Supplementary Material

Effects of an oral CRTH2 antagonist (AZD1981) on eosinophil activity and symptoms in chronic spontaneous urticaria

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Methods

Inclusion Criteria

Adults between 18 and 65 years with active CSU for 6 months or longer and who remained on symptomatic on a daily H_1-antihistamine at the current FDA-approved dose for at least 7 days prior to the screening visit were eligible. CSU was defined by the occurrence of hives for 3 days or more per week for at least 6 weeks without an underlying etiology or consistent physical trigger. All subjects were required to have normal liver function tests at baseline. Females were required to be surgically sterile or postmenopausal or use a highly effective form of birth control throughout the duration of the study such as an oral contraceptive (estrogen or implanted progesterone), or double barrier method contraception condom with spermatocide or copper banded intrauterine device. Females in certain categories (not sexually active as a lifestyle choice, vasectomized partner, tubal occlusion) were admitted at the discretion of the investigator on a case-by-case basis.

Exclusion Criteria

Exclusion criteria were as follows: use of oral or systemic corticosteroids, hydroxychloroquine, sulfasalazine, dapsone, methotrexate, cyclophosphamide, intravenous immunoglobulin, plasmapheresis, omalizumab or other monoclonal antibody therapies, cyclophosphamide, or any investigational drugs within 30 days of screening; active atopic dermatitis or other skin disease associated with pruritus which would require treatment with topical corticosteroids during the study; contraindications to diphenhydramine; use of doxepin daily or every other day during the study or 2 weeks prior to screening; history of drug or alcohol abuse (within 3 years); women who are pregnant (as determined by urinary human chorionic gonadotropin), breast-feeding, or of childbearing potential and not using acceptable contraception; or inability to comply with study and follow-up procedures. H_2-antihistamines and LTRAs were not allowed during the study unless patients were taking these medications for other conditions such as gastroesophageal reflux or asthma.

Symptom Assessments

Subject-reported outcomes were recorded in the Urticaria Patient Daily Diary using an electronic hand-held device (eDiary) for the duration of the study. Subjects recorded itch severity scores (0, none; 1, mild; 2, moderate; and 3, severe) and number of hives (0, none; 1, 1-6 hives; 2, 7-12 hives; and 3, > 12 hives) every morning and evening, which were averaged for the daily UAS and UAS7. Using this eDiary, subjects also recorded their sleep and daily activity interference (scored 0 = none to 3 = substantial), rescue medication use (number of tablets of 25 mg of diphenhydramine over 24 hours), presence of angioedema (yes or no), angioedema management, and contact with a health care provider for symptoms related to their CSU. Compliance with the eDiary was assessed throughout the study. During visits, patients completed the Dermatology Life Quality Index (DLQI) questionnaire at randomization (day 15) and end of double-blind treatment (day 42) as an assessment of the impact of their dermatology symptoms on their feelings, daily activities, leisure, work/school, personal relationships, and treatment. The 10 items of the DLQI are rated to produce a total score of 0 to 30, with higher scores indicating a reduced quality of life [E1].

Eosinophil and Basophil Surface Receptor Expression

Cell surface chemokine and CRTh2 receptor expression was assessed at baseline (day 15), end of treatment (day 42), and end of washout (day 56). Whole blood samples were collected in heparin

Vacutainer tubes (BD Bioscience) and washed in calcium free buffer, and examined with the appropriate IgG controls, CD123, or receptor antibodies as follows: CD203c ,CRTh2, and CCR1 (Miltenyi Biotec); CCR2 and CXCR1 (Novus Biologicals); CCR3, APC CCR3, CCR5, and TSLPR (eBioscience); CD11b (BD Bioscience); and CD16 (Beckman Coulter). Erythrocytes were lysed using a whole blood lysing reagent kit (Beckman Coulter) and leukocytes were suspended in 1% paraformaldehyde. Samples were run on a BD FACSCalibur flow cytometer. Basophils were gated on forward/side scatter, CD123 positivity, and BDCA-2 negativity. Eosinophils were gated on forward/side scatter, CCR3 positivity, and CD16 negativity.

Eosinophil Shape Change

Eosinophil shape change was assessed prior to randomization, end of treatment (day 42), and end of washout (day 56). Aliquots of whole blood were incubated for 15 minutes at 37°C with increasing doses of PGD_2 (P5172, Sigma-Aldrich, 10^{-8} to 10^{-5} M) or DMSO (vehicle), fixed, and analyzed by flow cytometry. Eosinophils were gated on forward/side scatter, $CCR3^+$, and $CD16^-$ cell populations. Cell movement was assessed using forward/side scatter with net cellular movement recorded (value at PGD_2 concentration minus value with buffer alone). Erythrocytes were lysed using a whole blood lysing reagent kit and leukocytes were suspended in 1% paraformaldehyde. Samples were run on a BD FACSCalibur flow cytometer.

