

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Development and evaluation of a sublingual tablet based on recombinant Bet v 1 in birch pollen-allergic patients

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Keywords

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Abstract

Background: Sublingual immunotherapy (SLIT) applied to type I respiratory allergies is commonly performed with natural allergen extracts. Herein, we developed a sublingual tablet made of pharmaceutical-grade recombinant Bet v 1.0101 (rBet v 1) and investigated its clinical safety and efficacy in birch pollen (BP)-allergic patients.

Methods: Following expression in *Escherichia coli* and purification, rBet v 1 was characterized using chromatography, capillary electrophoresis, circular dichroism, mass spectrometry and crystallography. Safety and efficacy of rBet v 1 formulated as a sublingual tablet were assessed in a multicentre, double-blind, placebo-controlled study conducted in 483 patients with BP-induced rhinoconjunctivitis.

Results: In-depth characterization confirmed the intact product structure and high purity of GMP-grade rBet v 1. The crystal structure resolved at 1.2 Å documented the natural conformation of the molecule. Native or oxidized forms of rBet v 1 did not induce the production of any proinflammatory cytokine by blood dendritic cells or mononuclear cells. Bet v 1 tablets were well tolerated by patients, consistent with the known safety profile of SLIT. The average adjusted symptom scores were significantly decreased relative to placebo in patients receiving once daily for 5 months rBet v 1 tablets, with a mean difference of 17.0–17.7% relative to the group treated with placebo ($P < 0.025$), without any influence of the dose in the range (12.5–50 µg) tested.

Conclusion: Recombinant Bet v 1 has been produced as a well-characterized pharmaceutical-grade biological drug. Sublingual administration of rBet v 1 tablets is safe and efficacious in patients with BP allergic rhinoconjunctivitis.

Abbreviations

(A)AdSS, (average) adjusted symptom score; AIT, allergen immunotherapy; ARMS, average rescue medication score; BP, birch pollen; EU, endotoxin unit; GMP, good manufacturing practices; LC-MS, liquid chromatography coupled to MS; moDC, monocyte-derived dendritic cell; MS/MS, tandem MS; MS, mass spectrometry; PBMC, peripheral blood mononuclear cell; rBet v 1, recombinant Bet v 1.0101 proteoform; RP-UHPLC-UV, reversed-phase ultra high-performance liquid chromatography with UV detection; SLIT, sublingual immunotherapy; TEAE, treatment-emergent adverse event.

Birch pollen (BP) is a frequent cause of allergic rhinoconjunctivitis within northern America and Europe, with around 100 million people sensitized to the BP major allergen Bet v 1 (1). For such patients with pollinosis, allergen immunotherapy (AIT) was shown to reorient inappropriate Th2 immune responses and consequently to alleviate allergy symptoms (2–9). In a recent clinical study (10), subcutaneous AIT with either a BP extract or purified recombinant Bet v 1 (rBet v 1) exhibited similar safety and efficacy profiles, suggesting that a recombinant allergen may be used instead of a complex biological extract. Such recombinant allergens are molecularly well defined and as such could be manufactured

with a better batch to batch consistency than natural extracts (11, 12). However, although recombinant allergens are already widely used for component-resolved *in vitro* diagnosis, the complexity of developing pharmaceutical-grade recombinant allergens has still precluded their broad application as immunotherapeutics. As of today, only few recombinant allergens have been tested via the subcutaneous route to treat successfully patients allergic to grass or BP (10–16). However, none of them has been tested sublingually, despite the interest raised by the sublingual route as an alternative to subcutaneous injections to perform AIT (2, 3, 5, 7–9).

Herein, we report the development and in-depth characterization of recombinant Bet v 1.0101 under conditions compliant with good manufacturing practices (GMP). This molecule was adopted by the European Pharmacopoeia Commission as a Chemical Reference Substance for the determination by ELISA of Bet v 1 content in allergen extracts (17, 18). We also present the clinical evaluation of this rBet v 1 allergen formulated as a sublingual tablet, confirming its safety and efficacy as a treatment of BP-allergic patients.

Material and methods

Cloning, expression, and purification of rBet v 1

Recombinant Bet v 1.0101 (thereafter named rBet v 1) was expressed in *Escherichia coli* and purified as described in the Supporting Information. The recombinant protein was then freeze-dried, sieved, and formulated as a sublingual tablet following direct compression (19).

Immunological and structural characterization of rBet v 1

Detailed information on analytical methods used, including chromatography, capillary electrophoresis, mass spectrometry (MS), circular dichroism, fluorescence spectroscopy, dynamic light scattering, and crystallography, is provided in the Supporting Information. Oxidized rBet v 1 was obtained following incubation with a 30% hydrogen peroxide solution. Immunological methods and assays used, based on basophils, peripheral blood mononuclear cells (PBMCs), or monocyte-derived dendritic cells (moDCs), are described in the Supporting Information.

