Discovery of epigenetic regulator I-BET762: lead optimization to afford a clinical candidate inhibitor of the BET bromodomains

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1. SAR tables with BZDR and ApoA1 data included

Table S1. SAR around the BZD ring RHS substituent.^a

Cmpd	BRD2 (FP) pIC ₅₀	BRD3 (FP) pIC ₅₀	BRD4 (FP) pIC ₅₀	PBMC (IL-6) pIC ₅₀	HepG2 ApoA1 Luciferase pEC ₁₇₀
7	5.9	6.2	6.3	6.8	6.7
8	4.6 ^b	4.9 ^b	4.7 ^b	NT	<4.7 ^b
9a	4.8	5.5	5.4	NT	5.3
9b	5.7	6.1	5.9	6.4	5.4
9c	4.5	5.2	5.2	4.8	5.1
13	5.6 ^{b,c}	5.7	5.2	5.3	5.7

^aAll results are means of $n \geq 2$ unless stated ^b $n=1$. ^cFollowing repeat testing, activity was observed but no pXC50 was defined on six separate test occasions due to low max response observed (<50% inhibition) at the highest concentration tested. NT = not tested.

Table S2. SAR around both phenyl rings of the BZD.^a

Cmpd	BRD2 (FP) pIC ₅₀	BRD3 (FP) pIC ₅₀	BRD4 (FP) pIC ₅₀	PBMC (IL-6) pIC ₅₀	Central BZDR pIC ₅₀	ApoA1 Luc pEC ₁₇₀
18a	4.9	5.5	5.5	5.7	NT	6.0
18b	5.5	5.8	5.9	6.2	7.3 ^b	6.4
18c	5.6	5.9	5.8	6.2	6.1 ^b	6.6
18d	6.1	6.1	6.3	6.9	NT	7.5
18e	5.0 ^b	5.3 ^b	5.3 ^b	5.0	NT	4.8
18f	5.5	6.0	6.0	6.3	<5	6.4
18g	5.7	6.0	5.9	6.5	<5	6.6 ^b
18h	5.8	6.0	5.9	NT	<5	6.8 ^b

^aAll results are means of n ≥ 2 unless stated ^bn=1. NT = not tested

Table S3. SAR around the BZD carbamate substituent.^a

Cmpd	R ² , R ³	BRD2 (FP) pIC ₅₀	BRD3 (FP) pIC ₅₀	BRD4 (FP) pIC ₅₀	PBMC (IL-6) pIC ₅₀	Central BZDR pIC ₅₀	ApoA1 Luc pEC ₁₇₀
7	H, Bn	5.9	6.2	6.3	6.8	6.2	6.7
19a	H, 4F-Bn	5.7	6	6.3	6.5	NT	7.2
19b	H, 3F-Ph	5.5 ^b	5.9 ^b	5.8 ^b	NT	NT	5.5 ^b
19c	H, Et	5.6	6	6.1	6.3	NT	5.8
(-)-19c	H, Et	4	4	4	NT	NT	5.0
(+)-19c	H, Et	5.9	6.3	6.4	6.7	NT	6.9
19d	4'-Cl, Et	5.6	5.9	6.1	NT	<5 ^b	6.9

^aAll results are means of n ≥ 2 unless stated ^bn=1. NT = not tested

Table S4. SAR around the BZD acetamide template^a

Cmpd	R ¹ , R ² X, R ³	BRD2 (FP) pIC ₅₀	BRD3 (FP) pIC ₅₀	BRD4 (FP) pIC ₅₀	PBMC (IL-6) pIC ₅₀	Central BZDR pIC ₅₀	ApoA1 Luc pEC ₁₇₀
13	H, H NH, Bn	5.6 ^{b,c}	5.7	5.2	5.3	<5 ^b	5.7
28a	8-OMe, 4'-Cl NH, 4F-Ph	6.0	6.3	6.3	5.7	<5 ^b	6.9
28b	8-OMe, 4'-Cl NH, 2-thiazole	6.0	6.3	6.4	6.0	<5 ^b	6.8
28c	8-OMe, 4'-Cl NH, 2-pyridyl	6.1	6.4	6.4	6.5	NT	6.9

