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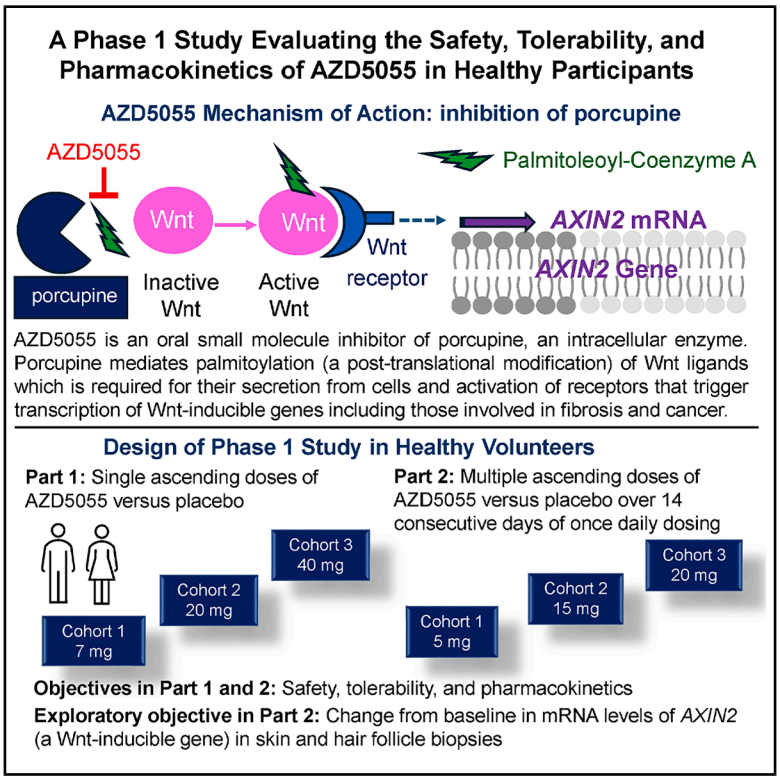
Graphical abstract



Highlights

- Oral dosing with AZD5055 for 14 days was well tolerated by healthy volunteers
- The pharmacokinetic properties of AZD5055 support once daily oral dosing
- AZD5055 treatment reduced *AXIN2* mRNA levels in skin and hair follicle biopsies
- AZD5055 treatment reduced serum levels of Wnt7a and Wnt16

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In brief

Health sciences; Medicine; Medical specialty; Internal medicine; Oncology; Drugs; Pharmacology.

Zhou et al., 2025, iScience *28*, 112602
June 20, 2025
https://doi.org/10.1016/j.isci.2025.112602

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A phase 1 study evaluating the safety, tolerability, and pharmacokinetics of the porcupine inhibitor, AZD5055

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https://doi.org/10.1016/j.isci.2025.112602

SUMMARY

Excessive Wnt signaling contributes to the development of fibrotic diseases and cancer. Here, we report the findings of a phase 1 study evaluating AZD5055, an orally administered porcupine inhibitor, which inhibits Wnt signaling. The primary objective was to evaluate the safety and tolerability of AZD5055 in healthy volunteers. Secondary and exploratory objectives were the pharmacokinetics and pharmacodynamics of AZD5055, respectively. Sixty healthy volunteers were randomized to receive placebo or AZD5055 in single ascending doses of 7, 20, or 40 mg (part 1), or multiple ascending doses of 5, 15, or 20 mg once daily over 14 consecutive days of dosing (Part 2). AZD5055 was safe and well tolerated in both study parts. AZD5055 exposure increased dose-proportionally with a pharmacokinetic profile enabling once daily dosing. AZD5055 effectively inhibited Wnt signaling in skin, hair follicles, and serum samples. Thus, AZD5055 has therapeutic potential in Wnt-driven fibrotic diseases and cancers.

INTRODUCTION

Tissue remodeling, regeneration, and repair maintain homeostasis in adult tissues. The wingless-related integration site (Wnt) pathway makes crucial contributions to these processes in the intestines, lung, skin, hair follicles, and bone.[1] Excessive Wnt signaling drives the chronic, aberrant wound healing process that leads to fibrosis in the lungs, heart, and kidneys.[2] Wnt signaling contributes to fibrosis by activating fibroblasts, promoting epithelial cell reprograming (including epithelial apoptosis, hyperplasia, and metaplasia, and epithelial-to-mesenchymal transition), and inducing the differentiation of macrophages into a pro-fibrotic phenotype.[2] In addition, chronic fibrosis increases the risk of cancer developing in various organs.[3,4] This increased cancer risk is due to shared molecular pathways contributing to fibrogenesis and oncogenesis including Wnt ligand-driven

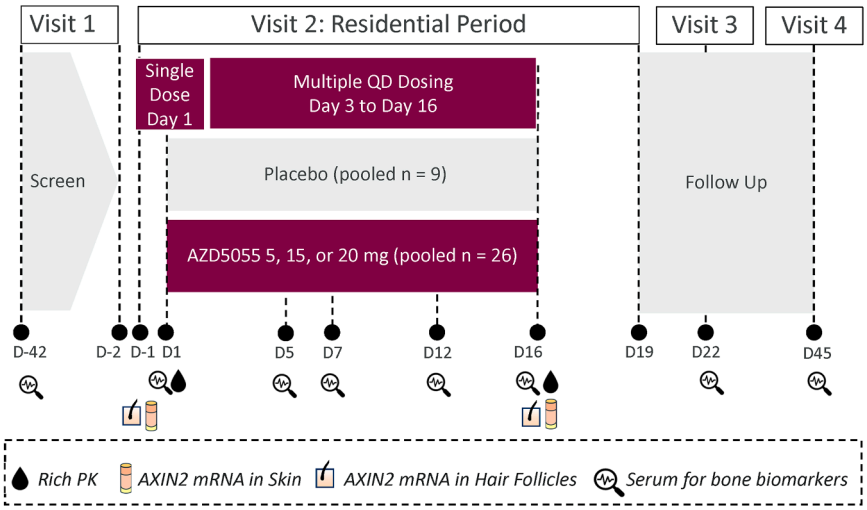

Figure 1. Design of part 2 of the study

The figure illustrates the design and the timing of the sampling for PK and biomarkers in part 2 of the study. Rich PK sampling was conducted on days 1 and 16 pre-dose and 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 48, and 72 (day 16 only) hours post-dose. Trough samples were taken on days 4, 5, 6, 9, and 12. Skin and hair follicle biopsies were taken on day −1 and 8 h ± 2 h after the last dose on day 16.

deposition of extracellular matrix resulting in stiffening of the tumor stroma, which promotes tumor cell growth and survival.[5] Exaggerated Wnt signaling has also been linked to the pathogenesis of cancers that are not secondary to fibrogenesis by promoting cancer cell proliferation and self-renewal of cancer stem cells, inducing tumor cell epithelial-to-mesenchymal transition, and promoting evasion of anti-tumor immune responses by tumor cells.[6–8]

Porcupine is a membrane-bound cysteine N-palmitoyl transferase and is located within the endoplasmic reticulum of cells in most tissues. Porcupine mediates a post-translational modification (palmitoylation) of Wnt ligands which is essential for their secretion from cells, and hence their signaling via Wnt ligand receptors.[9] Thus, inhibition of porcupine has therapeutic potential in a range of fibrotic diseases and cancers that are linked to excessive Wnt signaling. In support of this hypothesis, pharmacological inhibition of porcupine improves outcomes in nonclinical models of cancer, systemic sclerosis, and cardiac injury.[10–12] Several inhibitors targeting components of the Wnt pathway have been or are currently being evaluated in early clinical studies in patients with advanced cancers.[13]

AZD5055 is an orally available, potent, and selective inhibitor of porcupine.[14] AZD5055 treatment reduced collagen deposition in the lungs of bleomycin-challenged mice indicating that porcupine and Wnt signaling are potential therapeutic targets for fibrotic diseases.[15]

Herein, we present results from a randomized, double-blind, placebo-controlled phase 1 study of AZD5055 (ClinicalTrials.gov: NCT05134727). The aim of this study was to evaluate the safety, tolerability, and pharmacokinetics (PK) of AZD5055 following single and multiple ascending doses in healthy participants. We also report pharmacodynamic (PD) assessments of AZD5055 as exploratory endpoints.

Overall, AZD5055 was generally safe and well tolerated by healthy participants over 14 consecutive days of dosing and effectively reduced Wnt pathway signaling as assessed by significant reductions in mRNA levels of the Wnt-target gene *AXIN2*[16] in skin and hair follicle samples. Some of these results have been presented in the form of an abstract.[17]

RESULTS

Participant disposition and baseline characteristics

Sixty healthy participants between 18 and 55 years of age (inclusive) were evaluated at a phase 1 Unit in Baltimore (United States) including 24 participants in part 1 and 36 participants in part 2. The design and timing of the sampling for PK and exploratory endpoints in part 2 are shown in Figure 1. The participant disposition in part 1 and part 2 are shown in Figures 2 and 3, respectively. All participants in part 1 completed the study. In part 2, 5 participants discontinued study treatment due to an adverse event (AE) (n = 3), or behavioral issues (n = 2), and 1 participant withdrew consent before receiving any treatment. The baseline characteristics of the participants are shown in Tables 1 and 2. Most participants were male in both parts of the study.