Clinical evaluation of a sublingual rBet v 1 tablet in patients with BP-induced allergic rhinoconjunctivitis

A randomized, double-blind, placebo-controlled phase II study was conducted in seven European countries and Russia (ClinicalTrials.gov no. NCT00901914) after approval by national ethics committees and regulatory authorities. The study was compliant with European Good Clinical Practices guidelines. Following written informed consent, patients with BP allergic rhinoconjunctivitis were selected based on a positive skin prick test (with a wheal diameter > 3 mm) or *in vitro* IgE reactivity (> 0.7 kU/l) to BP allergens and randomized to receive sublingually either placebo or active tablets

(the latter containing 12.5, 25 or 50 µg of freeze-dried cGMP-grade rBet v 1) once daily. Patient demographics and baseline characteristics are described within Table 1. Study subjects were excluded from participation if they were symptomatic to non-birch-related allergens during the BP season, if they had AIT with pollen from birch and/or another Betulaceae within the previous 10 years, or if they had non-birch-related asthma. Treatment was initiated 4 months before the expected start of the BP season and continued until about 1 week after the season. Safety was assessed on all patients who received at least one dose of treatment, following adverse event monitoring and safety laboratory testing. The primary efficacy endpoint was the average adjusted symptom score (AAdSS), reflecting both symptom severity and the use of rescue medication (20). AAdSS is subject specific and is calculated as the average of the daily adjusted symptom scores (AdSSs) over the evaluation period. Each day, the AdSS adjusts the daily RTSS for rescue medication usage. If a patient took rescue medication on one day, the AdSS is defined as the RTSS of that day or the AdSS of the day before, whichever is higher. The next day, the AdSS is defined as the RTSS of that day or the AdSS of the day before, whichever is higher. Rescue medications (oral and ophthalmic antihistamines and nasal corticosteroids) were provided for the management of severe ARC according to a stepwise regimen. Investigators could provide oral corticosteroids if participants remained symptomatic despite these treatments. The average rescue medication score (ARMS) was also evaluated as a read-out of efficacy. The daily rescue medication score (RMS) was derived as follows: 0 = no rescue medication taken; 1 = use of antihistamines (oral and/or ophthalmic); 2 = use of nasal corticosteroids; 3 = use of oral corticosteroids. If more than one class of rescue medication was taken on a particular day, the highest score was retained for the score of that day. Analysis of covariance or ANCOVA was used as a general linear model combining an analysis of variance with a linear regression. ANCOVA is the usual method to compare one continuous variable between two or more groups, taking into account the variability of other variables named covariates.

Further details on the trial design, inclusion criteria, treatment, and rescue medication usage are provided in the Fig. S1.

Results

Expression and purification of pharmaceutical-grade rBet v 1

Escherichia coli cells were transformed with the pMW172-Bet v 1 plasmid and incubated at 37°C in complex growth medium, with rBet v 1 expression induced by adding 0.1 mM isopropyl-beta-thio-galactoside. Using reversed-phase ultra high-performance liquid chromatography (RP-UHPLC-UV), the presence of three main proteins was detected in the cytoplasm of transformed bacteria (Fig. 1A). Masses of these three molecules were determined using MS with an accuracy below 2 ppm and found to correspond to theoretical masses of (i) intact Bet v 1 for the main peak, (ii) a similar molecule

Table 1 Patient demographics and baseline characteristics (FAS)

Variable	12.5 µg N = 115	25 µg N = 114	50 µg N = 108	Placebo N = 118	Total N = 455
Gender [n (%)]					
Male	67 (58.3)	50 (43.9)	52 (48.1)	50 (42.4)	219 (48.1)
Female	48 (41.7)	64 (56.1)	56 (51.9)	68 (57.6)	236 (51.9)
Age (years)					
Mean (SD)	37.5 (10.70)	37.5 (11.17)	35.7 (12.10)	36.9 (11.69)	36.9 (11.41)
[95% CI]	[35.5, 39.5]	[35.4, 39.5]	[33.3, 38.0]	[34.8, 39.1]	[35.9, 38.0]
Min–Max	18–59	19–64	18–63	18–64	18–64
BMI (kg/m ²)					
Mean (SD)	25.81 (4.124)	24.96 (4.455)	24.56 (3.772)	24.96 (4.278)	25.08 (4.182)
[95% CI]	[25.05, 26.58]	[24.14, 25.79]	[23.84, 25.28]	[24.18, 25.74]	[24.70, 25.47]
Min–Max	17.0–38.4	16.2–43.1	18.1–33.3	16.8–43.3	16.2–43.3
RRTSS					
Mean (SD)	14.2 (1.65)	14.0 (1.54)	14.0 (1.63)	14.2 (1.67)	14.1 (1.62)
[95% CI]	[13.9, 14.5]	[13.7, 14.3]	[13.7, 14.3]	[13.9, 14.5]	[13.9, 14.2]
Min–Max	12–18	12–18	12–18	12–18	12–18
FEV ₁					
Mean (SD)	103.5 (12.74)	100.1 (11.28)	102.2 (12.24)	103.0 (12.67)	102.2 (12.28)
[95% CI]	[101.2, 105.9]	[98.0, 102.1]	[99.9, 104.5]	[100.7, 105.3]	[101.1, 103.3]
Min–Max	81–132	80–133	78–137	81–138	78–138
Sensitization status [n (%)]					
Mono-sensitized	14 (12.2)	12 (10.5)	10 (9.3)	11 (9.3)	47 (10.3)
Polysensitized	101 (87.8)	102 (89.5)	98 (90.7)	107 (90.7)	408 (89.7)

