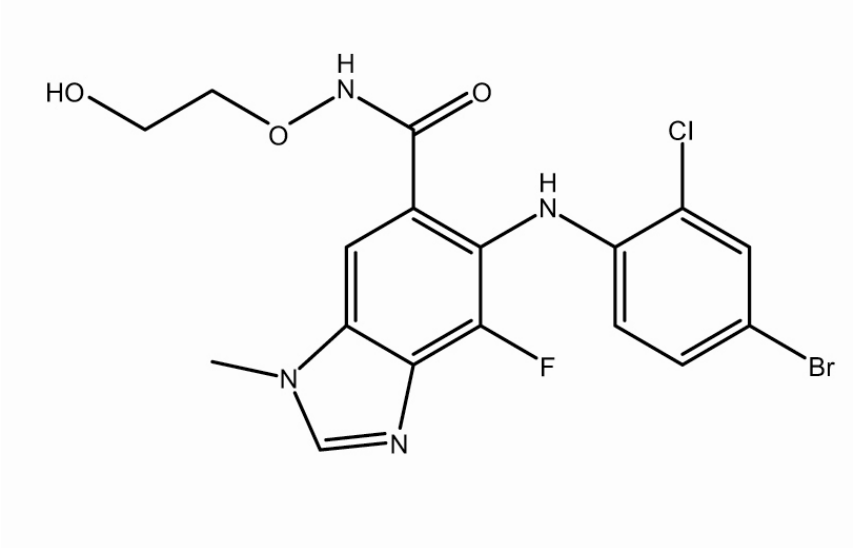
Supplementary Figure S1. Chemical structure of AZD6244: Chemical structure of 6-(4-Bromo-2-chloro-phenylamino)-7-fluoro-3-methyl-3H-benzoimidazole-5-carboxylic acid (2-hydroxy-ethoxy)-amide



Supplementary Patients and Methods

Pharmacokinetic analysis

Blood samples for the determination of plasma AZD6244 and N-desmethyl AZD6244 concentrations in Part A of the study were taken from all patients on Days 1 and 8 (prior to dosing, 5, 15 and 30 minutes and 1, 1 hour 30 minutes, 2, 4, 8 and 12 hours post-dose on Days 1 and 8, and 24 hours post-dose on Day 1). In Part B, blood samples were taken on Days 1 and 8 (prior to dosing, 5, 15 and 30 minutes and 1, 1 hour 30 minutes, 2, 4, 8, 12 and 24 hours post-dose) and on Days 15 and 22 (prior to dosing, and 1, 2 and 4 hours post-dose).

Non Compartmental Analysis

The maximum plasma concentrations (C_{max}) and the time to reach the maximum plasma concentrations (t_{max}) were determined by visual inspection of the plasma concentration-time profiles. Where more than one maxima occurred, the reported value was assigned to the first occurrence. The area under the plasma concentration-time curve from zero to the time of the last quantifiable plasma concentration, AUC(0-t), was calculated by the linear trapezoidal rule. For the

first single dose, the area from the last quantifiable drug concentration to infinite time, AUC, was calculated by the following formula: AUC(0-t)+*Ct*/λ*z*, where Ct is the last quantifiable concentration and λz is the terminal rate constant, calculated by log-linear regression of the terminal portion of the concentration-time profile where there were sufficient data (i.e. there were at least 3 points in the terminal phase). The terminal half-life (t½) was calculated from the equation Ln(2)/lz. Where the data allowed, the apparent oral clearance (CL/F), the apparent volume of distribution based on the terminal phase (Vz/F) and at steady-state Vss were calculated. Following multiple doses, the AUC over the dosing period (AUC_T), was also calculated.

