PKCζ    | 99 100                | Yes(h)   | 62 99                 |
| PKCη    | 106 112               | ZAK(h)   | 97 111                |
| PKCθ    | 91 94                 | ZAP-70(h) | 93 111               |
| PKCθ2   | 109 114               | ZIP(h)   | 89 115                |
| PKCθ3   | 93 104                | ATM(h)   | 106 123               |
| PKG1α   | 91 94                 | ATR/ATRIP(h) | 104 98           |
| PK1(h)  | 94 92                 | PDK Kinase (p120g) | 102 97           |
| PK3(h)  | 105 104               | PDK Kinase (p110d) | 96 101           |
| PK4(h)  | 83 90                 | PDK Kinase (p110a) | 96 101           |
| PKA(x)  | 89 84                 | PDK2a(h)  | 93 106                |
| PKG2(h) | 98 99                 | PDK2g(h)  | 96 95                 |
| PKI(h)  | 108 91                | PFK2a(h)  | 102 100               |
| PKG(h)  | 102 79                | PPK1a(h)  | 106 103               |
| PKR(h)  | 97 100                | PPK5(g)   | 96 99                 |
| RIN(h)  | 90 90                 |         |                      |
| PTK4(h) | 99 93                 |         |                      |
| Pyk2(h) | 101 114               |         |                      |
| Ret(h)  | 110 95                |         |                      |
| RIPK1(h) | 96 93                |         |                      |
| RIPK2(h) | 100 98              |         |                      |
**Table S3.** Effects of 10 µM BAY-850 and BAY-460 on GPCR activities. Measurements were performed at the Cerep/Eurofins GPCR panel (www.eurofinspanlabs.com).

| Compound ID | Assay                      | % of Control | Assay                      | % Inhibition of Control Agonist |
|------------|---------------------------|--------------|---------------------------|---------------------------------|
| BAY-850    | 5-HT1A (h) (agonist effect)| 10.2         | 5-HT1A (h) (agonist effect)| 15                              |
| BAY-850    | 5-HT2A (h) (agonist effect)| -6.2         | 5-HT1A (h) (agonist effect)| 20                              |
| BAY-460    | 5-HT2B (h) (agonist effect)| -3.2         | 5-HT2B (h) (agonist effect)| 28                              |
| BAY-850    | 5-HT6 (h) (agonist effect) | 3            | 5-HT6 (h) (agonist effect) | 6                               |
| BAY-460    | 5-HT6 (h) (agonist effect) | 4.1          | 5-HT6 (h) (agonist effect) | 11                              |
| BAY-850    | A2B (h) (agonist effect)   | -0.6         | A2B (h) (agonist effect)   | -4                              |
| BAY-460    | A2B (h) (agonist effect)   | -3.1         | A2B (h) (agonist effect)   | 8                               |
| BAY-850    | A3 (h) (agonist effect)    | 34.6         | A3 (h) (agonist effect)    | -10                             |
| BAY-460    | A3 (h) (agonist effect)    | 5.2          | A3 (h) (agonist effect)    | -9                              |
| BAY-850    | alpha 1A (h) (agonist effect)| 0.6       | alpha 1A (h) (agonist effect)| 78                              |
| BAY-460    | alpha 1A (h) (agonist effect)| -3.4      | alpha 1A (h) (agonist effect)| 56                              |
| BAY-850    | alpha 2A (h) (agonist effect)| 5.2        | alpha 2A (h) (agonist effect)| 10                              |
| BAY-460    | alpha 2A (h) (agonist effect)| -3.7      | alpha 2A (h) (agonist effect)| 5                               |
| BAY-850    | beta 1 (h) (agonist effect) | 5.5         | beta 1 (h) (agonist effect) | -7                              |
| BAY-460    | beta 1 (h) (agonist effect) | 2.8         | beta 1 (h) (agonist effect) | -23                             |
| BAY-850    | beta 2 (h) (agonist effect) | 0.4         | beta 2 (h) (agonist effect) | 45                              |
| BAY-460    | beta 2 (h) (agonist effect) | -0.3        | beta 2 (h) (agonist effect) | -27                             |
| BAY-850    | CB1 (h) (agonist effect)   | 36.2         | CB1 (h) (agonist effect)   | 16                              |
| BAY-460    | CB1 (h) (agonist effect)   | -55.8        | CB1 (h) (agonist effect)   | 2                               |
| BAY-850    | D1 (h) (agonist effect)    | -2.9         | D1 (h) (agonist effect)    | -13                             |
| BAY-460    | D1 (h) (agonist effect)    | 1.8          | D1 (h) (agonist effect)    | 1                               |
| BAY-850    | D2S (h) (agonist effect)   | 14.6         | D2S (h) (agonist effect)   | 21                              |
| BAY-460    | D2S (h) (agonist effect)   | 86.1         | D2S (h) (agonist effect)   | 5                               |
| BAY-850    | EP3 (h) (agonist effect)   | 8.6          | EP3 (h) (agonist effect)   | -2                              |
| BAY-460    | EP3 (h) (agonist effect)   | 10.5         | EP3 (h) (agonist effect)   | 0                               |