BMI, Body mass index; CI, confidence interval; FAS, full analysis set; FEV₁, forced expiratory volume in 1 second; Max, maximum; Min, minimum; N, number of patients in each treatment group; RRTSS, retrospective rhinoconjunctivitis total symptom score; SD, standard deviation.

incremented with one methionine and (iii) a form comprising an additional formylmethionine. The latter two forms, representing around 20% of total protein content, correspond to nonmature allergens, as protein synthesis in bacteria is initiated with N-formylmethionine, with subsequent processing to the mature form by a deformylase and methionine aminopeptidase (21). Liquid chromatography–tandem MS (LC–MS/MS) peptide mapping confirmed the protein sequences of both the intact molecule and the two nonmature isoforms, with either a methionine (Fig. S2A) or a formylmethionine at the N-terminus. The amount of these impurities was reduced to <0.1% of total allergen content by replacing the complex growth medium with a defined broth consisting of salts, glucose, and magnesium.

When switching from a complex to defined medium, we observed a new peak eluted at 3.5 min by RP-UHPLC–UV analysis (Fig. 1B, arrow). MS characterization revealed that this peak is due to a small molecule with an experimental mass (MH⁺ ion) of 304.06386 Da (Fig. S2B) corresponding to the unique C₁₅H₁₃O₄NS formula, thereafter named P303. The latter co-purified with rBet v 1, consistent with the identification of P303 as a metabolite from *E. coli* noncovalently bound to rBet v 1. The purification process was optimized to decrease P303 to a concentration below 26 ng/ml (i.e., the current lowest level of detection) in the final bulk product. The endotoxin concentration was confirmed to be <0.002 EU/mg of protein.

Production of rBet v 1 at the 1500 l scale involved storage of over 10 l of final purified bulk product in sterile plastic bags with some air trapped in the bag. During prolonged storage, a prepeak was evidenced by RP-UHPLC–UV, eluting at around 4 min (Fig. 1C). MS/MS analyses confirmed that this distinct form of the molecule (always representing <1% of total allergen content) corresponds to rBet v 1 oxidized on methionine 139.

Characterization of rBet v 1

Using LC–MS, the intact mass of rBet v 1 was confirmed with an accuracy below 1 ppm (Fig. 1D), whereas the sequence was entirely covered using LC–MS/MS peptide mapping (data not shown). Hydrophobic heterogeneity and charge heterogeneity were assessed using RP-UHPLC–UV (Fig. 1E) and isoelectric focusing (Fig. 1F), respectively, confirming that purified rBet v 1 is highly homogenous, with an experimental pI of 5.45 (in agreement with the theoretical value 5.39). The secondary structure was investigated using circular dichroism (Fig. 1G), indicating the presence of both α -helical content and β -sheet regions. The quaternary structure was evaluated using size exclusion chromatography with UV (Fig. 1H) and multiangle light scattering detections (data not shown), revealing a monomeric molecule with a deduced mass around 18 kDa, confirmed by dynamic light scattering measurement (Fig. S2C). A qualitative assessment of the tertiary structure was obtained