28d	8-OMe, 4'-Cl	6.0	6.3	6.3	NT	NT	6.8
	NH, 3-pyridyl						
28e	8-OMe, 4'-Cl	5.9	6.3	6.3	NT	<5 ^b	6.6
	NH, cyclopropylmethyl						
1	8-OMe, 4'-Cl	6.1	6.4	6.2	6.5	<5 ^b	6.7
	NH, ethyl						
29	9-OMe, 4'-Cl	5.7 ^b	5.8 ^b	5.7 ^b	6.2	NT	6.1
	NH, ethyl						
30	8-OMe, 3'-F	5.5	6.0	5.9	NT	NT	6.2
	NH, ethyl						
27	8-OMe, 4'-Cl	5.5	5.4	5.1 ^b	<4	NT	<5.0
	O, H						
26	8-OMe, 4'-Cl	6.2	6.6	6.6	6.9	NT	7.2
	O, methyl						
28g	8-OMe, 4'-Cl	6.7	6.6	6.4	7.4	NT	7.4
	O, butyl						

^aAll results are means of n ≥ 2 unless stated ^bn=1. ^cFollowing repeat testing, activity was observed but no pXC50 was defined on six separate test occasions due to low max response observed (<50% inhibition) at the highest concentration tested.

2. Supplementary Methods

2.1 Chemistry

(S)-Methyl 3-(((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-4-((2-(4-chlorobenzoyl)-5-methoxyphenyl)amino)-4-oxobutanoate

HRMS (M+H)⁺ calc for C₃₄H₃₀³⁵ClN₂O₇ 613.1741; found 613.1814. LC/MS: (M+H)⁺= 613, Rt 4.05 min.

(S)-Methyl 2-(5-(4-chlorophenyl)-8-methoxy-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)acetate

Mp = 164°C. ¹H NMR (300 MHz, CDCl₃) δ ppm 8.46 (s, 1 H), 7.51 - 7.42 (m, 2 H), 7.39 - 7.30 (m, 2 H), 7.22 (d, *J* = 8.9 Hz, 1 H), 6.73 (dd, *J* = 8.9 and 2.5 Hz, 1 H), 6.61 (d, *J* = 2.5 Hz, 1 H), 4.19 (dd, *J* = 7.6 and 6.5 Hz, 1 H), 3.89 (s, 3 H), 3.75 (s, 3 H), 3.46 (dd, *J* = 17.0 and 7.6 Hz, 1 H), 3.18 (dd, *J* = 17.0 and 6.5 Hz, 1 H). HRMS (M+H)⁺ calc for C₁₉H₁₈³⁵ClN₂O₄ 373.0955; found 373.0943. LC/MS: (M+H)⁺= 373, Rt 3.09 min.

(S)-Methyl 2-(5-(4-chlorophenyl)-8-methoxy-2-thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)acetate

^1H NMR (300 MHz, CDCl_3) δ ppm 10.21 (s, 1 H), 7.48 - 7.40 (m, 2 H), 7.37 - 7.30 (m, 2 H), 7.24 (d, J = 8.9 Hz, 1 H), 6.79 (dd, J = 8.9 and 2.5 Hz, 1 H), 6.69 (d, J = 2.5 Hz, 1 H), 4.41 (dd, J = 7.2 and 6.8 Hz, 1 H), 3.89 (s, 3 H), 3.74 (s, 3 H), 3.68 (dd, J = 17.0 and 7.2 Hz, 1 H), 3.35 (dd, J = 17.0 and 6.8 Hz, 1 H).

Methyl 2-((4*S*)-6-(4-chlorophenyl)-9-methoxy-1-methyl-4*H*-benzo[*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-4-yl)acetate

Method C was used with temperature kept at 0°C when reacting with hydrazine. LC/MS: $(\text{M}+\text{H})^+ = 411$, Rt 2.86 min.

2-((4*S*)-6-(4-Chlorophenyl)-9-methoxy-1-methyl-4*H*-benzo[*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-4-yl)acetic acid

^1H NMR (300 MHz, CDCl_3) δ ppm 7.52 - 7.45 (m, 2 H), 7.38 (d, $J = 8.7$ Hz, 1 H), 7.34 (m, 2 H), 7.00 (dd, $J = 8.7$ and 2.4 Hz, 1 H), 6.96 (d, $J = 2.4$ Hz, 1 H), 4.58 (dd, $J = 7.0$ and 6.8 Hz, 1 H), 3.95 (s, 3 H), 3.68 (dd, $J = 16.7$ and 7.0 Hz, 1 H), 3.59 (dd, $J = 16.7$ and 6.8 Hz, 1 H), 2.67 (s, 3 H), 2.02 (s, 3 H). LC/MS: $(\text{M}+\text{H})^+ = 397$, Rt 2.17 min.