Pharmacodynamic analysis

Inhibition of ERK phosphorylation in the lymphocyte population of peripheral blood cells which had been stimulated *ex vivo* with TPA (also known as Phorbol 12-myristate 13-acetate; Sigma-Aldrich # P8139) was used as a surrogate pharmacodynamic biomarker for inhibition of MEK1/2. Blood samples were taken from all patients on Day 1 in Part A and on Days 1 and 8 in Part B (prior to dosing and 1, 4, 8 and 24 hours post dose).

Sample preparation

The Ras/Raf/MEK/ERK signalling pathway was stimulated using TPA, which was diluted to 10 mg/mL in dimethyl sulphoxide (DMSO; Sigma-Aldrich # D5879), aliquoted and stored at –20°C. A working concentration (250µg/mL TPA

diluted in ethanol) was prepared from the stock solution on each day of sample preparation. This was stored at 4°C and used to treat all samples from the same patient over the 24 hour period. All centrifugation steps in this method were performed at 300g for 5 minutes at room temperature. At each timepoint, two 2mL blood samples were collected in 2mL ethylenediaminetetraacetic acid (EDTA)-containing blood collection tubes. Each sample was transferred to a 15mL graduated polypropylene conical centrifuge tube and treated with either 20µL of working concentration TPA or 20µL of 1:40 DMSO in ethanol. Samples were gently mixed, incubated at 37°C for 10 minutes then fixed using 2mL freshly prepared 4% (w/v) paraformaldehyde (Sigma-Aldrich #441244) in calcium and magnesium free phosphate buffered saline (PBS) per tube for 10 minutes at room temperature with occasional mixing. Red cells were lysed by adding 10mL 0.14% (w/v) Triton-X100 (Sigma-Aldrich #X100-100ML) in PBS to each tube and incubating for 30 minutes at room temperature with gentle agitation. Following centrifugation, the supernatants were discarded and each sample was resuspended in 10mL of 0.1% (w/v) Triton-X100 and incubated for a further 5–10 minutes at room temperature with gentle agitation. Samples were centrifuged again, the supernatant discarded and cells were washed once in 10mL PBS. The supernatant was discarded and cells were gently resuspended in 10mL of chilled methanol and stored at -20°C prior to analysis.

Immunofluorescence staining for pERK

For Part A of the study all samples from the same patient were analysed together. For Part B all samples from the same day of sampling (day 1 or day 8) were analysed together. Quality control (QC) samples (blood from healthy volunteers obtained from CPU, AstraZeneca, Alderley Park, UK, treated with either vehicle or agonist) were stained and analysed concurrently to samples from patients.

ERK phosphorylation was detected in lymphocytes using indirect immunofluorescence staining, analysed by flow cytometry. Where sample size was sufficient, non-specific binding was assessed in half the sample using an isotype control primary antibody. PBS containing 4% (v/v) foetal calf serum (FCS [PBS-FCS]) was used for wash steps (4mL per tube) and antibody dilutions (100µL per tube). All centrifugation steps were performed at 300g for 5 minutes at room temperature and all antibody incubations were performed for 30 minutes at room temperature with shaking at 400rpm. Samples were warmed to room temperature and transferred to 5mL polystyrene round-bottom test tube (BD Biosciences #352054). Methanol was removed by centrifugation and non-specific binding was blocked by resuspending samples in 4mL PBS-FCS for 10 minutes. Following centrifugation, the supernatants were discarded and samples were incubated with either 1.25µg/mL rabbit anti-human phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Cell Signalling Technology #9101) or 1.25µg/mL rabbit IgG (Sigma-Aldrich #I5006). Samples were subsequently washed 3 times by centrifugation and resuspended in 6µg/mL fluorescein (FITC)-conjugated goat anti-

rabbit IgG (Sigma-Aldrich #F1262) and incubated in the dark. To remove unbound antibody, samples were washed once in PBS-FCS and once in PBS then resuspended in a suitable volume of PBS ready for analysis by flow cytometry.