Basophil Isolation and Histamine Release

Basophil studies were performed as described [E2] at baseline (day 14), end of treatment (day 42), and end of washout (day 56). Venous blood was drawn into syringes containing 5 mM EDTA and basophils enriched by Percoll density-gradient sedimentation. Basophil histamine release (HR) was stimulated using polyclonal goat anti-human IgE (0.01-1 µg/mL, The Johns Hopkins University Dermatology, Allergy and Clinical Immunology (DACI) Reference Laboratory, Baltimore, MD) and fMLP (1 µmol, Sigma-Aldrich) in calcium- containing buffers in duplicate samples for 45 minutes at 37°C. Spontaneous HR was measured in the supernatant of unstimulated cells in buffer for 45 minutes. An equivalent aliquot of unstimulated cells were lysed in perchloric acid to determine the total leukocyte histamine content derived from 1 mL of blood. This value reflects the numbers of blood basophils present in the circulation. Stimulated HR was measured in cell-free supernatants using an automated fluorimetric assay, and reported as a percentage of the total lysate after subtraction of the spontaneous HR.

Pharmacodynamics Studies

Serum drug levels were obtained throughout the study (Covance). The trough concentration was measured approximately 8-10 hours after the last dose of AZD1981 during the midpoint of treatment (day 28). A peak level was obtained at the end of treatment (day 42).

Safety Data

The safety of AZD1981 was be assessed using the following outcome measures: incidence and severity of treatment-emergent adverse events and serious adverse events, clinical laboratory measures, and vital signs. In particular we measured CBCs with differential at baseline, week 6, and week 8 and liver function tests were measured at baseline, week 4, week 6, and week 8 based on past trial experience of dose-related toxicity [E3]. Vital signs and a physical examination were obtained at each visit, and a focused review of systems was performed at each visit.

Study Endpoints

The primary efficacy endpoint was the change in diary-based clinical symptoms as measured by the UAS7 from baseline (placebo run-in, days 8 to 14) to the last week (days 36 to 42) in the treatment period. Secondary endpoints included the following: the safety of AZD1981; the pharmacodynamics of AZD1981 through an *ex-vivo* eosinophil shape change assay; the pharmacokinetics of AZD1981 through a serum bioassay; and quality of life benefit as measured by DLQI. Safety analyses included AEs, SAEs, laboratory abnormalities, and physical examination abnormalities. Exploratory endpoints included the following: the change in circulating leukocyte population numbers that are targeted by CRTh2 such as blood basophils, eosinophil and lymphocyte numbers by automated analysis; basophil HR and whole blood histamine content; and pruritus-free and hive-free days (based on eDiary) during treatment period.

Results

Basophil Histamine Content and Release

Basopenia and suppressed basophil HR through the IgE receptor have been established in CSU. Total leukocyte histamine content, a reflection of blood basophil presence, was stable in subjects at the end of treatment compared to baseline (**Fig. S2A**). There was no significant change in spontaneous HR in AZD1981-treated or placebo-treated subjects (**Fig. S2B**). Prior work has shown that CSU patients can be classified as “CSU Non-Responders” (CSU-NR) and “CSU Responders” (CSU-R) based on their basophil HR profiles. At baseline, CSU-R patients possess an anti-IgE induced HR profile similar to normal subjects while CSU-NR patients have diminished anti-IgE induced HR [E2, E4]. There was no significant change in anti-IgE HR with AZD1981 treatment (**Fig. S2C and S2D**) or in fMLP-stimulated HR (not shown), regardless of subgroup analysis based on HR release profile.

Serum Drug Levels

In vivo levels of AZD1981 approximated the expected range of 0.1- 1 μM as directly measured at the midpoint and/or end of active treatment (**Table S2**).

References (Supplementary Material)

(E1) Finlay AY and Khan GK: Dermatology Life Quality Index (DLQI)--a simple practical measure for routine clinical use. Clin.Exp.Dermatol. 1994; 19: 210-216.

(E2) Vonakis BM, Vasagar K, Gibbons SP,Jr, Gober L, Sterba PM, Chang H and Saini SS: Basophil FcepsilonRI histamine release parallels expression of Src-homology 2-containing inositol phosphatases in chronic idiopathic urticaria. J.Allergy Clin.Immunol. 2007; 119: 441-448.

(E3) Snell N, Foster M and Vestbo J: Efficacy and safety of AZD1981, a CRTH2 receptor antagonist, in patients with moderate to severe COPD. Respir.Med. 2013; 107: 1722-1730.