(S)-Methyl 2-(5-(3-fluorophenyl)-7-(methoxy)-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)acetate

LC/MS: $(\text{M}+\text{H})^+ = 357$, Rt 2.83 min.

(S)-Methyl 2-(5-(3-fluorophenyl)-7-(methoxy)-2-thioxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)acetate

LC/MS: (M+H)⁺ = 373, Rt 3.11 min.

(S)-Methyl 2-(2-(2-acetylhydrazono)-5-(3-fluorophenyl)-7-methoxy-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)acetate

LC/MS: (M+H)⁺ = 413, Rt 2.66 min.

Methyl 2-((4S)-6-(3-fluorophenyl)-8-methoxy-1-methyl-4H-benzo[f][1,2,4]triazolo[4,3-a][1,4]diazepin-4-yl)acetate

The crude intermediate (assumed 9.3 mmol) was suspended in THF (50 mL) and AcOH (50 mL) was added at RT. The reaction mixture was stirred at this temperature for 2 days before being concentrated under reduced pressure. The crude mixture was suspended in DCM and washed with sat. NaHCO₃ solution. The organic layer was dried over Na₂SO₄, filtered and concentrated in vacuo and the resulting solid was triturated in i-Pr₂O and filtered. The crude product was purified by flash-chromatography on silica gel (DCM/MeOH : 95/5) to give the title compound as a brown solid. LC/MS: (M+H)⁺ = 395, Rt 2.71 min.

***a*][1,4]diazepin-4-yl)acetic acid**

¹H NMR (300 MHz, DMSO-*d*₆) δ ppm 12.44 (br s, 1 H), 7.81 (d, *J* = 8.9 Hz, 1 H), 7.47 (m, 1 H), 7.40 (m, 1 H), 7.36 - 7.29 (m, 3 H), 6.88 (d, *J* = 2.8 Hz, 1 H), 4.43 (dd, *J* = 7.5 and 6.8 Hz, 1 H), 3.79 (s, 3 H), 2.55 (s, 3 H). LC/MS: (M+H)⁺ = 381, Rt 2.08 min.

***b*][1,4]diazepin-3(11*H*)-one 30**

¹H NMR (300 MHz, CDCl₃) δ ppm 7.49 (m, 2 H), 7.42 - 7.30 (m, 3 H), 7.13 (dd, *J* = 8.9 and 2.9 Hz, 1 H), 7.10 - 6.99 (m, 1 H), 6.69 (dd, *J* = 9.7 and 2.9 Hz, 1 H), 4.41 (m, 1H), 4.13 (dd, *J* = 8.1 and 2.1 Hz, 1 H), 3.74 (s, 3 H), 3.36 (dd, *J* = 18.1 and 2.1 Hz, 1 H), 3.18 (dd, *J* = 18.1 and 8.1 Hz, 1 H). LC/MS: *m/z* 355 [M+H]⁺, *Rt* = 2.56 min.

2.2 Vibrational Circular Dichroism

Theoretical Analysis

- Model used for calculation:

reduced structure model with R-configuration

- Conformational Search: Yes (PCModel)

- Level of theory used for frequency calculation: B3LYP/6-31G*

- Conformational Averaging: Yes-4 unique conformers (1, 2, 4 and 5)

- Lorentzian band width: 6 cm^{-1}

- Frequency scale factor: 0.97

Experimental:

- Spectrometer: Bomem ChiralIR VCD spectrometer operated at 4 cm^{-1}

- Frequency range: 2100-800

- PEM calibration: PEM calibrated at 1400 cm^{-1}

- Scan method: Single 90-minute block scan

- Solvent: CDCl_3

- Concentration: $\sim 8\text{ mg}/125\text{ }\mu\text{L}$

- Baseline Correction Method: enantiomer correction

- Additional processing: No

2.3 Fluorescent Anisotropy (FP) Assay

A mixture of 5- and 6- isomers of Alexa Fluor 488-*N*-(5-aminopentyl)-2-[(4*S*)-6-(4-chlorophenyl)-1-methyl-8-(methoxy)-4*H*-[1,2,4]triazol

o[4,3-*a*][1,4]benzodiazepin-4-yl]acetamide were synthesized as an fluorescent probe for the acetyl-lysine site of BET proteins.