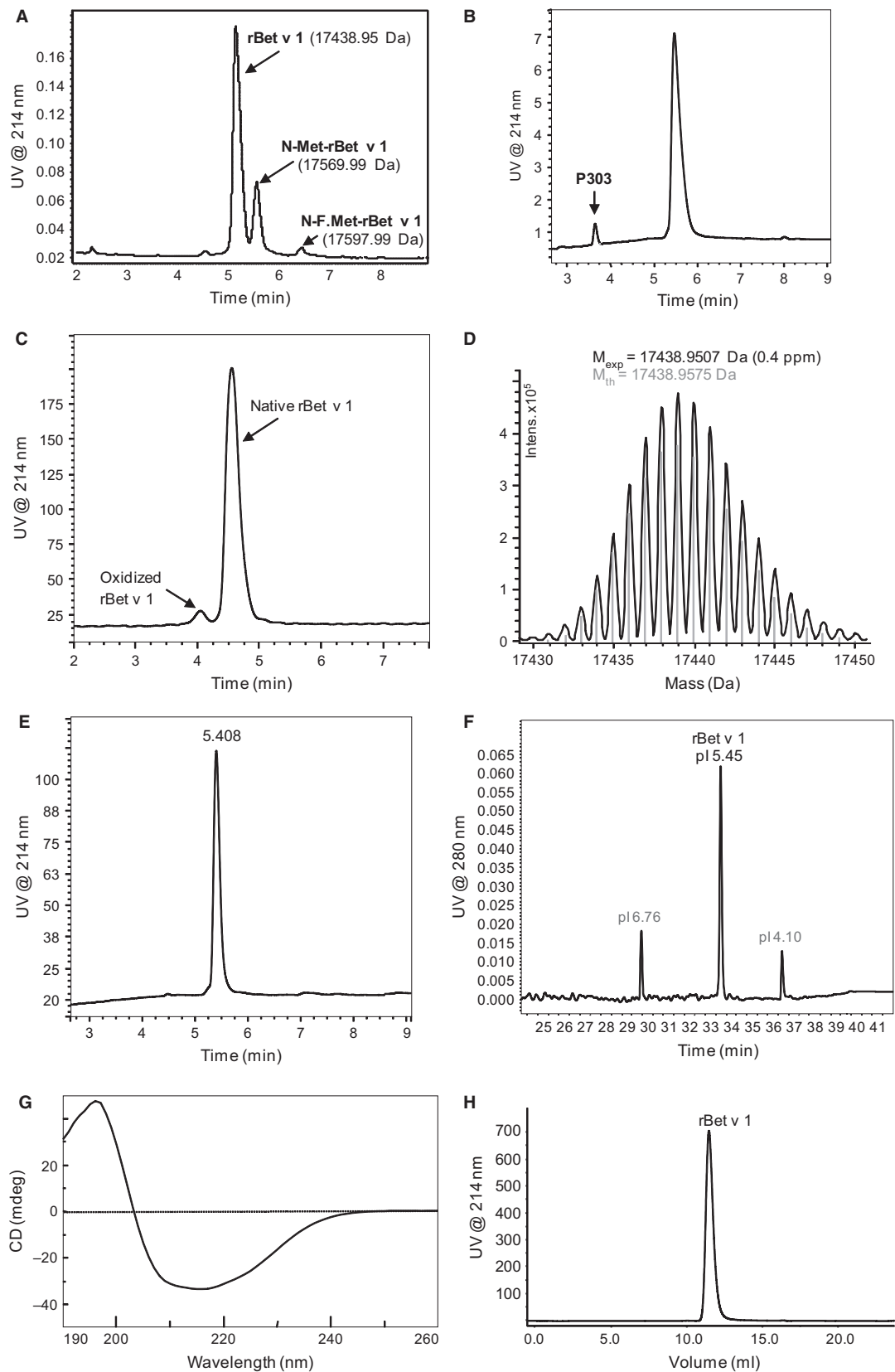


Figure 1 Physico-chemical and structural characterization of purified rBet v 1. RP-UHPLC-UV chromatograms of rBet v 1 expressed in complex growth medium (A, N-F. Met: N-formylmethionine) and defined growth medium (B). (C) Chromatogram of rBet v 1 stored in bags. (D) Overlay of deconvoluted theoretical (grey bars) and experi-

mental (black) MS spectra of rBet v 1. (E) RP-UHPLC chromatogram with UV detection at 214 nm. (F) Capillary isoelectric focusing electropherogram at 280 nm showing the rBet v 1 peak and pI markers at 6.76 and 4.10. (G) Circular dichroism spectrum acquired at 20°C. (H) Size exclusion chromatogram with UV detection at 214 nm.

using the fluorescent hydrophobic probe 1-anilino-8-naphthalene-sulphonate, confirming its binding properties (Fig. S2D).

The crystal structure of rBet v 1 in complex with P303 was solved to a 1.2 Å resolution. The allergen adopts the expected Bet v 1 fold, consisting of two short α -helices and a seven-stranded antiparallel β -sheet wrapping around a long C-terminal α -helix (Fig. 2A), in agreement with previously published structures (22, 23). The *E. coli* metabolite P303 is contained within a cavity, where it establishes hydrophobic contacts with the protein, principally through residues Phe22, Phe30, Tyr83, and Ile102 (Fig. 2B), without altering the binding site conformation. Our results confirm that the cavity is large enough (Fig. 2C) to accommodate various ligands (22–24), including quercetin-3-*O*-sophoroside, that is, the main natural ligand of Bet v 1 (25).