Acquisition and analysis by flow cytometry

Sample acquisition was conducted on a FACSCalibur or FACSCanto II flow cytometer using CellQuestPro or FACSDiVa software, respectively (all BD Biosciences). Voltages for forward scatter and side scatter were optimised for each set of samples then maintained throughout their acquisition. The lymphocyte population was gated and for each sample a minimum of 10,000 gated events were collected. The position of the lymphocyte gate was maintained for all samples from the same patient or healthy volunteer during sample acquisition. The percentage of lymphocytes staining positive for pERK was determined for each timepoint by setting a gate to discriminate between positive and negative cells for each patient using the pre-dose vehicle treated pERK stained sample and allowing 1% ± 2% to fall in the positive area. For the analytical run to pass, agonist treated pERK stained QC samples were required to have 90% ± 10% cells staining positive and for each sample 10,000 ± 50% lymphocytes were required for data to be used. For pharmacokinetic/pharmacodynamic analysis, the percentage inhibition of pERK was calculated with respect to the pre-dose value for all agonist-treated samples.

SupplementaryTables

Supplementary Table S1 Patients with the most common all-causality AEs (≥10% across all patient groups) by preferred term: safety analysis set

	AZD6244 BID dose, number (%) of patients												
	Part A								Part B		Part A+B		
	25mg (n=6)		50mg (n=7)		75mg (n=7)		100mg (n=8)		75mg (n=28)		75mg (n=35)		Total no. of patients (n=56)
Adverse event	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	
Fatigue	2 (33.3)	1 (16.7)	4 (57.1)	0	3 (42.9)	2 (28.6)*	7 (87.5)	1 (12.5)	14 (50.0)	4 (14.3)	17 (48.6)	6 (17.1)	38 (67.9)
Acneiform dermatitis	2 (33.3)	0	6 (85.7)	0	2 (28.6)	1 (14.3)	5 (62.5)	1 (12.5)†	17 (60.7)	1 (3.6)	19 (54.3)	2 (5.7)	35 (62.5)
Nausea	4 (66.7)	0	3 (42.9)	1 (14.3)	6 (85.7)	0	4 (50.0)	1 (12.5)	11 (39.3)	0	17 (48.6)	0	30 (53.6)
Diarrhoea	2 (33.3)	0	2 (28.6)	0	6 (85.7)	0	4 (50.0)	0	13 (46.4)	0	19 (54.3)	0	27 (48.2)
Peripheral oedema	1 (16.7)	0	2 (28.6)	0	5 (71.4)	0	4 (50.0)	0	11 (39.3)	1 (3.6)	16 (45.7)	1 (2.9)	24 (42.8)
Cough	3 (50.0)	0	1 (14.3)	0	4 (57.1)	0	0	0	11 (39.3)	0	15 (42.9)	0	19 (33.9)
Dry skin	0	0	1 (14.3)	0	3 (42.9)	0	3 (37.5)	0	12 (42.9)	0	15 (42.9)	0	19 (33.9)
Exertional dyspnoea	2 (33.3)	0	3 (42.9)	0	2 (28.6)	0	0	0	8 (28.6)	1 (3.6)	10 (28.6)	1 (2.9)	16 (28.6)
Anorexia	2 (33.3)	0	1 (14.3)	0	0	0	2 (25.0)	0	10 (35.7)	0	10 (28.6)	0	15 (26.8)
Constipation	1 (16.7)	0	4 (57.1)	0	2 (28.6)	0	1 (12.5)	0	7 (25.0)	0	9 (25.7)	0	15 (26.8)
Vomiting	3 (50.0)	0	1 (14.3)	1 (14.3)	2 (28.6)	0	2 (25.0)	0	4 (14.3)	2 (7.1)	6 (17.1)	2 (5.7)	15 (26.8)
Abdominal pain	0	0	3 (42.9)	0	3 (42.9)	0	1 (12.5)	0	5 (17.9)	0	8 (22.9)	0	12 (21.4)
Pruritus	1 (16.7)	0	4 (57.1)	0	3 (42.9)	0	1 (12.5)	0	4 (14.3)	0	7 (20.0)	0	13 (23.2)
Dizziness	1 (16.7)	0	2 (28.6)	0	1 (14.3)	0	2 (25.0)	0	5 (17.9)	0	6 (17.1)	0	11 (19.6)