The binding of compounds to His6-tagged Brd 2, 3 and 4 was assessed using a Fluorescence Anisotropy Binding Assay. Generally the method involves incubating the bromodomain protein (BRD 2 (1-473), BRD 3 (1-435) or BRD 4 (1-477)), fluorescent ligand and a variable concentration of test compound together to reach thermodynamic equilibrium under conditions such that in the absence of test compound the fluorescent ligand is significantly (>50%) bound and in the presence of a sufficient concentration of a potent inhibitor the anisotropy of the unbound fluorescent ligand is measurably different from the bound value. All data was normalized to the mean of 16 high and 16 low control wells on each plate. A four parameter curve fit of the following form was then applied: $y = a + ((b - a) / (1 + (10^x / 10^c)^d))$ Where 'a' is the minimum, 'b' is the Hill slope, 'c' is the pIC50 and 'd' is the maximum.

Protocol for Bromodomain BRD 2: All components were dissolved in buffer composition of 50 mM HEPES pH 7.4, 150 mM NaCl and 0.5 mM CHAPS with final concentrations of BRD 2, $2 \times K_d$, fluorescent ligand 5nM. 10 μ L of this reaction mixture was added using a micro multidrop to wells containing 100 nL of various concentrations of test compound or DMSO vehicle (1% final) in Greiner 384 well Black low volume microtitre plate and equilibrated in the dark for 60 mins at room temperature. Fluorescence anisotropy was read in Envision (lex= 485 nm, IEM = 530 nm; Dichroic =505 nm).

Protocol for Bromodomain BRD 3: All components were dissolved in buffer of composition 50 mM HEPES pH 7.4, 150 mM NaCl and 0.5 mM CHAPS with final concentrations of BRD 3 $2 \times K_d$, fluorescent ligand 5 nM. 10 μ L of this reaction mixture was added using a micro multidrop to wells containing 100 nL of various concentrations of test compound or DMSO vehicle (1% final) in Greiner 384 well Black low volume microtitre plate and equilibrated in the dark for 60 mins at room temperature. Fluorescence anisotropy was read in Envision (lex= 485 nm, IEM = 530 nm; Dichroic =505 nm).

Protocol for Bromodomain BRD 4: All components were dissolved in buffer of composition 50 mM HEPES pH 7.4, 150 mM NaCl and 0.5 mM CHAPS with final concentrations of BRD 4 $2 \times K_d$, fluorescent ligand 5 nM. 10 μ L of this reaction mixture was added using a micro multidrop to wells containing 100 nL of various concentrations of test compound or DMSO vehicle (1% final) in Greiner 384 well Black low volume microtitre plate and equilibrated in the dark for 60 mins at room temperature. Fluorescence anisotropy was read in Envision (lex= 485 nm, IEM = 530 nm; Dichroic -505 nm).

2.4 IL-6 PBMC Assay Using Mesoscale Discovery Technology (electrochemiluminescence):

Cell Preparation:

The plating media (500 mL DMEM, 50ml Heat Inactivated Australian Foetal Bovine Serum, 5ml Penicillin/Streptomycin, 5 mL Glutamax) was pre-warmed in a 37°C water bath for at least 20 minutes prior to thawing frozen Peripheral Blood Mononuclear Cells (PBMCs). The compound plates were prepared before commencing cell preparation – two replicates of each master plate are required to account for PBMC donor variability. Sufficient vials of PBMCs from two different donors were selected from the -140°C freezer and placed in the water bath until the cells were just defrosted. In the primary cell biosafety cabinet, the PBMCs were transferred to 50ml centrifuge tubes, with each donor being processed separately. Warm

media was added drop wise to each tube of cells, and they were then centrifuged at 1600 rpm for 5 minutes at ambient temperature. The supernatant was discarded and the PBMCs resuspended in a suitable volume of warm media. 300 μ L of cell suspension was removed for counting on the Cedex cell counter (Innovatis). The viable cell count and percentage viability for each donor were entered into a spreadsheet which calculated the correct volumes of cell suspension, warm media and Lipopolysaccharide required to make up sufficient plating suspension for the compound plates. 50 μ L of cell suspension was added to each well of the compound plates using a Multidrop Combi (Thermo). The seeded plates were then lidded and placed in the humidified primary cell incubator for 18-24 hours at 37°C, 5% CO₂.