Safety and tolerability

Treatment-emergent AEs (TEAEs) are summarized in Table 3. In part 1, 4 (22.2%) participants in the pooled AZD5055 group experienced at least one TEAE and 1 (16.7%) participant in the pooled placebo group had one TEAE (Table 3). In the AZD5055 cohorts, 2 (33.3%) participants who received 7 mg, 1 (16.7%) participant who received 20 mg, and 1 (16.7%) participant who received 40 mg experienced at least one TEAE. All TEAEs were CTCAE grade 1 (mild) in intensity and had resolved by the final follow-up visit (Table S1). The TEAEs that were considered possibly related to AZD5055, as assessed by the investigator, were dizziness, non-sustained ventricular tachycardia (nsVT), wheezing, upper abdominal pain, nausea, and vomiting which were reported in a total of 3 (16.7%) participants across all AZD5055 doses (Table S1).

In part 2, at least one TEAE was reported in 21 (80.8%) participants in the pooled AZD5055 group and 5 (55.6%) participants in the pooled placebo group (Table 3). In the pooled AZD5055 group, the TEAEs were either CTCAE grade 1 in intensity and reported by 16 (61.5%) participants or CTCAE grade 2 in intensity and reported in 5 (19.2%) participants (Table S2). In the pooled placebo group, all the TEAEs reported in 5 (55.6%) participants were CTCAE grade 1 in intensity. The TEAEs considered possibly related to AZD5055, as assessed by the investigator, were dysgeusia, headache, photophobia, nsVT, and breath odor and were reported in a total of 7 (26.9%) participants across all AZD5055 doses (Table S2).

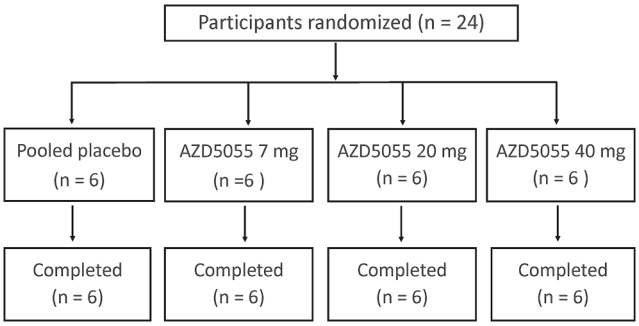

Figure 2. Disposition of participants in part 1 of the study
Disposition of the participants is shown for part 1.

No deaths, serious adverse events (SAEs) or AEs of CTCAE grade 3 or higher were reported in this study. Three participants in part 2 discontinued AZD5055 treatment due to a TEAE including 2 participants who received 5 mg of AZD5055 due to nsVT (see below) and 1 participant who received 15 mg of AZD5055 due to an AE of coronavirus infection. There were no clinically significant changes in clinical laboratory results, vital signs, physical examinations, ECGs, or oxygen saturation in either part 1 or part 2.

Three participants experienced one TEAEs each of nsVT during the study. One participant who received 20 mg of AZD5055 in part 1 experienced a grade 2 TEAE of nsVT. Two participants who received 5 mg of AZD5055 in part 2 both experienced grade 2 TEAEs of nsVT and discontinued treatment in part 2. All participants with TEAEs of nsVT were asymptomatic, and all events were detected during the telemetry analysis. The nsVT episode of the participant who received 20 mg of AZD5055 in part 1 was detected 48 h post dose and was 5 beats in duration with left bundle branch block (LBBB) morphology and a mean heart rate of 150 bpm. Both participants in part 2 had nsVT events that were detected ~11 h after either the initial or the second 5 mg dose. One of the latter nsVT episodes was 7 beats in duration with LBBB morphology and a mean heart rate of 130 bpm and the other was 3 beats in duration with RBBB morphology and a mean heart rate of 154 bpm. None of the 3 nsVT episodes had any features consistent with a drug-induced arrhythmia or met pre-defined study stopping criteria. All 3 participants underwent evaluation with a cardiologist (including echocardiograms) and no clinically significant findings were observed in any of the 3 participants. The baseline and on-treatment ECGs for these participants were normal. From the pharmacokinetic data, AZD5055 had a median time to maximum plasma concentration AZD5055 (t_{max}) of 1–1.5 h and a half-life of 10–11 h after a single dose and 12–17 h after the multiple doses (Figures 4A and 4B). Therefore, the onset times for the 3 nsVT in relation to time after AZD5055 dosing did not support a direct drug exposure-related onset mechanism for any of these nsVT events.

Pharmacokinetics

Single ascending doses

The PK profile of orally administered AZD5055 was characterized by an initial rapid absorption with a median time of maximum concentration (t_{max}) of 1–1.5 h, a bi-phasic elimination from plasma with a mean terminal elimination half-life of 10–11 h. The plasma concentration-time data after a single dose of AZD5055 are shown in Figure 4A. PK parameters are provided in Table S3. A power-model approach indicated that AZD5055 behaves dose-proportionally after single doses in the studied dose range (7–40 mg).

Multiple ascending doses

After 14 consecutive days of once daily treatment in part 2, AZD5055 systemic exposure increased in a dose-proportional manner (Figure 4B). A power-model analysis of the data from the 5–20 mg doses revealed that the 90% confidence intervals (CI) of the slope included 1 for both the area under the curve (AUC) (0.87–1.19) and the peak plasma concentration (C_{max}) (0.96–1.34). PK parameters are provided in Table S4. The mean terminal half-life following repeated dosing was 12–17 h (Figure 4B) which was slightly longer than that observed following a single dose. Steady state conditions for AZD5055 in plasma were reached within 5 doses (Figure 4B). The accumulation ratio was low ($\leq$ 2) following once daily dosing and data indicated limited time-dependency in PK. Approximately 3% of the AZD5055 dose was excreted unchanged in urine resulting in a mean renal clearance of 0.2–0.3 L/h.

Exploratory endpoints

Exploratory endpoints included analysis of circulating bone turnover biomarkers as Wnt signaling regulates bone formation and resorption.[18] In addition, AZD5055-mediated inhibition of Wnt pathway activity was assessed by quantifying changes in *AXIN2* mRNA expression in skin and hair follicle samples and a high-throughput proteomic analysis of serum samples.

Bone turnover biomarkers

Wnt signaling inhibits osteoclast activation and increases osteoblast activity.[18] Thus, AZD5055 has the potential to increase bone resorption and decrease bone formation. Accordingly, we evaluated the effect of AZD5055 treatment on established biomarkers of bone resorption (carboxy-terminal cross-linked telopeptide of type I collagen [CTX-1]) and bone formation (procollagen type I N propeptide [P1NP], bone-specific alkaline phosphatase [BSAP], and osteocalcin).[19] In placebo-treated participants, there was a mean ~15% increase in serum CTX-1 levels and ~10% and ~5% reduction from baseline in serum P1NP and BSAP levels on study day 16 (the final day in the residential treatment period), respectively (Figure 5). These changes returned to or toward baseline by the out-patient follow-up visit on study day 45 and likely reflect the known effect of reductions in physical activity that is associated with the residential period in phase 1 studies on bone turnover markers.[20] Following multiple dose administration of AZD5055 for 14 days at the 15 mg or 20 mg dose levels, there was an estimated increase in serum CTX-1 levels of 17.5% (95% CI, 30.75 to 1.8) and 32.8% (95% CI, 43.5 to 20.1), respectively, compared with the placebo group. Changes in serum CTX-1 levels were similar in the 5 mg AZD5055 and placebo groups. In the 15 mg and 20 mg AZD5055 dose groups, there was an estimated decrease in serum P1NP of 56.3% (95% CI, −29.0 to −89.5) and 111.5% (95% CI, −74.5 to −56.4), respectively, when compared with the placebo group. The changes in serum

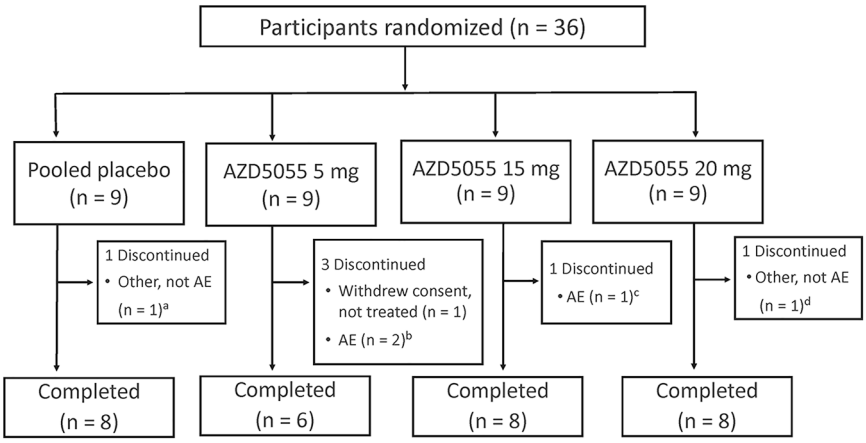

Figure 3. Disposition of participants in part 2 of the study

Disposition of the participants is shown for part 2. Reason for discontinuation: [a,d]behavioural issues; [b]AE of ventricular tachycardia/nsVT; [c]AE of coronavirus infection.