Lastly, functional studies confirmed that native and oxidized forms of rBet v 1 do not induce the production of any proinflammatory cytokine by blood dendritic cells or mononuclear cells (Fig. S3).

Safety and efficacy of sublingual immunotherapy with rBet v 1

Recombinant Bet v 1 was formulated as a fast disintegrating sublingual tablet (i.e., releasing the allergen in ≤ 2 min). Such sublingual tablets were tested in a pre-seasonal scheme in a cohort of 483 patients with BP-induced rhinoconjunctivitis, randomized (Figs 3A and S1) to receive placebo ($n = 120$) or either one of the 3 rBet v 1 doses (12.5 μg : $n = 123$; 25 μg : $n = 120$; 50 μg : $n = 120$). The full analysis set

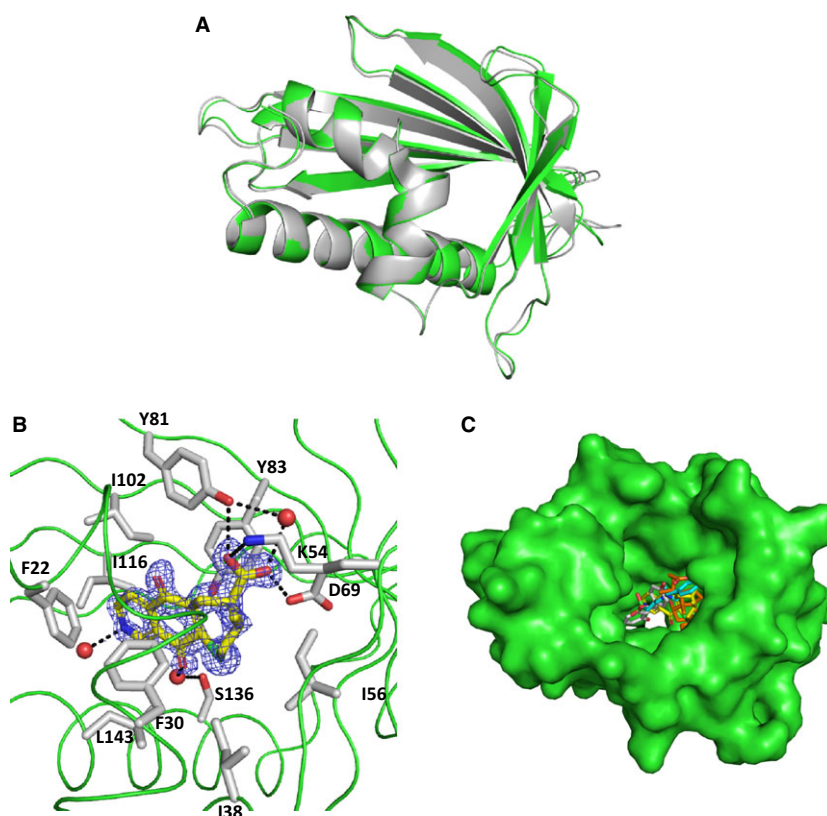


Figure 2 Crystal structure of rBet v 1. (A) The structure of rBet v 1 (green) was superposed to the previously published structure of Bet v 1-A (PDB entry: 4A88, grey). (B) Close-up view of the P303 binding site in the same orientation as in A, with the experimental $2F_o - F_c$ electron density contoured at 0.7σ . P303 and interacting rBet v 1 residues are coloured in yellow and white, respectively. Water molecules and hydrogen bonds are represented as red spheres and

dashed lines, respectively. (C) Structures of Bet v 1 in complex with small ligands were superposed to rBet v 1 (green) in complex with P303 (yellow), in the same orientation as in A and B. Previously described ligands are shown, including NDSB-265 (PDB entry: 4A8G) coloured in cyan, DXC (PDB entry: 4A83) in orange, and naringenin (PDB entry: 4A87) in white. For the purpose of clarity, only rBet v 1 is represented and the residues 22–30 were deleted.

consisted of 455 participants (placebo: $n = 118$; 12.5 μg : $n = 115$; 25 μg : $n = 114$; 50 μg : $n = 108$), 28 participants being excluded for not having at least one AAdSS while on

treatment. Patient demographics and baseline characteristics are summarized in Table 1. Overall, 90% of patients were polysensitized.