Assay:

The activity in the IL-6 assay was determined by using Mesoscale Discovery (MSD) technology to measure electrochemiluminescence. 10 μ l of cell supernatant was transferred to a low-profile 384-well MSD plate, pre-coated with human IL-6 capture antibody using the Cybiwell. The plates were then sealed and placed on a shaker at 700rpm at room temperature for 2 hours. A 1% solution of MSD Blocker A was prepared by dissolving solid Blocker A in PBS, and this was then used to dilute the anti-human IL-6 antibody labelled with MSD SULFO-TAGTM reagent to a working solution of 1 μ g/mL (a 1 in 50 dilution). 10 μ l of the working antibody solution was then added to each well of the MSD plates using a Multidrop Combi. The plates were then re-sealed and returned to the shaker for another 2 hours at 700rpm at room temperature. The plates were then washed three times with PBS, ensuring there was no liquid remaining in the plate after washing. 35 μ L of 2X MSD Read Buffer T (stock 4X MSD Read Buffer T was diluted 50:50 with de-ionised water) was then added to each well using a Multidrop Combi and the plates were then read on the MSD Sector Imager 6000. The resulting data was used for data analysis.

2.5 ApoA1 Luciferase Assay

A transcriptional screen on HepG2 cells was used for primary screening of compounds. Briefly, HepG2 cells were transfected with a plasmid encoding firefly luciferase under the control of 1123 bp of the human apoA-1 promoter region and 232 bp after the transcription start site (-1162, +232), and the 3'-UTR of the human apoA-1 gene (+1037, +1091).

Stably transfected cells were obtained by adding 500 µg/ml of Geneticin into culture medium. A stably transfected HepG2 cell line was chosen for screening, and grown in BME medium containing 10% heat inactivated fetal calf serum glutamine, sodium pyruvate, non essential amino acids, penicillin-streptomycin and 100 µg/mL of geneticin at 37°C in a 95% air - 5% CO₂ humidified atmosphere. Compounds were dissolved in DMSO, added onto cells in BME medium containing 1% FCS and all other additives. After 16 to 20 hour incubation, luciferase activity was quantified in cell lysates by adding Bright GlowTM substrate (Promega). Results are expressed as % of controls, and the concentration of compounds leading to a 70% increase in luciferase activity over controls is determined.

2.6 Central BZD Receptor Binding Assay

Anesthetized male Han Wistar rats (350 g) were sacrificed by cervical dislocation and the brain was excised from cranium. Brains were homogenized in 5 volumes of phosphate buffer saline pH 7.4 (PBS– Invitrogen) using an Ultra-Turrax (IKA-France). Homogenates were centrifuged twice (30 000g – 30 min – 4°C). Pellets were resuspended in 50 mM Tris-HCl and stored frozen at -80°C.

For inhibition studies, brain homogenates (1.5 mg/mL) were incubated in PBS in presence of [³H]-flunitrazepam (2 nM - 220 Bq - PerkinElmer) and compounds of interest (DMSO 0.1%) for 1 hour at 4°C under constant agitation. The final incubation volume was 200 µL. The reaction was stopped by filtration on 96 well-plates (Millipore MANOB). Filters were rinsed

4 times with PBS at 4°C. After drying, filters were dampened with 50 µL scintillation reagent (Optiphase HiSafe III - PerkinElmer). Radioactivity was quantified using a MicroBeta (PerkinElmer). Under these conditions, Diazepam's IC₅₀ was 20 nM (data not shown).

2.7 *In vivo* pharmacokinetic studies in rat

This study is conducted to evaluate the pharmacokinetic parameters and the oral bioavailability of compounds in the male Sprague-Dawley rat. The study is carried out with two groups of three male Sprague-Dawley rats. In the first group, the animals (fed) are dosed by bolus intravenous administration at a target dose of 1mg/kg. In the second group, the animals (fasted) are dosed orally with a suspension at a target dose of 5mg/kg. Blood samples are collected by tail vein sampling at various time points up to 24 hours following administration. Plasma is isolated by centrifugation and plasma concentrations are quantified by LC/MS/MS (LLQ = 5.00 ng/mL). Non-compartmental methods are used for pharmacokinetic data analysis.