| Compound ID | Assay                | % of Control Agonist Response | Assay                | % Inhibition of Control Agonist Response |
|-------------|----------------------|------------------------------|----------------------|-----------------------------------------|
| BAY-850     | H1 (h) (agonist effect) | -5.1                         | H1 (h) (antagonist effect) | 18                                      |
| BAY-460     | H1 (h) (agonist effect) | -2.7                         | H1 (h) (antagonist effect) | -5                                      |
| BAY-850     | H2 (h) (agonist effect) | -4.7                         | H2 (h) (antagonist effect) | -4                                      |
| BAY-460     | H2 (h) (agonist effect) | -2                           | H2 (h) (antagonist effect) | -24                                     |
| BAY-850     | H3 (h) (agonist effect) | 38.2                         | H3 (h) (antagonist effect) | 9                                       |
| BAY-460     | H3 (h) (agonist effect) | 9.2                          | H3 (h) (antagonist effect) | 10                                      |
| BAY-850     | kappa (KOP) (agonist effect) | -15.2                      | kappa (KOP) (antagonist effect) | 62                                      |
| BAY-460     | kappa (KOP) (agonist effect) | -0.8                       | kappa (KOP) (antagonist effect) | 22                                      |
| BAY-850     | M1 (h) (agonist effect) | 2.4                          | M1 (h) (antagonist effect) | 65                                      |
| BAY-460     | M1 (h) (agonist effect) | -0.5                         | M1 (h) (antagonist effect) | 44                                      |
| BAY-850     | M4 (h) (agonist effect) | 1.5                          | M4 (h) (antagonist effect) | 28                                      |
| BAY-460     | M4 (h) (agonist effect) | -0.7                         | M4 (h) (antagonist effect) | 31                                      |
| BAY-850     | MC4 (h) (agonist effect) | -1.8                         | MC4 (h) (antagonist effect) | 80                                      |
| BAY-460     | MC4 (h) (agonist effect) | -1.2                         | MC4 (h) (antagonist effect) | 4                                       |
| BAY-850     | motilin (h) (agonist effect) | 40.4                      | motilin (h) (antagonist effect) | 13                                      |
| BAY-460     | motilin (h) (agonist effect) | -2.2                       | motilin (h) (antagonist effect) | 50                                      |
| BAY-850     | mu (MOP) (h) (agonist effect) | 70.6                      | mu (MOP) (h) (antagonist effect) | 13                                      |
| BAY-460     | mu (MOP) (h) (agonist effect) | 40.3                       | mu (MOP) (h) (antagonist effect) | 7                                       |
| BAY-850     | NK1 (h) (agonist effect) | -3                           | NK1 (h) (antagonist effect) | 41                                      |
| BAY-460     | NK1 (h) (agonist effect) | -1.3                         | NK1 (h) (antagonist effect) | 32                                      |
| BAY-850     | P2Y2 (h) (agonist effect) | 0                            | P2Y2 (h) (antagonist effect) | -25                                     |
| BAY-460     | P2Y2 (h) (agonist effect) | -1.1                         | P2Y2 (h) (antagonist effect) | 0                                       |
| BAY-850     | sst4 (h) (agonist effect) | 7.2                          | sst4 (h) (antagonist effect) | 12                                      |
| BAY-460     | sst4 (h) (agonist effect) | 15.7                         | sst4 (h) (antagonist effect) | 4                                       |
Table S4. Physicochemical and in vitro PK properties of BAY-850 and BAY-460

| Assay Type               | Parameter       | Unit     | Compound ID | BAY-850 | BAY-460 |
|--------------------------|-----------------|----------|-------------|---------|---------|
| Solubility               | pH 6.5          | mg/L     |             | >3.07E03| 6       |
| LogD (HPLC)              | pH 7.5          |          |             |         |         |
| Permeability (Caco2)     | Papp(A-B)       | nm/sec   |             | 39      | 37      |
|                          | Papp(B-A)       | nm/sec   |             | 11      | 60      |
|                          | efflux          | ratio    |             | 0.3     | 1.6     |
| Metabolic stability (CLb, in vitro) | rat hepatocytes | L/(h kg) |             | 1.3     | 3.7     |
|                          | mouse liver microsomes | L/(h kg) |             | 1.2     | 5       |
|                          | rat liver microsomes | L/(h kg) |             | 3.5     | 3.9     |
|                          | human liver microsomes | L/(h kg) |             | 1.5     | 1.2     |
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