P1NP levels in the 5 mg AZD5055 and placebo groups were similar and small in magnitude. There were reductions in serum osteocalcin levels at the 15 and 20 mg doses and in BSAP levels at the 20 mg AZD5055 dose. However, at lower doses, serum levels of both bone turnover biomarkers (BTBs) were comparable to those in the placebo group. These changes had trended back toward baseline values by the follow-up visit on study day 45.

Target engagement

AZD5055-mediated target engagement on Wnt pathway activity was assessed by quantifying changes in mRNA levels of *AXIN2* (a Wnt-inducible gene) in skin and hair follicle samples. *AXIN2* mRNA expression was significantly reduced from baseline after 14 consecutive days of dosing in both sample types at all dose levels (Figures 6A and 6B) in AZD5055-treated compared with placebo-treated participants. The median *AXIN2* mRNA levels were reduced by ~50%–60% in skin and hair follicle samples in both the 15 and 20 mg AZD5055 cohorts.

A high-throughput proteomic approach was used to further characterize the effect of AZD5055 on a selected panel of serum proteins. Among the 250 proteins analyzed, the only two Wnt ligands included in the panel, Wnt7a and Wnt16, were both significantly reduced by AZD5055 treatment compared with levels in the placebo group (Figure 6C). Serum Wnt7a levels were reduced on study day 16 for all AZD5055 dose levels evaluated and were returning toward baseline values 3 days after the last dose on study day 19 (Figure 6D). Serum Wnt16 levels were reduced on study day 16 at the 15 and 20 mg AZD5055 dose levels and were returning toward baseline on study day 19 (Figure 6E). Thus, AZD5055 treatment rapidly reduces circulating levels of two Wnt ligands, and these changes are rapidly reversible after stopping therapy.

DISCUSSION

This phase 1 clinical study evaluated a potent and orally available porcupine inhibitor having potential to treat diseases associated with heightened Wnt signaling such as chronic fibrotic diseases and various cancers.[14] AZD5055 was generally safe and well tolerated in healthy participants at doses up to 40 mg in part 1 and 20 mg in part 2 of the study, with a half-life permitting a convenient once daily dosing regimen. This study evaluated whether a porcupine inhibitor inhibits Wnt signaling in multiple compartments. AZD5055 treatment led to robust inhibition of Wnt signaling in skin, hair follicle, and blood samples in part 2 of the study. Two AZD5055-responsive Wnt ligands (Wnt7a and Wnt16) were identified in blood samples that may have potential to serve as non-invasive biomarkers of porcupine inhibition.

No deaths or SAEs were reported. All TEAEs were CTCAE grade 1 in intensity in part 1, and most TEAEs were CTCAE grade 1 and the remainder was CTCAE grade 2 in part 2. There were no clinically significant changes in laboratory values, vital signs, physical examinations, or oxygen saturation or ECGs. As this was a phase 1 clinical study, the monitoring for cardiac safety was rigorous and included lengthy telemetry after the initial dose and around steady state. The intense telemetric monitoring allowed for detection of a few asymptomatic very short monomorphic nsVTs of 3–7 beats in duration. The PK profiles for the participants with nsVT did not differ significantly from those of the other participants in the cohort and it is noteworthy that there was no temporal proximity of the events to the highest drug exposure (t_{max}). The arrhythmias also did not start close to the vulnerable ECG period in the T-U waves of the preceding beat, and there were no QTcF or QRS prolongations. Thus, there was no evidence to support a proarrhythmic effect of AZD5055 in any of these participants. Short nsVTs can appear in healthy participants without underlying demonstrable cardiac pathology.[21] After completion of the study, all participants with AEs of nsVTs underwent cardiac evaluation by cardiologists (including echocardiograms) which did not identify any previously unknown cardiac pathology in any of these participants. However, as a precaution, the clinical study protocol for a subsequent study in a diseased patient population will include stringent exclusion criteria based on heart disease, stringent cardiovascular stopping criteria, and frequent ECG monitoring.

Dysgeusia is a common AE reported for other porcupine inhibitors which is likely due to the contributions of Wnt signaling to taste bud renewal.[22] Other phase 1 studies evaluating other porcupine inhibitors (e.g., ETC-159 and WNT-974) in patients with advanced solid tumors reported dysgeusia in up to ~50% of participants.[23,24] However, there were only 2 TEAEs of dysgeusia reported in the current study, one at the 20 mg dose the other at the 15 mg dose of AZD5055 in part 2 and both were CTCAE grade 1 in intensity. Taste bud cells are continuously renewed and studies in rodents have reported

Table 1. Baseline characteristics of participants in part 1

	Statistic	AZD5055 7 mg (*n* = 6)	AZD5055 20 mg (*n* = 6)	AZD5055 40 mg (*n* = 6)	Pooled AZD5055 (*n* = 18)	Pooled Placebo (*n* = 6)
Demographics						
Age (years)	Median (Range)	30.5 (22–37)	35 (27–39)	43 (22–55)	34.5 (22–55)	31 (23–42)
Female	n (%)	1 (16.7)	0	1 (16.7)	2 (11.1)	0
Male	n (%)	5 (83.3)	6 (100)	5 (83.3)	16 (88.9)	6 (100)
White	n (%)	4 (66.7)	1 (16.7)	3 (50.0)	8 (44.4)	1 (16.7)
Black or African American	n (%)	2 (33.3)	4 (66.7)	3 (50.0)	9 (50.0)	5 (83.3)
Asian	n (%)	0	1 (16.7)	0	1 (5.6)	0
Hispanic or Latino	n (%)	1 (16.7)	1 (16.7)	3 (50.0)	5 (27.8)	2 (33.3)
Not Hispanic or Latino	n (%)	5 (83.3)	5 (83.3)	3 (50.0)	13 (72.2)	4 (66.7)
Anthropometrics						
Height (cm)	Mean (SD)	179.2 (9.8)	176.8 (6.5)	172.3 (4.6)	176.1 (7.4)	176.7 (8.7)
Weight (kg)	Mean (SD)	82.6 (9.7)	78.6 (11.4)	77.4 (9.0)	79.5 (9.7)	84.1 (8.9)
BMI (kg/m^2)	Mean (SD)	25.8 (2.6)	25.2 (3.6)	26.1 (3.0)	25.7 (2.9)	27.0 (1.9)
Medical history						
Myopia	n (%)	4 (66.7)	1 (16.7)	2 (33.3)	7 (38.9)	0
Previous fracture (any)	n (%)	3 (50)	1 (16.7)	1 (16.7)	5 (27.8)	0
Previous surgery (any)	n (%)	2 (33.3)	0	2 (33.3)	4 (22.2)	1 (16.7)

The table shows the baseline demographic and anthropometric characteristics of participants in Part 1.

that the average taste bud renewal cycle is 10–14 days in duration.[25] Thus, it is possible that dysgeusia will be more frequently reported as an AE in subjects dosing with AZD5055 for longer than 14 days.

AZD5055 PK was characterized by rapid absorption following oral administration and bi-phasic elimination. Both C_{max} and AUC were dose-proportional after single (7–40 mg) and multiple (5–20 mg) doses. Accumulation following repeated dosing was low, and urinary excretion of AZD5055 was negligible.