2.8 FaSSIF solubility

This assay is a high throughput method to determine the FaSSIF solubility of compounds by precipitation. Compounds are dissolved in DMSO at 2.5 mg/mL and then diluted in Fast State Simulated Intestinal Fluid (FaSSIF pH 6.5) at 125 µg/mL (final DMSO concentration is 5%). After 16h of incubation at 25°C, the suspension is filtered. The concentration of the compound is determined by a fast HPLC gradient. The ratio of the peak areas obtained from the standards and the sample filtrate is used to calculate the solubility of the compound.

2.9 Microsomal clearance

This primary *in vitro* test is carried out to assess the propensity of a compound to be metabolized by liver microsome enzymes from Xenotech (KA, USA). On an automated

Tecan Genesis platform, liver microsomes, 0.1 M potassium phosphate 50 mM pH 7.4 (or 0.4% w:v sodium carbonate) buffer and compound are pre-incubated at 37°C. The metabolic reaction conducted at 37°C is started with the addition of co-factor NADPH and time points are serially sampled at 0, 3, 6, 12, 30 min. Reaction is stopped with cold acetonitrile. Final incubation concentrations are 0.5 mg/mL microsomal protein, 0.5 µM compound and 0.44 mM NADPH. Samples are analyzed by LCMS/MS. The resultant area is used for the determination of the intrinsic clearance of compound (CL_{int}, in µL/min/mg protein).

2.10 Hepatocyte Stability

The following assay method was used for incubations with mouse, rat, dog, cynomolgous monkey and human hepatocytes:

A 5 mM stock solution of GSK525762 was prepared and diluted to 100 µM with DMSO. An aliquot was taken and further diluted to 1 µM with Williams Medium E (WME) and pre-warmed at 37 °C. The cryopreserved hepatocytes for each species were thawed in the manner recommended by the supplier. Cell concentration and viability was ascertained using the Trypan blue exclusion method. The cell suspension was then diluted to 1.4 million cells per mL with pre-warmed (37°C) WME. 300 µL of cell suspension was added to each well of a 12 well plate to which 300 µL of 1 µM GSK525762 was also added to give a final incubation concentration of 0.5 µM GSK525762 and 0.7 million cells/mL. The incubations were maintained at 37°C for the duration of the experiment. At set intervals (0, 10, 20, 30, 45, 60, 75, 90, 105 and 120 minutes) 25 µL of cell suspension was transferred to a 96 well block containing 100 µL of stopping solution containing internal standard. No compound controls, replacing GSK525762 with 300 µL of WME were performed, with samples being taken at 120 minutes only. No hepatocyte controls, replacing 300 µL of cell suspension with 300 µL of WME were also performed, with samples being taken at 0 and 120 minutes only.

Incubations were repeated with 0.05 μ M GSK525762 using mouse, rat, dog and human hepatocytes. Samples were analysed by LC-MS/MS.

2.11 Ames Test

The bacterial mutation assay or Ames Test, detects gene mutations (base pair substitutions and frameshift mutations) at specific histidine or tryptophan gene loci in genetically modified strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2 *uvrA* (pKM101). Assays were performed in the presence and absence of an exogenous mammalian (rat) oxidative metabolism system (S9 mix), to mimic mammalian metabolism [Ames, 1975; Garner, 1972; Green, 1984, Maron, 1983].

The test methodology was based on established procedures for bacterial mutagenicity testing [Ames, 1973; Ames, 1975; Garner, 1972; Green, 1984, Green, 1976; Maron, 1983].

2.12 Methods for crystal structures of Brd4-BD1 with GW548493X and Etizolam

E. coli expressed de-His-tagged Brd4-BD1(44-168) was generated as previously described. The protein was at ~10 mg/mL in 10mM HEPES pH 7.5, 100mM NaCl and purified to homogeneity using a HisTrap column followed by gel filtration, Tev protease cleavage and gel filtration using a Sephadex 75 column. Compound was added to the protein at 3:1 excess and spun prior to co-crystallisation in 120 nL +120 nL sitting drops @ 20°C using MRC plates. Brd4-BD1 GW548493X crystals were grown over a well solution of 0.2M CaCl_2 , 0.1M Mes pH6.5, 20% PEG6000. Those of Brd4-BD1 with Etizolam over a solution of 1M LiCl, 0.1M NaAc (unbuffered), 30% PEG6K. Crystals were briefly transferred into cryo buffer consisting of the well solution with 20% ethylene glycol before flash freezing in liquid nitrogen. Data from a single crystal of Brd4-BD1 GW548493X was collected at the ESRF Grenoble synchrotron (id29) and processed to 1.55 Å using XDS (Acta Cryst. (2010) D66, 133-144)