(E4) Eckman JA, Hamilton RG, Gober LM, Sterba PM and Saini SS: Basophil phenotypes in chronic idiopathic urticaria in relation to disease activity and autoantibodies. J.Invest.Dermatol. 2008; 128: 1956-1963.

FIGURE S1. Consort flow diagram showing details of subject enrollment and randomization into treatment groups.

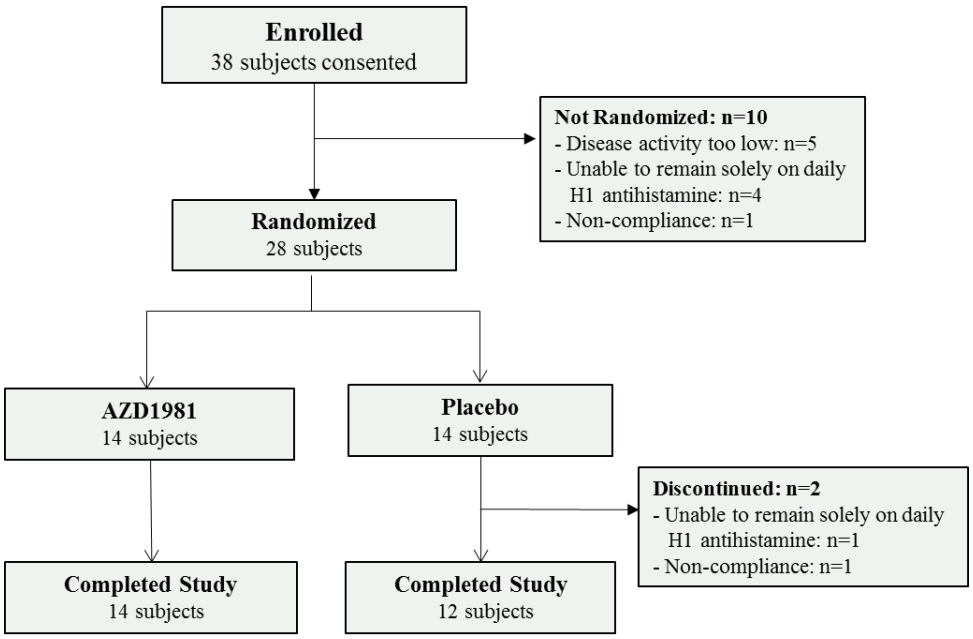
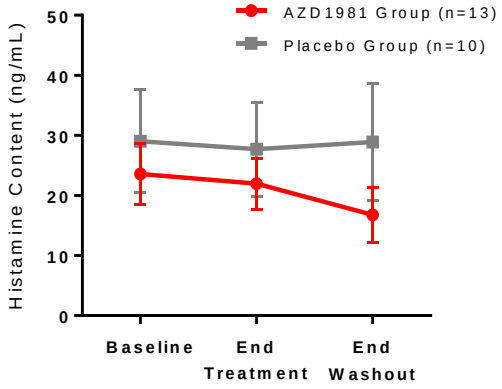
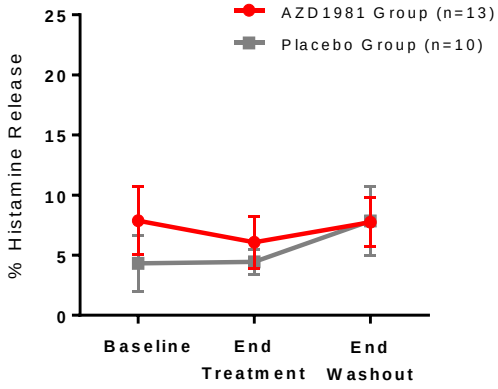


FIGURE S2. The effect of AZD1981 on histamine content and release. (**A**) Total leukocyte histamine content and (**B**) spontaneous histamine release are shown. Changes in anti-IgE mediated histamine release are depicted based on basophil histamine release profiles. Anti-IgE stimulated histamine release is shown for **(C)** CSU anti-IgE responders and **(D)** CSU anti-IgE non-responders at baseline, end of treatment, and washout. Values are shown as mean ± SEM