The Wnt pathway makes crucial contributions to adult bone homeostasis, a dynamic process involving the activity of osteoblasts and osteoclasts.[18] The canonical (β-catenin-dependent) Wnt pathway promotes bone formation by driving the commitment of mesenchymal stem cells to the osteoblast lineage, and

Table 2. Baseline characteristics of participants in part 2

	Statistic	AZD5055 5mg (*n* = 8)	AZD5055 15 mg (*n* = 9)	AZD5055 20 mg (*n* = 9)	Pooled AZD5055 (*n* = 26)	Pooled Placebo (*n* = 9)
Demographics						
Age (years)	Median (Range)	37 (28–52)	37 (20–44)	33 (26–48)	36 (20–52)	33 (24–53)
Female	n (%)	0	1 (11.1)	0	1 (3.8)	0
Male	n (%)	8 (100)	8 (88.9)	9 (100)	25 (96.2)	9 (100)
White	n (%)	4 (50.0)	5 (55.6)	2 (22.2)	11 (42.3)	3 (33.3)
Black or African American	n (%)	4 (50.0)	3 (33.3)	6 (66.7)	13 (50.0)	6 (66.7)
Asian	n (%)	0	1 (11.1)	1 (11.1)	2 (7.7)	0
Hispanic or Latino	n (%)	1 (12.5)	2 (22.2)	2 (22.2)	5 (19.2)	1 (11.1)
Not Hispanic or Latino	n (%)	7 (87.5)	7 (77.8)	7 (77.8)	21 (80.8)	8 (88.9)
Anthropometrics						
Height (cm)	Mean (SD)	177.6 (7.5)	178.2 (8.2)	175.4 (5.3)	177.1 (6.9)	174.7 (9.8)
Weight (kg)	Mean (SD)	84.3 (8.3)	85.0 (13.1)	80.9 (9.7)	83.4 (10.4)	77.3 (13.4)
BMI (kg/m^2)	Mean (SD)	26.8 (2.6)	26.7 (2.8)	26.2 (2.2)	26.5 (2.5)	25.2 (2.4)
Medical history						
Myopia	n (%)	2 (25)	1 (11.1)	4 (44.4)	7 (26.9)	0
Previous fracture (any)	n (%)	1 (12.5)	1 (11.1)	0	2 (7.7)	0
Previous surgery (any)	n (%)	4 (50)	2 (22.2)	2 (22.2)	8 (30.8)	3 (33.3)

The table shows the baseline demographic and anthropometric characteristics of participants in Part 2.

Table 3. Summary of participants with TEAEs in the AZD5055-and placebo-treated groups in each cohort of the as-treated population

	Part 1 SAD cohorts		Part 2 MAD cohorts	
	AZD5055 *n* = 18	Placebo *n* = 6	AZD5055 *n* = 26	Placebo *n* = 9
Participants with ≥1 TEAE	4 (22.2)	1 (16.7)	21 (80.8)	5 (55.6)
Participants with ≥1 TEAE of grade 1 severity	4 (22.2)	1 (16.7)	16 (61.5)	5 (55.6)
Participants with ≥1 TEAE of grade 2 severity	0	0	5 (19.2)	0
Participants with ≥1 TEAE of grade 3 severity	0	0	0	0
Participants with ≥1 TESAE	0	0	0	0
Participants with ≥1 TEAE leading to discontinuation of investigational product[a]	0	0	3 (11.5)	0
Nervous system disorders	1 (5.6)	0	7 (26.9)	0
Dizziness	1 (5.6)	0	0	0
Dysgeusia	0	0	2 (7.7)	0
Headache	0	0	5 (19.2)	0
Cardiac disorders	1 (5.6)	0	2 (7.7)	0
Ventricular tachycardia	1 (5.6)	0	2 (7.7)	0
Respiratory, thoracic and mediastinal disorders	1 (5.6)	0	2 (7.7)	2 (22.2)
Wheezing	1 (5.6)	0	0	0
Cough	0	0	0	2 (22.2)
Epistaxis	0	0	1 (3.8)	0
Nasal discomfort	0	0	1 (3.8)	0
Gastrointestinal disorders	1 (5.6)	0	1 (3.8)	0
Abdominal pain upper	1 (5.6)	0	0	0
Nausea	1 (5.6)	0	0	0
Vomiting	1 (5.6)	0	0	0
Breath odor			1 (3.8)	0
General disorders and administration site conditions	2 (11.1)	1 (16.7)	11 (42.3)	2 (22.2)
Application site scab	1 (5.6)	0	0	0
Medical device site dermatitis	1 (5.6)	1 (16.7)	11 (42.3)	1 (11.1)
Sensation of foreign body	0	0	1 (3.8)	0
Vessel puncture site haematoma	0	0	0	1 (11.1)
Infections and infestations	0	0	2 (7.7)	0
Coronavirus infection	0	0	1 (3.8)	0
Folliculitis	0	0	1 (3.8)	0
Eye disorders			2 (7.7)	0
Photophobia	0	0	1 (3.8)	0
Vision blurred	0	0	1 (3.8)	0
Skin and subcutaneous tissue disorders	0	0	1 (3.8)	1 (11.1)
Dermatitis contact	0	0	0	1 (11.1)
Erythema	0	0	1 (3.8)	0
Musculoskeletal and connective tissue disorders	0	0	1 (3.8)	0
Costochondritis	0	0	1 (3.8)	0
Injury, poisoning and procedural complications	0	0	5 (19.2)	1 (11.1)
Limb injury	0	0	1 (3.8)	0
Post procedural erythema	0	0	1 (3.8)	0
Post procedural inflammation	0	0	1 (3.8)	0
Procedural pain	0	0	2 (7.7)	0

(*Continued on next page*)

Table 3. *Continued*

	Part 1 SAD cohorts		Part 2 MAD cohorts	
	AZD5055 *n* = 18	Placebo *n* = 6	AZD5055 *n* = 26	Placebo *n* = 9
Skin abrasion	0	0	1 (3.8)	0
Suture rupture	0	0	0	1 (11.1)

The table shows TEAEs by system organ class and preferred term (MedDRA version 25.1). Participants are counted once for each system organ class and preferred term irrespective of the number of events.

[a]Reason for discontinuation: AE of ventricular tachycardia/nsVT (*n* = 2) or coronavirus infection (*n* = 1). All data are n (%). Abbreviations used: TEAE, treatment-emergent adverse event; TESAE, treatment-emergent serious adverse event.

by inducing the proliferation of osteoblast precursors and the differentiation and survival of osteoblasts.[26] This pathway also reduces bone resorption by attenuating osteoclastogenesis.[26] Consequently, treating patients chronically with AZD5055 could lead to on-target effects on bone by inhibiting osteoblasts and activating osteoclasts. BTBs are used to monitor therapeutic responses to drugs being evaluated in clinical studies to treat patients with osteoporosis.[27] Thus, to begin to assess the effects of AZD5055 on bone turnover, circulating BTBs were measured. In part 2, dosing with AZD5055 led to dose-dependent increases in levels of CTX-1 that were associated with dose-dependent decreases in circulating levels of the bone formation biomarkers, P1NP and BSAP (and to a lesser extent osteocalcin).[27] Treatment with ETC-159 (a porcupine inhibitor) also increased circulating CTX-1 levels in patients with advanced cancer.[13] Treatment with ETC-159 and WNT974 (another porcupine inhibitor) caused loss of bone mineral density when administered to mice.[28,29] In addition, in an open-label Ph1b/2 clinical study in which 20 patients with Wnt-dependent metastatic colorectal cancer were treated with WNT974 for a median of 22 (range 0–80) weeks, 45% of the patients developed fragility fractures.[30] Thus, long-term dosing with AZD5055 could reduce bone density and increase the risk of fragility fractures. However, a nonclinical study reported that co-treating mice with ETC-159 and alendronate (a bone anti-resorptive agent) for 4 weeks prevented the loss of bone mineral density occurring in mice treated with ETC159 alone.[28] Thus, additional longer-duration studies should evaluate the effects of chronic dosing with AZD5055 on bone mineral density and, if reductions in bone mineral density develop, whether this effect can be effectively mitigated with bone anti-resorptive agents.

We evaluated whether AZD5055 inhibits Wnt signaling in 3 different compartments in the same individuals including hair follicle and skin biopsies (as Wnt signaling contributes to hair follicle development[31] and skin homeostasis[32]) and blood samples. Significant reductions in *AXIN2* mRNA levels in both hair follicle and skin biopsies confirmed that AZD5055 inhibited Wnt signaling. A prior phase 1 study of another porcupine inhibitor (WNT974) measured *AXIN2* mRNA levels in skin biopsies and demonstrated reductions in *AXIN2* mRNA levels that were of similar magnitude (mean ~50%–60% reduction) to those reported in our study.[23] However, this prior study lacked a placebo arm, and measured only change from baseline in a limited number of patients treated with each dose of the inhibitor. A strength of our study is that we evaluated samples from enough patients (6–8 per group) to demonstrate statistically significant and placebo-corrected changes from baseline at 3 dose levels. Based on these findings, the recommended dose for future clinical studies will be 5 and 15 mg once daily. Dosing at higher doses was not supported by the exposure limits set by non-clinical toxicology studies. In addition, minimal additional gain in target inhibition was predicted at doses higher than 15 mg. The magnitude of inhibition of Wnt signaling needed to achieve clinical efficacy in oncologic or fibrotic indications is not known but could be evaluated in future clinical studies in diseased populations. It is possible that partial inhibition of Wnt signaling is both sufficient for clinical efficacy and desirable for limiting on-target adverse effects on crucial Wnt-mediated homeostatic mechanisms in bone, skin, hair follicles, and other organs. Future clinical studies could evaluate whether AZD5055-mediated reductions in *AXIN2* mRNA levels in accessible tissues can be used as a surrogate endpoint that predicts a clinically meaningful benefit from AZD5055 treatment in patients with fibrotic or oncologic diseases.