and SCALA(CCP4). Data from a single crystal of Brd4-BD1 with Etizolam was collected at the Diamond synchrotron (i04-1) and processed to 1.87 Å using XDS (Acta Cryst. (2010) D66, 133-144) and SCALA(CCP4). Molecular replacement solution was performed with a previously refined in house apo structure using Phaser (CCP4). In both cases the P2₁2₁2₁ cell contained a single molecule in the ASU. Model building was performed using Coot and refined using refmac (CCP4, Acta Cryst. (1994) **D50**, 760-763 1994) and buster (Global Phasing Ltd.). The difference density after molecular replacement and initial refinement of the protein allowed both ligands to be modelled unambiguously. The statistics for the data collection and refined co-ordinates are given in Table S5. The final models are deposited to the protein data bank under the accession codes 4c66 and 4c67.

Table S5. Data collection and refinement statistics (Molecular replacement)

	BRD4-BD1 GW548493X (4c67)	BRD4-BD1 ETIZOLAM (4c66)
Data collection		
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	37.121, 44.248, 78.470	35.464, 44.664, 78.664
α , β , γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution (Å)	44.25-1.55 (1.60-1.55) *	19.67-1.87 (1.97-1.87) *
<i>R</i> _{merge}	0.030 (0.051)	0.106 (0.392)
<i>I</i> / σ <i>I</i>	39.8 (17.7)	9.6 (3.9)
Completeness (%)	94.7 (67.9)	99.6 (99.7)
Redundancy	5.7(3.6)	5.6(5.9)
Refinement		
Resolution (Å)	44.25-1.55	19.67-1.87
No. reflections	104759 (4435)	60452 (9138)
No. _{work} / _{free} / _{test} / _{total} / _{uniqu}	18392 (1249)	10798 (1542)
<i>R</i> _{work} / <i>R</i> _{free}	0.163/0.186	0.217/0.2656
No. atoms	1367	1190
Protein	1085	1048
Ligand/ion	20/8	23/0
Water	254	119
B-factors		
Protein	11.2	34.4
Ligand/ion	7.4/32.0	26.3/0.0
Water	25.6	35.5
R.m.s deviations		
Bond lengths	0.0051	0.011
Bond angles (°)	1.015	1.379

*Highest resolution shell is shown in parenthesis. (Collection on a single crystal)

2.13 Rat DTH Experimental Protocol

The *in vivo* study described in this publication was carried out on behalf of GlaxoSmithKline at external laboratories: KWS BioTest Ltd, School of Medical Sciences, University Walk, Bristol, BS8 1TD, UK. Pharmacokinetic analysis of blood samples by LC-MS/MS was carried out at GlaxoSmithKline, Stevenage, UK.

Male Sprague-Dawley rats were immunised by subcutaneous administration of keyhole limpet hemocyanin in complete Freund's adjuvant (KLH/CFA) on Day 0. Rats were orally (po) dosed with either vehicle (1 % methyl cellulose (400 cp) containing 0.2 % (w/v) Sodium Dodecyl Sulphate in a 1 mL dose volume), rapamycin (3 mg/kg po), or GSK525762A (3 or 10 mg/kg po) once daily from day 0 to day 8 (total of 9 days). Seven days after immunisation (on Day 7), all animals received an intradermal (id) injection of 20 μ L of a 1 mg/mL solution of KLH in NaCl 0.9 % in the left ear pinna. An equal volume of vehicle was injected (id) in the contralateral ear. Twenty-four hours later, on Day 8, ear thickness was measured using callipers and increases expressed as the difference in thickness between the challenged and unchallenged ears. In order to measure blood levels of GSK525762, compound dosed animals were bled on Day 0 and Day 8 at 0.5 h and 4 h after compound administration, ie: on first and last day of dosing. Data were analysed by ANOVA followed by multiple comparisons using Tukey's post hoc test; $P < 0.05$ was regarded as statistically significant.