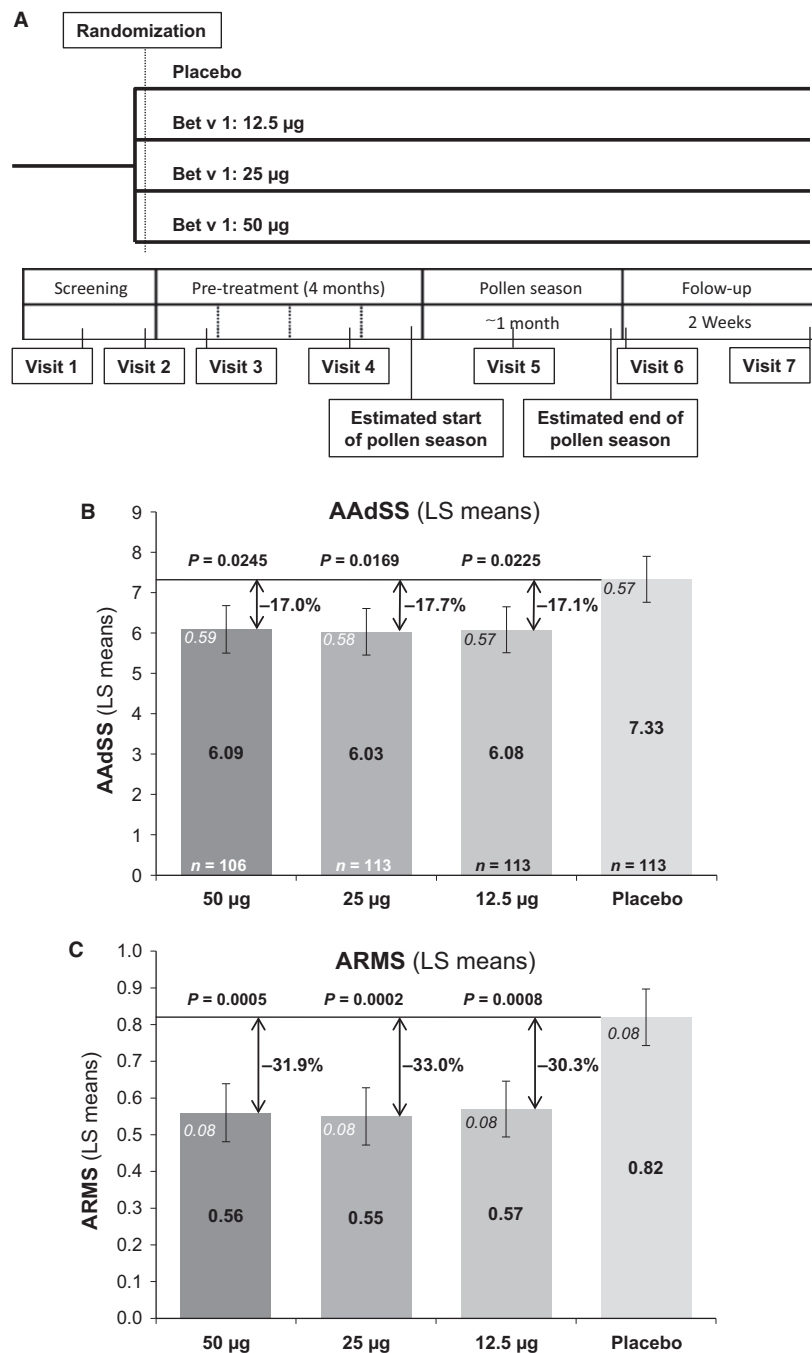


Figure 3 Design and clinical efficacy of sublingual rBet v 1 tablets. (A) The AAdSS was assessed using an analysis of covariance (ANCOVA) with treatment and pooled study centre as main effects, and age, gender, birch seasonal asthma status, and sensitization status (mono- vs polysensitized at baseline) as covariates. To control the overall type I error rate at 5%, treatment comparisons were performed using a step-down approach (50 μg rBet v 1 vs Placebo,

then 25 μg vs Placebo, then 12.5 μg vs Placebo). AAdSS (B) and ARMS (C) are shown for groups of patients receiving either placebo or rBet v 1 (12.5, 25, 50 μg) tablets. P -value (P), number of patients (n), least squares (LS), and standard errors are indicated in italics. Relative LS mean difference: $[(50 \mu\text{g} - \text{Placebo})/\text{Placebo}] \times 100$, $[(25 \mu\text{g} - \text{Placebo})/\text{Placebo}] \times 100$, or $[(12.5 \mu\text{g} - \text{Placebo})/\text{Placebo}] \times 100$, as appropriate.