Palmar-plantar erythrodysaesthesia syndrome	2 (33.3)	0	1 (14.3)	0	1 (14.3)	1 (14.3)	1 (12.5)	0	4 (14.3)	0	5 (14.3)	1 (2.9)	10 (17.9)
Dry mouth	0	0	1 (14.3)	0	1 (14.3)	0	1 (12.5)	0	5 (17.9)	0	6 (17.1)	0	8 (14.3)
ALT increased	1 (16.7)	0	1 (14.3)	0	0	0	0	0	4 (14.3)	1 (3.6)	4 (11.4)	1 (2.9)	7 (12.5)
Dysphonia	1 (16.7)	0	2 (28.6)	0	1 (14.3)	0	0	0	3 (10.7)	0	4 (11.4)	0	7 (12.5)
Headache	1 (16.7)	0	0	0	1 (14.3)	0	0	0	4 (14.3)	0	5 (14.3)	0	6 (10.7)
Hypertension	1 (16.7)	0	1 (14.3)	1 (14.3)	0	0	2 (25.0)	0	1 (3.6)	2 (7.1)	1 (2.9)	2 (5.7)	8 (14.3)
Musculoskeletal chest pain	0	0	0	0	0	0	1 (12.5)	0	5 (17.9)	0	5 (14.3)	0	6 (10.7)
Epistaxis	1 (16.7)	0	0	0	1 (14.3)	0	1 (12.5)	0	3 (10.7)	0	4 (11.4)	0	6 (10.7)
Insomnia	1 (16.7)	0	0	0	2 (28.6)	0	2 (25.0)	0	1 (3.6)	0	4 (11.4)	0	6 (10.7)
Pyrexia	0	0	2 (28.6)	0	1 (14.3)	0	2 (25.0)	0	0	1 (3.6)	1 (2.9)	1 (2.9)	6 (10.7)

ALT, alanine aminotransferase; BID, twice daily; n, number of patients.

Part B includes 75mg Hyd-Sulfate capsule and 100mg free-base suspension dosing at days 1 or 8.

Supplementary Table S2 Summary of AZD6244 pharmacokinetic parameters in Part B

AZD6244 dose and formulation	Summary statistic	C_{max} ng/mL	t_{max} h	AUC ng.h/mL	AUC_{0-24} ng/h/mL	$t_{1/2}$ h	CL/F L/h	Vss/F L
75mg Hyd-Sulfate capsule (single dose)	n	27	27	18	26[†]	18	18	18
	Mean*	1307	1.50	4736	4402[†]	7.83	16.8	106
	CV%	76.3	NC	37.3	34.6	NC	NC	NC
	Minimum	177	0.48	2430	2100[†]	4.12	7.75	60.3
	Maximum	3030	2.28	9670	8260	14.0	30.8	301
100mg free-base suspension (single dose)	n	28	28	19	28	21	19	19
	Mean*	523	1.08	2700	2260	9.66	44.4	365
	CV%	91.0	NC	69.0	66.9	NC	NC	NC
	Minimum	81.0	0.50	980	680	4.79	12.4	76.7
	Maximum	1770	4.05	8040	7680	32.0	102	1230

BID, twice daily; CV, coefficient of variation; n, number of observations; NC, not calculated/non calculable.

[a]Geometric mean for C_{max}, AUC and AUC_{0-12}; arithmetic mean for $t_{1/2}$; median for t_{max}.

[†]AUC_{0-24} was not calculable for one patient as a second dose of AZD6244 75mg Hyd-Sulfate capsule was given in error 12 hours post dose; available data to 8 hour post dose suggest that the geomean and minimum presented are likely to be overestimated.