A high-throughput proteomic assay was used to further explore the effect of AZD5055 on the serum proteome in part 2. Among 250 proteins analyzed, AZD5055 treatment induced significant reductions from baseline in levels of Wnt7a and Wnt16 (the only Wnt ligands that were included in the panel). Our results suggest that circulating levels of Wnt ligands have potential to serve as PD biomarkers for monitoring porcupine inhibition in a clinical setting. Further clinical studies should determine whether these biomarkers show similar trends in patient populations treated with AZD5055 or other porcupine inhibitors.

Other porcupine inhibitors have been evaluated in preclinical models of cancer and/or are currently under clinical development for Wnt-driven cancers.[33] For example, RXC004 has been evaluated in preclinical models of colorectal and pancreatic and other cancer models in which it inhibits tumor cell proliferation by inducing epithelial differentiation and suppressing immune cell evasion by tumor cells by activating the host immune response.[34] ETC-159 induces tumor regression in patient-derived xenograft models by decreasing tumor cell proliferation and the expression of stem cell markers, and by promoting differentiation of tumor cells from an adenocarcinoma phenotype to a mucinous phenotype.[35] CGX1321 also induces tumor cell differentiation and promotes tumor cell apoptosis in rodent models of cancer.[36] Efficacy data in clinical studies of porcupine inhibitors are limited. However, an open-label phase 1 study of WNT974 in participants with various advanced cancers reported that WNT974 treatment reduced tumor size in 74% of participants.[23]

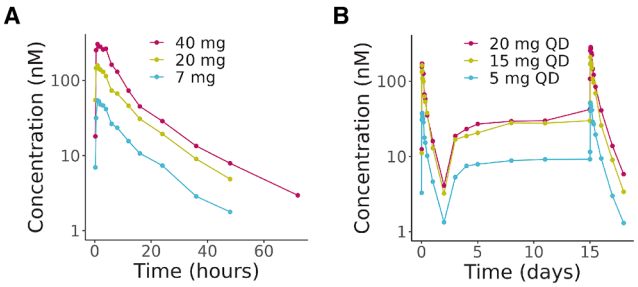

Figure 4. The observed AZD5055 plasma concentrations

Geometric means of AZD5055 plasma concentrations after single dose administration ($N = 6$ per cohort) in (A) and day 1 dose administration followed by once daily dosing on days 3–16 for different doses of AZD5055 in (B) ($n = 8$ for 5 mg QD; and $n = 9$ for each of the 15 and 20 mg QD doses).

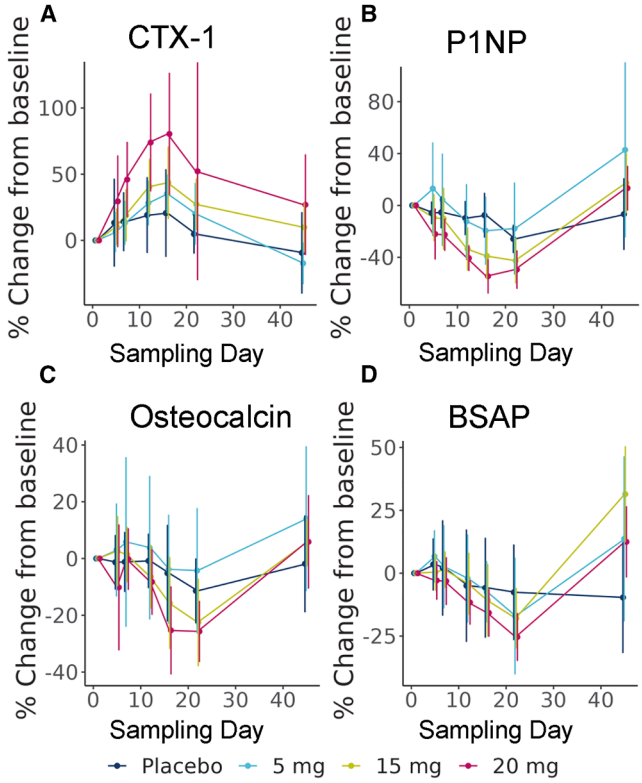

Figure 5. The effect of AZD5055 on changes in serum bone turnover biomarkers in healthy volunteers in part 2

The plots in (A-D) show the percentage change from baseline (y axis) at different time points (x axis) for the circulating bone degradation biomarker CTX-1 (in A) and bone formation biomarkers (P1NP in B, osteocalcin in C and BSAP in D). Treatment with once daily AZD5055 started on day 1 (no dose was given on day 2) and continued on days 3–16. The y axis indicate the mean percentage changes in biomarker levels compared to baseline values. The error bars indicate the standard deviation of the mean (SD); $n = 8$ in the placebo group; and $n = 6$ in the AZD5055 5 mg group and $n = 8$ in each of the AZD5055 15 and 20 mg groups.

Chronic fibrotic diseases are other potential therapeutic indications for these molecules as excessive Wnt signaling has been implicated in their pathogenesis. Chronic fibrosis can affect many organs including the lung, liver, and heart and is a major cause of morbidity and mortality worldwide.[37] Idiopathic pulmonary fibrosis (IPF), the most common chronic fibrotic interstitial lung disease, has a median survival of only 3–5 years after diagnosis.[38] Wnt signaling has been strongly linked to the pathogenesis of IPF as exuberant activation of Wnt pathway genes including Wnt-1 inducible signaling protein-1 (WISP-1), have been demonstrated in lung samples from patients with IPF.[39,40] Elevated expression of low-density lipoprotein receptor-related protein 5 (a co-receptor for Wnt ligands) is associated with IPF progression as assessed by categorical relative decline in forced vital capacity or diffusing capacity for carbon monoxide clinical endpoints.[41] Interestingly, our study identified Wnt7a protein levels as a circulating AZD5055-responsive biomarker, and another study reported that Wnt7a expression is upregulated in basal epithelial cells from patients with IPF, and that Wnt7a promotes pulmonary fibrosis in bleomycin-challenged mice.[42] Antibody-mediated neutralization of WISP-1 in bleomycin-challenged mice reduced pulmonary fibrosis and improved survival,[40] highlighting the therapeutic potential of porcupine and other Wnt inhibitors in IPF.

Chronic liver diseases are a major global burden.[43] Chronic hepatic injury results in fibrosis by inducing hepatic stellate cells (HSC) to transdifferentiate into hepatic myofibroblasts that produce interstitial collagens.[44] Hepatic fibrosis has been linked to excessive Wnt signaling as elevated β-catenin levels occur in human fibrotic livers.[45] Polymorphism in key Wnt pathway genes in males are associated with hepatic fibrosis secondary to chronic hepatitis C viral infection.[46] Inhibition of Wnt signaling with a selective inhibitor of the cyclic adenosine monophosphate-response element-binding protein-binding protein (CBP)/β-catenin (PRI-724) in a murine model of hepatic fibrosis suppressed fibrosis by promoting HSC apoptosis and increasing levels of extracellular matrix (ECM) protein-degrading matrix metalloproteinases.[47]

Myocardial fibrosis is associated with poor outcomes.[48] Wnt signaling has been strongly linked to myocardial fibrosis as Wnt ligands promote *trans*-differentiation of cardiac fibroblasts into ECM protein-producing myofibroblasts and elevated circulating levels of Wnt5a occur in patients with heart failure.[49,50] In addition, genetic deletion of β-catenin in cardiac fibroblasts in mice subjected to cardiac pressure overload resulted in decreased interstitial fibrosis and cardiomyocyte hypertrophy.[51]

There is an emerging body of evidence that porcupine inhibitors have anti-fibrotic effects. In experimental models of fibrosis, WNT974 prevented progression of skin fibrosis[11] and promoted heart repair following cardiac injury by reducing collagen deposition.[10] AZD5055 has been shown to have anti-fibrotic effects *in vitro* by increasing differentiation of alveolar epithelial type II cells into alveolar type I cells thereby promoting normal epithelial repair.[14] Thus, AZD5055 has therapeutic potential across a broad range of fibrotic indications associated with high unmet medical need. However, to the best of our knowledge, porcupine inhibitors have not yet been evaluated in clinical studies in patients with fibrotic diseases.