Table S6. Raw data for ear measurements, including differences between left-right ears
(n=10/group)

Ear Swelling Measurement (mm)				
Treatment group	Animal ID	Right ear	Left ear	Difference
Vehicle	1.1	0.59	1.09	0.50
Vehicle	1.2	0.65	1.12	0.47
Vehicle	1.3	0.68	1.19	0.51
Vehicle	1.4	0.70	1.70	1.00
Vehicle	1.5	0.66	1.32	0.66
Vehicle	1.6	0.67	1.28	0.61
Vehicle	1.7	0.70	1.57	0.87
Vehicle	1.8	0.68	1.41	0.73
Vehicle	1.9	0.67	1.44	0.77
Vehicle	1.10	0.68	1.53	0.85
Rapamycin 3 mg/kg	2.1	0.61	1.04	0.43
Rapamycin 3 mg/kg	2.2	0.68	0.98	0.30
Rapamycin 3 mg/kg	2.3	0.68	0.89	0.21
Rapamycin 3 mg/kg	2.4	0.67	0.85	0.18
Rapamycin 3 mg/kg	2.5	0.70	0.84	0.14
Rapamycin 3 mg/kg	2.6	0.63	1.01	0.38
Rapamycin 3 mg/kg	2.7	0.63	0.81	0.18
Rapamycin 3 mg/kg	2.8	0.63	0.77	0.14
Rapamycin 3 mg/kg	2.9	0.67	0.94	0.27
Rapamycin 3 mg/kg	2.10	0.75	0.99	0.24
I-BET762 3 mg/kg	4.1	0.67	1.01	0.34
I-BET762 3 mg/kg	4.2	0.63	1.14	0.51
I-BET762 3 mg/kg	4.3	0.69	1.08	0.39
I-BET762 3 mg/kg	4.4	0.64	1.16	0.52
I-BET762 3 mg/kg	4.5	0.67	1.12	0.45
I-BET762 3 mg/kg	4.6	0.62	1.14	0.52
I-BET762 3 mg/kg	4.7	0.70	1.28	0.58
I-BET762 3 mg/kg	4.8	0.68	1.40	0.72
I-BET762 3 mg/kg	4.9	0.63	1.13	0.50
I-BET762 3 mg/kg	4.10	0.65	1.04	0.39
I-BET762 10 mg/kg	5.1	0.70	1.13	0.43
I-BET762 10 mg/kg	5.2	0.72	0.87	0.15
I-BET762 10 mg/kg	5.3	0.75	1.06	0.31
I-BET762 10 mg/kg	5.4	0.68	0.90	0.22
I-BET762 10 mg/kg	5.5	0.71	0.84	0.13
I-BET762 10 mg/kg	5.6	0.65	0.99	0.34
I-BET762 10 mg/kg	5.7	0.66	0.95	0.29
I-BET762 10 mg/kg	5.8	0.70	1.01	0.31
I-BET762 10 mg/kg	5.9	0.69	1.11	0.42
I-BET762 10 mg/kg	5.10	0.63	1.05	0.42

Table S7. Blood Concentrations of GSK525762 in the Male Sprague Dawley Rat following Oral Gavage Administration of GSK525762A at a Target Dose of 3 mg/kg

Animal ID	Day	Timepoint (h)	Blood Conc. (ng/mL)	Mean blood Conc. (ng/mL)
4.1	0	0.5	26.6	27.5 +/- 4.2
4.2	0	0.5	23.8	
4.3	0	0.5	32.1	
4.4	0	4.0	5.3	4.1 +/- 1.1
4.5	0	4.0	3.8	
4.6	0	4.0	3.2	
4.7	8	0.5	32.2	102.6 +/- 74.9
4.8	8	0.5	181.3	
4.9	8	0.5	94.2	
4.1	8	4.0	27.0	26.3 +/- 7.0
4.2	8	4.0	19.0	
4.3	8	4.0	32.9	

Table S8. Blood Concentrations of GSK525762 in the Male Sprague Dawley Rat following Oral Gavage Administration of GSK525762A at a Target Dose of 10 mg/kg

Animal ID	Day	Timepoint (h)	Blood Conc. (ng/mL)	Mean blood Conc. (ng/mL)
5.1	0	0.5	710.8	751.5 +/- 92.6
5.2	0	0.5	686.2	
5.3	0	0.5	857.5	
5.4	0	4.0	57.9	41.9 +/- 13.8
5.5	0	4.0	33.6	
5.6	0	4.0	34.3	
5.7	8	0.5	197.1	

5.8	8	0.5	206.2	196.3 +/- 10.3
5.9	8	0.5	185.6	
5.1	8	4.0	374.2	
5.2	8	4.0	95.3	253.9 +/- 143.3
5.3	8	4.0	292.1	