A majority of participants (86.0% in the placebo group; 90.2%, 96.7%, and 95.8% in the 12.5, 25, and 50 µg groups, respectively) reported at least one treatment-emergent adverse event (TEAE), occurring during or after administration of the first dose of the investigational product (Table 2). Percentages of participants reporting drug-related TEAEs were higher in the active treatment groups than in the placebo group (i.e., 29.8% in the placebo group vs 73.0, 84.2, and 87.5% in the 12.5, 25, and 50 µg groups, respectively). Most common

TEAEs were primarily local reactions, including oral pruritus, mouth oedema, throat irritation, and ear pruritus (Table 2). Eight participants reported serious adverse events, none of which considered related to the investigational product. A total of 23 participants (four in the placebo group; three in the 12.5 µg group; four in the 25 µg group; 12 in the 50 µg group) prematurely withdrew from the study due to adverse events, the majority of which being application site reactions considered to be related to treatment (Table 2).

Table 2 Incidence of TEAEs reported by at least 5% of patients by SOC and PT

System organ class Preferred term	12.5 µg N = 122		25 µg N = 120		50 µg N = 120		Placebo N = 121		Total N = 483	
	n	%	n	%	n	%	n	%	n	%
Patient with at least one TEAE	110	90.2	116	96.7	115	95.8	104	86.0	445	92.1
Respiratory, thoracic, and mediastinal disorders	63	51.6	67	55.8	69	57.5	55	45.5	254	52.6
Throat irritation	40	32.8	39	32.5	42	35.0	7	5.8	128	26.5
Sneezing	16	13.1	19	15.8	18	15.0	23	19.0	76	15.7
Nasal discomfort	15	12.3	16	13.3	16	13.3	21	17.4	68	14.1
Cough	12	9.8	17	14.2	21	17.5	15	12.4	65	13.5
Rhinitis	15	12.3	14	11.7	18	15.0	17	14.0	64	13.3
Nasal congestion	10	8.2	15	12.5	12	10.0	19	15.7	56	11.6
Rhinitis allergic	10	8.2	10	8.3	8	6.7	12	9.9	40	8.3
Dyspnoea	6	4.9	13	10.8	10	8.3	8	6.6	37	7.7
Wheezing	7	5.7	8	6.7	8	6.7	4	3.3	27	5.6
Asthma	1	0.8	3	2.5	2	1.7	8	6.6	14	2.9
Pharyngeal oedema	4	3.3	2	1.7	6	5.0	1	0.8	13	2.7
Gastrointestinal disorders	75	61.5	90	75.0	87	72.5	28	23.1	280	58.0
Oral pruritus	57	46.7	61	50.8	64	53.3	11	9.1	193	40.0
Oedema mouth	9	7.4	13	10.8	10	8.3	0	0.0	32	6.6
Stomatitis	4	3.3	5	4.2	7	5.8	3	2.5	19	3.9
Glossodynia	3	2.5	7	5.8	7	5.8	1	0.8	18	3.7
Oral discomfort	3	2.5	4	3.3	6	5.0	1	0.8	14	2.9
Dyspepsia	3	2.5	4	3.3	6	5.0	0	0.0	13	2.7
Infections and infestations	43	35.2	46	38.3	47	39.2	49	40.5	185	38.3
Nasopharyngitis	22	18.0	23	19.2	20	16.7	27	22.3	92	19.0
Rhinitis	3	2.5	8	6.7	9	7.5	12	9.9	32	6.6
Respiratory tract infection	6	4.9	3	2.5	6	5.0	3	2.5	18	3.7
Influenza	7	5.7	1	0.8	3	2.5	3	2.5	14	2.9
Nervous system disorders	19	15.6	22	18.3	20	16.7	25	20.7	86	17.8
Headache	17	13.9	21	17.5	20	16.7	20	16.5	78	16.1
Ear and labyrinth disorders	16	13.1	14	11.7	21	17.5	2	1.7	53	11.0
Ear pruritus	14	11.5	14	11.7	20	16.7	2	1.7	50	10.4
General disorders and administration site conditions	9	7.4	7	5.8	10	8.3	6	5.0	32	6.6
Chest discomfort	5	4.1	6	5.0	5	4.2	5	4.1	21	4.3
Musculoskeletal and connective tissue disorders	18	14.8	4	3.3	6	5.0	4	3.3	32	6.6
Back pain	8	6.6	1	0.8	2	1.7	0	0.0	11	2.3
Skin and subcutaneous tissue disorders	7	5.7	9	7.5	7	5.8	10	8.3	33	6.8
Eye disorders	45	36.9	50	41.7	45	37.5	46	38.0	186	38.5
Eye pruritus	19	15.6	23	19.2	22	18.3	20	16.5	84	17.4
Conjunctivitis allergic	19	15.6	16	13.3	19	15.8	15	12.4	69	14.3
Lacrimation increased	10	8.2	10	8.3	11	9.2	11	9.1	42	8.7
Conjunctivitis	8	6.6	10	8.3	8	6.7	10	8.3	36	7.5
Immune system disorders	18	14.8	15	12.5	15	12.5	17	14.0	65	13.5
Seasonal allergy	15	12.3	13	10.8	11	9.2	12	9.9	51	10.6

N, Number of patients with event; N, number of patients in each treatment group; %, percentage of patients with event based on the total number of patients in each treatment group; PT, preferred term; SOC, system organ class; TEAE, treatment-emergent adverse event.