In conclusion, AZD5055 had an acceptable safety and tolerability profile over 14 days of consecutive dosing in healthy volunteers with a half-life supporting a convenient once daily oral dosing regimen. Inhibition of Wnt signaling in multiple

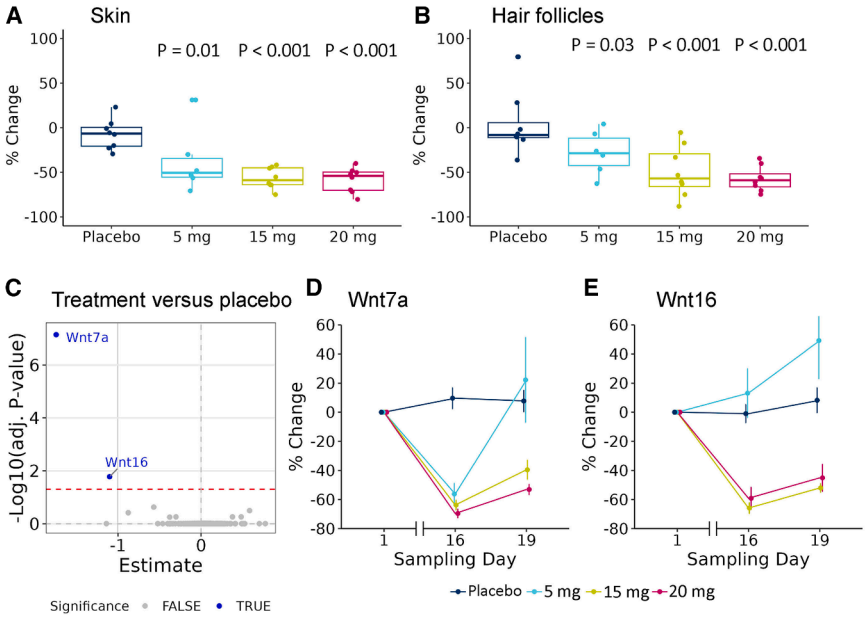

Figure 6. AZD5055-mediated target engagement in skin and hair follicles and a high throughput proteomic analysis of serum samples

(A and B) Percentage change in *AXIN2* mRNA expression from baseline by treatment group in skin biopsies (A) and hair follicle samples (B). Quantitative RT-PCR for *AXIN2* mRNA levels were normalized to the geometric mean of three housekeeping genes (*HPRT1*, *IPO8*, and *POLR2A*). Change from baseline relative to placebo in *AXIN2* gene expression data ($2^{-\Delta\Delta}$Ct) were calculated for each healthy participant.

(C) Volcano plot showing serum proteins that were statistically significantly affected by treatment with AZD5055 on day 16 when compared with the placebo group. The x axis indicates the estimate of coefficients of interaction between treatment and time point (day 16) that contributed to the protein levels in a mixed linear regression model. The y axis indicates –log10 adjusted *p* value of the coefficient.

(D and E) Percentage changes in Wnt7A (D) and Wnt16 (E) protein levels compared to baseline levels by treatment group on days 1, 16, and 19. In (C–E), proteomic data were generated from serum samples taken on day 1 pre-treatment, day 16 (15 days of once daily treatment in total) and day 19 (3 days post last dose). Error bars indicate the standard error of the mean (SEM). For the placebo group, $n = 8$ on day 1, $n = 8$ on day 16, and $n = 7$ on day 19; for the 5 mg treatment group, $n = 6$ on day 1, $n = 6$ on day 16, and $n = 5$ on day 19; for the 15 mg treatment group, $n = 8$ on day 1, $n = 8$ on day 16, and $n = 8$ on day 19; for the 20 mg treatment group, $n = 8$ on day 1, $n = 8$ on day 16, and $n = 8$ on day 19.

compartments in humans was achieved with 14 days of once daily dosing with 5–20 mg of AZD5055. However, the magnitude of inhibition of Wnt signaling needed to achieve efficacy in oncologic and fibrotic diseases needs to be determined in future clinical studies and longer-duration clinical studies are warranted to investigate the efficacy and safety of AZD5055 in these diseases.

Limitations of the study

Our study has several limitations including small sample sizes, short duration of treatment, and the predominantly male study population. Most of the healthy volunteers who enrolled in the study were males with a mean age of 35 years who are likely to be younger than patients with cancer or chronic fibrosis, which may reduce the generalizability of our findings. Another limitation of our study is that the number of female participants who were enrolled in this study was not sufficient to perform an analysis of the influence of sex on any of the outcomes evaluated. The influence of sex on the safety, tolerability, and efficacy of AZD5055 could be evaluated in future clinical studies in patients with diseases affecting both sexes. In addition, this study did not provide longer-term safety data (beyond 14 days of dosing) which is especially relevant to the potential risk of loss in bone density that is known to be associated with inhibition of Wnt signaling in man. Previous clinical studies of Wnt or porcupine inhibitors that included prophylactic treatment with anti-resorptive agents did not report any clinically significant bone loss or fragility fractures.[52,53] Longer-duration studies of AZD5055 in diseased populations are needed to further evaluate the safety of AZD5055 and should include a robust safety monitoring and therapeutic mitigation plan to minimize the potential risk of loss of bone density and the development of fragility fractures.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Caroline A. Owen, MD, PhD. Email: caroline.owen@astrazeneca.com.

Materials availability

This study did not generate any reagents.

Data and code availability

Data: All data reported in this paper will be shared by the lead contact, Caroline A. Owen (caroline.owen@astrazeneca.com) upon request.
Code: This paper does not report original code.
Other: Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

We thank the participants who took part in the clinical study of AZD5055. This study was sponsored by AstraZeneca BioPharmaceuticals R&D.

AUTHOR CONTRIBUTIONS

Conceptualization: C.A.O., X.-H.Z, S.P., E.L., E.H., and M.G.B.; methodology: C.A.O., N.F., K.H., Z.L., Z.B., A.P., Z.B., J. A., M.L.W., and S.P.; Investigation: C.A.O., E.L., X.-H.Z., O.S., C.G., B.G., and R.G.; statistical analysis: S.N.; writing – original draft: E.L. and C.A.O.; writing – review and editing: All authors; supervision: R.G. and C.A.O.

DECLARATION OF INTERESTS

All authors, except R.G., are employed by AstraZeneca, receive salaries from AstraZeneca and may own stock in AstraZeneca. Some of the authors employed by AstraZeneca have patent applications/registrations related to this

work. R.G. is employed by Parexel which was contracted by AstraZeneca to conduct the study.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2025.112602.

Received: November 11, 2024
Revised: March 11, 2025
Accepted: May 2, 2025

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Immunoassays		
CTX1, beta-Crosslaps ECLIA, COBAS Roche	Nordic Biosciences	07026960190 Nordic cat. 9001EX01. Roche cat 09005781190
Osteocalcin ECLIA, COBAS Roche	Nordic Biosciences	07027591190 Nordic cat. 9002EX01. Roche cat 07027591190
P1NP ECLIA, COBAS Roche	Nordic Biosciences	07027940190 Nordic cat. 9003EX01. Roche cat 07027940190
BSAP ELISA	Nordic Biosciences	Nordic cat. 9103AX01. IDS cat AC-20F1
Critical commercial assays		
NULISAseq™ Inflammation Panel 250	Alamar Biosciences	https://alamarbio.com/products-and-services/nulisa-inflammation-panel/
Oligonucleotides		
qRT-PCR primers		N/A
AXIN2	ThermoFisher	Hs00610344_m1
HPRT1	ThermoFisher	Hs02800695_m1
IPO8	ThermoFisher	Hs00914057_m1
POLR2A	ThermoFisher	Hs00172187_m1
Software and algorithms		
R 4.1.0		N/A
Phoenix® WinNonlin® Version 8.1	Certara	Add link https://www.certara.com/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Study participants

Human participants

This Phase 1 study (NCT05134727) was conducted exclusively on the homo sapiens species. The study evaluated healthy adult men and adult women of non-child-bearing potential. All races and ethnicities were eligible to enroll. For Part 1, men and women between 18 and 55 years of age (inclusive) were eligible. For Part 2, men between 18 and 49 years of age (inclusive) and women between 18 and 55 years or age (inclusive) were eligible. Data on the age, sex, race, race, and ethnicity of the participants who enrolled are provided in Table 1. Data on the ancestral origin of the study participants are not available as no samples were collected to perform this analysis. Healthy adult participants were selected to avoid interference from disease processes or other drugs. The study was conducted in compliance with International Council for Harmonisation Good Clinical Practice guidelines.

Animals

No animals other than homo sapiens were evaluated in this study.

Plants

No studies of plants were included in this manuscript.

Microbe strains

No studies of microbial strains were included in this manuscript.

Cell lines

No cells lines were studied in this manuscript.

Primary cell cultures

No primary cell cultures were studied in this manuscript.

Ethics

This study was reviewed and approved by the US Food and Drug Administration (FDA) and an Institutional Review Board (Advarra, Inc. USA). All participants signed informed consent forms prior to the start of the study.

Study design

This randomized, double-blind, placebo-controlled phase I study (NCT05134727) in healthy participants was conducted at a single site (Parexel Early Phase Clinical Unit, Baltimore, Maryland, United States) between November 18, 2021 and March 31, 2023.