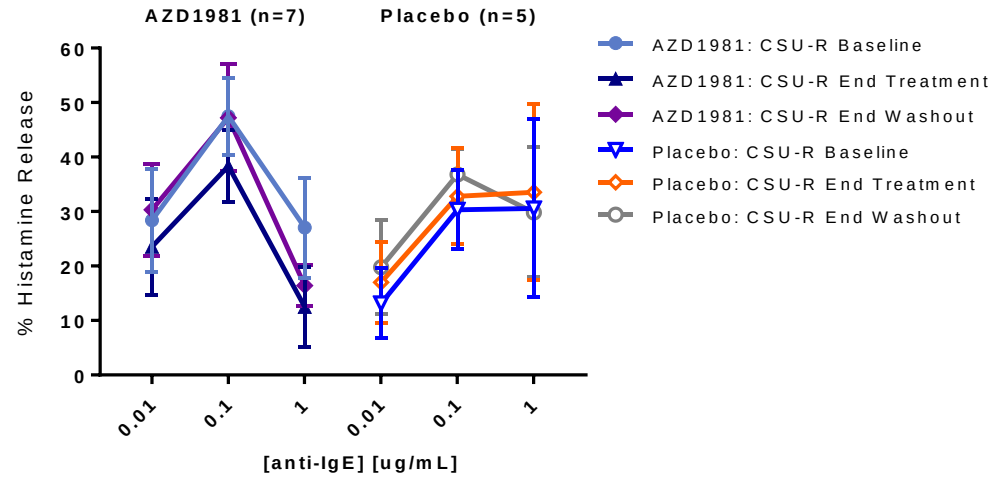
A. Total Leukocyte Histamine Content



B. Spontaneous Histamine Release



C. Anti-IgE Histamine Release: CSU-R Phenotype



D. Anti-IgE Histamine Release: CSU-NR Phenotype

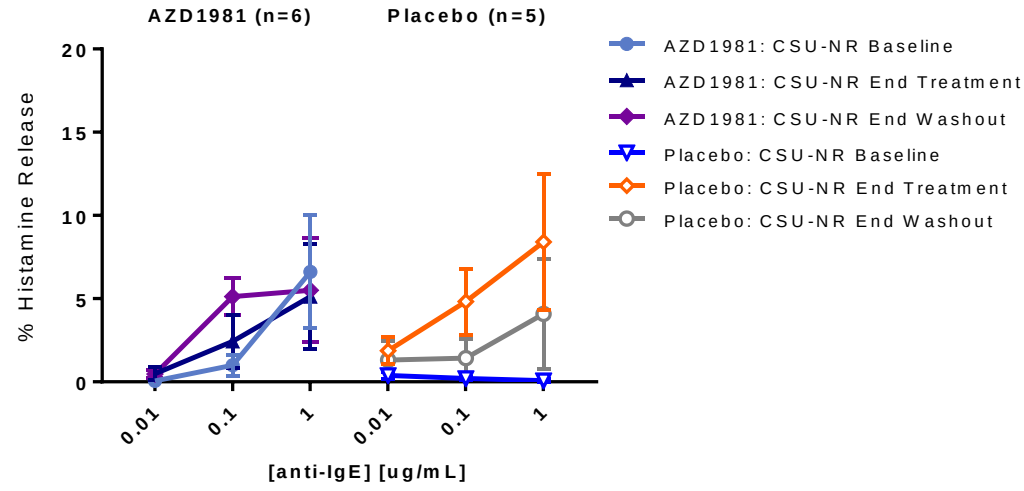


TABLE S1A. Area under the curve for PGD_2-mediated eosinophil shape change in AZD1981-treated subjects (n=13).

Timepoint	Mean AUC	Timepoint Comparison	*P* value
1 - Baseline	1521.317 ± 138.146	1 vs 3	.02
2 - Baseline + 1 uM AZD1981	1441.034 ± 142.960	1 vs 2	.0005
3 - End of Treatment (AZD1981)	1435.540 ± 137.752	3 vs 4	.04
4 - End of Washout	1508.367 ± 116.085	1 vs 4	.07

1 Mean is reported with standard deviation
2 One patient was excluded due to a dispensing error during the washout period.

TABLE S1B. Area under the curve for PGD_2-mediated eosinophil shape change in placebo-treated subjects (n=10).

Timepoint	Mean AUC	Timepoint Comparison	*P* value
1 - Baseline	1472.867 ± 184.401	1 vs 3	.37
2 - Baseline + 1 uM AZD1981	1393.638 ± 173.223	1 vs 2	.002
3 - End of Treatment (Placebo)	1456.602 ± 157.485	3 vs 4	.27
4 - End of Washout	1406.221 ± 108.539	1 vs 4	.76

3 Mean is reported with standard deviation.
4 Two subjects were excluded due to lack of end of washout data secondary to flow cytometer malfunction and
5 unscheduled visit.

TABLE S2. Plasma concentrations of AZD1981 in active subjects

Subject no.	Trough (μmol/L)	Peak (μmol/L)
1	.056	.096
2	1.33	3.20
3	.067	.592
4	.071	.126
5	.253	.626
6	.910	2.24
7	.944	2.42
8	.238	2.21
9	.016	1.52
10	.220	2.44
11	.594	1.13
12	.141	2.37
13	.056	.568
14	.164	.354
Mean ± SD	.361 ± .4004	1.42 ± 1.004