The design was supported by and based on the nonclinical data package that was submitted to the FDA. This study consisted of two parts. Part 1 was a single ascending dose (SAD) study with 3 cohorts of healthy participants dosed with either a single dose of oral AZD5055 (7, 20, or 40 mg) or placebo. In each SAD cohort, 6 healthy participants were randomized to receive AZD5055, and 2 were randomized to receive placebo. Part 2 was a multiple ascending dose (MAD) study with 3 cohorts of subjects dosed once daily with either oral AZD5055 or placebo over a total of 15 days (on day 1 and on days 3–16). In the initial MAD cohort, 6 healthy participants were randomized to receive 5 mg AZD5055, and 2 were randomized to receive placebo. In the second and third MAD cohorts, 8 healthy participants were randomized to receive 15 or 20 mg AZD5055, respectively, and 3 to placebo. Data from placebo-treated participants in each cohort in Part 1 and Part 2 were pooled to generate separate pooled placebo groups for Part 1 and Part 2.

Single oral doses of 7, 20 or 40 mg of AZD5055 were studied in Part 1 of the study and once daily dosing with 5, 15 or 20 mg of AZD6793 for 14 days were studied in Part 2 of the study. The dose escalation increments were set to allow for minimum exposure overlap between doses without exceeding the pre-defined exposure limits. In Part 1 of the study, a SAD approach was performed to investigate the safety and PK of AZD5055 administered as single dose. Sentinel dosing was incorporated as a mitigation strategy and these participants were enrolled (1 on active treatment and 1 on placebo for all SAD cohorts) and monitored for adverse events for 6 days post the dose for SAD cohorts before continuing to the next dose level.[54]

In Part 2, AZD5055 was administered as multiple doses after the data from a corresponding or higher total daily dose in the SAD study had been reviewed by the safety review committee and concluded it to be safe and well tolerated. Each MAD cohort was initiated with a sentinel cohort (2 participants on active and 1 on placebo) and all participants were monitored for 6 days post the last dose before the next dose escalation. In addition, all participants in MAD cohorts were followed up until 29 days post the last dose to monitor the changes in bone turnover biomarkers. Multiple dosing allowed for steady state to be reached to further investigate the safety, tolerability, and PK of AZD5055. The duration of consecutive daily dosing for Part 2 was 14 days as this duration was deemed to be needed for a robust assessment of the potential effect of AZD5055 on bone turnover biomarkers.

Stopping rules for dose escalation are described in the clinical study protocol (CSP), provided in supplemental information, and included the systemic exposure of AZD5055, adverse event, and laboratory, bone, and cardiovascular criteria.

Participants

Healthy males and females of non-childbearing potential of any race or ethnicity weighing at least 50 kg with a body mass index between 18 and 30 kg/m^2 (inclusive) were included in this study. For Part 1, men and women between 18 and 55 years of age (inclusive) were eligible. For Part 2, men between 18 and 49 years of age (inclusive) and women between 18 and 55 years or age (inclusive) were eligible. Key exclusion criteria for Part 1 and Part 2 included a history or presence of any clinically important disease or disorder that might influence the results of the study; any clinically important abnormalities in rhythm, conduction or morphology of the resting ECG and any clinical important abnormalities in the 12-lead ECG that may interfere with the interpretation of QTc interval changes; a history of osteoporosis, osteomalacia, Paget's disease of the bone, thyrotoxicosis, rheumatoid arthritis, Cushing's disease or a pathological fracture; and a history of traumatic fracture within 6 months of the screening visit. Other key exclusion criteria included any abnormal values related to liver or renal function. For Part 2, postmenopausal women with a T score less than −1 as measured by dual energy X-ray absorptiometry (DXA) were excluded. Male and pre-menopausal women with a *Z* score of less than −1.5 as measured by DXA were also excluded. The complete list of inclusion and exclusion criteria is available in the supplemental information.

Randomization and masking

Sentinel dosing was included in each cohort in both parts. In Part 1, 2 sentinel participants were randomized in each cohort in a 1:1 ratio to receive either AZD5055 or placebo. In Part 2, 3 sentinel participants in each cohort were randomized in a 2:1 ratio to receive either AZD5055 or placebo. Following the sentinel dosing, the remaining participants were randomized in a 5:1 ratio for Part 1 and either 5:1 ratio (cohort 1) or a 3:1 ratio (cohorts 2 and 3) in Part 2. Randomization codes were generated using the AZRand system and assigned sequentially as eligibility of participants for randomization was confirmed.

Treatment and dose selection rationale

The starting dose was selected based on data from the pre-clinical toxicological studies, according to FDA Guidance for Industry "Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers" (July 2005). The exposure limits were pre-defined by the maximum exposure obtained in the non-clinical toxicological studies.

In Part 1, the selected starting dose was 7 mg as this dose was predicted to be a pharmacological active dose based on pre-clinical data using rodent models of fibrosis, as presented in CSP section 3.2.2.4. However, the observed exposure in the participants at the 7 mg dose was higher than the predicted systemic exposure. Thus, a lower dose range than had been planned, was subsequently explored. The subsequent doses (20 mg and 40 mg) were escalated up to the pre-defined exposure limit which was 2.5 μmol/L for Cmax and 3.9 μmol*h/L for AUC. The exposure limits were based on 50% of the NOAEL in the 1-month dog toxicology study, as presented in the CSP section 3.2.2.6. Participants received a single oral dose of AZD5055 or placebo on Day 1 and were resident

at the clinical unit until at least 72 h after IMP administration. A follow-up visit 6 ± 1 day after the IMP administration was scheduled.

Based on the PK observed after single doses, 15 mg once daily was predicted to be a dose to achieve 24-h coverage of 3-fold the *in vitro* IC50 potency assay value with a trough AZD5055 concentration of 0.023 μmol/L, predicted to result in continuous inhibition of Porcupine >75% in magnitude across the dosing interval. This magnitude of inhibition of Porcupine is hypothesized to be sufficient to reduce the progression of pulmonary fibrosis in patients with IPF and progressive pulmonary fibrosis (a hypothesis that would be evaluated in subsequent clinical studies). The 3 × *in vitro* IC50 approach is supported by clinical data published on WNT974, a porcupine inhibitor currently in clinical development for the treatment of patients with advanced cancers.[23]

Based on the single dose data, the planned doses for Part 2 were 5, 15 and 30 mg, separated to reduce overlap in systemic exposure. However, due to variability in the PK parameters observed for the 15 mg dose group, the highest dose allowed by the exposure limits was 20 mg once daily for the third MAD cohort. In Part 2, participants received a single QD morning dose of AZD5055 or placebo on Day 1 and on Days 3 through Day 16 with no dosing on Day 2 to permit PK sampling for 48 h after the initial dose, leading to a total of 15 days of treatment with 14 consecutive days of dosing from Day 3 through Day 16. Participants were discharged on Day 19 at least 72 h after the last IMP dose, after all the samples were collected and all assessments were performed. Two follow-up visits occurred within 6 ± 1 day (for safety evaluations) and within 29 ± 2 days (for collection of bone turnover biomarkers) after the last AZD5055 dose.

Procedures

Adverse events, vital signs, physical examinations, electrocardiograms, telemetry, and clinical laboratory tests (hematology, clinical chemistry, and urinalysis) were collected throughout the study.

Blood was obtained throughout the study treatment for pharmacokinetic and exploratory biomarker analyses. Skin and hair follicle biopsies were taken in all subjects in Part 2 on Day-1 and Day 16 (8 ± 2 h after the last dose) for assessment of target engagement. Hair follicles were collected from the lower region of the back of the head using micropunch (2 mm) technique and skin biopsies (4 mm) were also collected from the same region.

Sample size determination

The sample size for Part 1 and 2 was estimated based on a risk mitigation strategy (i.e., exposing a minimum number of participants to the study drug), previous experience in Phase I studies, and the sample size needed to show significant differences in *AXIN2* mRNA expression in the AZD5055 versus placebo-treated patients based on data from other clinical studies of other porcupine inhibitors (Part 2 only).[23] Thus, it was estimated that 6 participants completing each cohort on active drug would provide 80% power to detect a difference in means of 70% in *AXIN2* gene expression levels in hair follicles assuming that the common standard deviation (SD) is 35% using a two-group t-test with a 5% two-sided significance level. The SD assumed is the upper 80% CI for the SD observed for percentage reduction in *AXIN2* mRNA levels in a clinical study of another porcupine inhibitor.[55] To enable an adequate analysis of the PK, safety, and pharmacodynamics of AZD5055, 6 evaluable subjects were selected in the AZD5055 arm for all cohorts in Part 1 and cohort 1 in Part 2. Eight evaluable subjects were selected in the AZD5055 treatment arms in cohorts 2 and 3 in Part 2. In the placebo groups, 6 evaluable participants were selected and pooled across all cohorts in Part 1 and 8 evaluable subjects were selected and pooled across all cohorts in Part 2 (2 in cohort 1 and 3 in each of cohorts 2 and 3).

METHOD DETAILS

Safety assessment

Safety and tolerability were assessed during the study by monitoring adverse events (AEs), vital signs, physical examination findings, electrocardiogram (ECG) findings, and laboratory tests (i.e., hematology, clinical chemistry, and urinalysis).

Pharmacokinetic assessment

Venous blood samples for analysis of plasma AZD5055 concentrations were taken on Day 1 in Parts 1 and 2 and on Day 16 in Part 2. In Part 1, samples were taken at pre-dose, 15 min, 30 min and at 1,1.5, 2, 3, 4, 6, 8, 12, 16, 18, 36, 48 and 72 (40 mg only) hours post-dose. In Part 2, sampling was the same excluding the 72 h sample on Day 1 and excluding the 16 and 36 h sample on Day 1 and 16. Trough samples were taken pre-dose on Days 4, 5, 6, 9 and 12. Urine for analysis of AZD5055 was collected on Day 1 in Part 1 and 2 and on Day 16 in Part 2 at intervals (0–6, 6–12, 12–24, 24–48 and 48–72 h) post dose. In Part 2 on Day 1, the last interval listed above for part 1 was omitted.

AZD5055 was quantified in human plasma and urine samples using validated bio-analytical methods at Labcorp Early Development Laboratories Inc., Madison, Wisconsin, US. The method employed protein precipitation followed by liquid chromatography with tandem mass spectrometric (LC-MS/MS) detection in the positive ion mode. The method for plasma was validated in the range of 1.00 (LLOQ) to 600 nmol/L and the method for urine was validated in the range of 100 (LLOQ) to 60000 nmol/L using a 25 μL sample volume. The intra-batch and inter-batch precision for both the plasma and urine quantification, reported as coefficient of variation (CV), were well below the accepted ≤15% (≤20% at LLOQ) at all levels. Intra-batch and inter-batch bias were well within the accepted 15% (±20% at LLOQ) of the nominal concentration at all levels. To verify the reliability of the reported sample analyte concentrations in plasma, and to support the accuracy and precision of measurements established with spiked control samples, incurred

sample reanalysis was also performed. It was observed that 100% (114 of 114) of the repeat results and original results were within 20% of the mean of the two values, which is well within the acceptance criteria in the current regulatory guidance.

Pharmacodynamics assessments

Target engagement

Target engagement was evaluated by measuring mRNA expression of *AXIN2*, which is downstream target of Wnt signaling. Skin and hair follicles samples were collected from subjects enrolled in Part 2 (MAD) on Day −1 (baseline) and on Day 16 (following 14 consecutive days of daily AZD5055 treatment) 8 ± 2 h post the last dose of AZD5055. The RNeasy Plus Universal Mini Kit (Qiagen) and the PicoPure RNA Isolation Kit (Qiagen) were used to extract RNA from skin and hair follicle tissues, respectively, using an optimized version of the manufacturer's protocol. Quantitative RT-PCR (QuantStudio 7 Flex Real-Time PCR System) was performed to analyze target gene expression using the primers included in the key resources table. Quantitative RT-PCR expression data for *AXIN2* mRNA were normalized to the geometric mean of three optimized housekeeping genes (*HPRT1*, *IPO8*, *POLR2A*). Change from baseline in *AXIN2* gene expression was calculated for each participant in Part 2 using the $2^{-\Delta\Delta Ct}$ method in skin and hair follicle biopsy samples. Data from 8 healthy participants who received placebo were pooled across the cohorts for null hypothesis testing of target engagement.

Serum high throughput proteomic analysis: Data processing and normalization

Serum samples (88 samples from 30 participants) were collected at baseline on Day 1 pre-dose, Day 16 pre-dose and on day 19 (3 days post last dose). Serum samples were stored at −80°C until analysis and were assessed for 250 protein analytes using the NULISAseq inflammation panel. NULISAseq assays were performed at Alamar Biosciences as described previously.[56] Briefly, serum samples stored at −80°C were thawed on ice and centrifuged at 10,000g for 10 min. Supernatant samples (10 μL) were plated in 96-well plates and analyzed with Alamar's Inflammation Panel 250 which targets mostly inflammation and immune response-related cytokines and chemokines. A Hamilton-based automation instrument was used to perform the NULISAseq workflow, starting with immunocomplex formation with DNA-barcoded capture and detection antibodies, followed by capturing and washing the immunocomplexes on paramagnetic oligo-dT beads, then releasing the immunocomplexes into a low-salt buffer, which were then captured and washed on streptavidin beads. Finally, the proximal ends of the DNA strands C on each immunocomplex were ligated to generate a DNA reporter molecule containing both target-specific and sample-specific barcodes. DNA reporter molecules were pooled and amplified by PCR, purified, and sequenced on Illumina NextSeq 2000.

For NULISAseq, sequencing data were processed using the NULISAseq algorithm (Alamar Biosciences). The sample- (SMI) and target-specific (TMI) barcodes were quantified, and up to two mismatching bases or one indel and one mismatch were allowed. Intra-plate normalization was performed by dividing the target counts for each sample well by that well's internal control counts. Inter-plate normalization was then performed using inter-plate control (IPC) normalization, wherein counts were divided by target-specific medians of the three IPC wells on that plate. Data were then rescaled, 1 was added and the data were log2 transformed to obtain NULISA Protein Quantification (NPQ) units for downstream statistical analysis.

Bone turnover biomarkers

In Part 2, participants fasted overnight prior to blood sample collection, which was obtained no later than 10 a.m. at screening and pre-dose on day 1, day 5, day 7, day 12, day 16 (last dose), day 22 (±1) and day 45 (±2). All post-baseline blood samples were collected at the same timepoint as the baseline sample. Bone turnover biomarkers were analyzed in serum samples at Nordic Bioscience (Herlev, Denmark) using Electrochemiluminescence immunoassays (ECLIA) assays to quantify carboxy-terminal cross-linked telopeptide of type 1 collagen (CTX1, beta-Crosslaps COBAS Roche), procollagen type 1 N propeptide (P1NP, COBAS Roche) and osteocalcin (COBAS Roche). Serum bone alkaline phosphatase (BSAP/Ostase/BAP) was quantified using a quantitative sandwich enzyme linked immunosorbent technique (ELISA, Nordic Biosciences). The intra-assay and inter-assay variation (coefficients of variation (CVs)) were $\leq 4\%$ and $\leq 6\%$ for the CTX1 assay, and $\leq 1\%$ and $\leq 7\%$ for the P1NP assay, and $\leq 3\%$ and $< 5\%$ for the ostecalcin assay, and $\leq 4\%$ and $< 11\%$ for the BSAP assay.

QUANTIFICATION AND STATISTICAL ANALYSIS

Evaluation of PK was performed using noncompartmental analysis (Phoenix WinNonlin version 8.1 (Certara USA, Inc., Princeton, NJ, 2018). AUC was calculated as AUC0–last+Clast/λz in which Clast is the last observed quantifiable concentration. λz is the rate constant estimated from individual linear regression of the terminal part of the log concentration versus time curve. The t½ was calculated by ln(2)/λz. Observations below LLOQ were set to missing and thus ignored in the analysis. Dose proportionality was analyzed based on a graphical analysis of dose-adjusted AUC and C_{max} and by using the power model approach. The intercept α and the slope β (in [AUC or C_{max}] = α*doseβ) together with associated 90% confidence intervals (CI) were estimated and presented for AUC and C_{max}, and dose proportionality was concluded if the CI of the slope included one and had a reasonable range.[57] The power model parameters were estimated using least-squares regression.

Continuous variables were summarized as means (SD), categorical variables as total numbers and percentages. For data presentation and statistical analyses, placebo groups from each dosing regimen were pooled. *AXIN2* gene expression data was summarized and analyzed as the fold change from baseline using the $2^{-\Delta\Delta CT}$ method in which ΔCT is the difference $\log_2$ normalized expression from the housekeeping genes. We used the ANOVA test for statistical analysis of the *AXIN2* gene expression data and

summarized the results as percent change from baseline relative to the placebo group with the standard error of the mean. We used MMRM (mixed model repeated measures) for statistical analysis of the bone biomarker data with fixed effect for treatment and random effect for subject and log-transformed baseline bone biomarker level as a covariate and treatment-by-visit interaction. The endpoint was defined as the log of the ratios of the post-baseline and baseline measurements. The results were back-transformed and presented as percent change from baseline for each treatment group with associated 95% confidence intervals. We used R4.1.0. for the statistical analysis.

ADDITIONAL RESOURCES

This trial is registered with ClinicalTrials.gov (NCT05134727).