The analysis of primary endpoint data revealed statistically significant differences for each of the active groups compared to placebo in the AAdSS ($P < 0.03$, Fig. 3B) and ARMS ($P < 0.001$, Fig. 3C). Compared to placebo, treatment with rBet v 1 tablets resulted in a relative LS mean difference in AAdSS of 17.0–17.7% and a 30.3–33.0% decrease in the ARMS, depending upon the dose tested. No significant differences were observed between the three doses of rBet v 1 used in the tablets, with respect to their impact on either the AAdSS or the ARMS (Fig. 3B,C).

Discussion

Currently, AIT relies upon standardized allergen extracts obtained from natural sources, prepared and characterized as per GMPs and the 2008 European Medicines Agency Guideline on allergen products. Allergens produced by recombinant DNA technologies are generating considerable interest, in that they should improve product consistency, eventually yielding better characterized and highly purified biologicals for AIT (11, 12, 26, 27).

In this regard, we previously reported the characterization of pilot-scale GMP-grade rBet v 1 (28). Herein, we describe the development and characterization of pharmaceutical-grade rBet v 1 produced at an industrial scale (1500 l) in *E. coli*. Highly purified rBet v 1 was obtained with very low amounts (i.e., <1% of total allergen content) of nonmature (i.e., with additional N-methionine or N-formylmethionine) or oxidized forms. The documentation of rBet v 1 purity, as well as primary, secondary, and quaternary structures, revealed a highly homogenous, intact, and monomeric product with the expected α -helical and β -sheet contents. Tertiary structure assessment, performed using a fluorescent hydrophobic probe, confirmed binding properties consistent with previously published results (24). We also documented the 3D structure, solved to 1.2 Å resolution, of rBet v 1 complexed with an internal ligand (i.e., the *E. coli* metabolite P303) within the hydrophobic pocket. These data are in agreement with previously published Bet v 1 structures (22, 23), thought to represent the molecule in its natural conformation involved in patient IgE sensitization. Importantly, the structure of our highly purified pharmaceutical-grade Bet v 1, free from any ligand and used for AIT, was confirmed to be strictly identical to the one of rBet v 1 complexed with the P303 internal ligand.

We subsequently assessed the biological activity on immune cells of native rBet v 1 (29). In light of the detection of trace amounts of oxidized Bet v 1, we also investigated the potency of a fully oxidized allergen. Specifically, we assessed the capacity of these two molecules to activate human moDCs, PBMCs, and basophils. We observed that native and oxidized rBet v 1 did not induce any secretion of pro-inflammatory or effector cytokines (i.e., IL-1 β , IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IFN γ , and TNF- α) by PBMCs or moDCs (Fig. S3). This observation is in contrast with other reports suggesting that rBet v 1 may have some immunomodulatory activities, most particularly when the hydrophobic

cavity is empty (30, 31). Whereas the reason for this discrepancy is presently unclear, the lack of pro-inflammatory activities of our rBet v 1 may be explained by its high homogeneity (no substitutions detected) and purity, with only trace amounts of contaminating host cell proteins (≤ 50 ng/mg allergen), DNA (≤ 10 ng/mg allergen), and endotoxins (≤ 0.002 EU/mg allergen). Native and oxidized rBet v 1 exhibited a similar capacity to induce the CD203c activation marker in basophils from BP-allergic patients, and consequently, oxidized rBet v 1 can be considered as a drug-related substance.

Following formulation into a fast disintegrating tablet, rBet v 1 was evaluated sublingually in a cohort of 483 adults with BP-related rhinoconjunctivitis, using three different doses of rBet v 1 (i.e., 12.5, 25, and 50 μ g). This study was the first clinical trial conducted with a recombinant allergen via the sublingual route. The application site reactions observed, including mainly oral pruritus and throat irritation, are similar to those previously observed with sublingual immunotherapy (SLIT) regimens based on natural allergen extracts (2, 3, 5, 7, 8, 32, 33). SLIT with rBet v 1 resulted in a significant decrease of 17.0–17.7% of AAdSS, vs placebo, in BP-allergic patients, as well as a 30.3–33% decrease in ARMS, without any significant difference between the three active treatment groups. The latter observation could possibly be explained by the relative saturation of antigen-presenting cells with the lower dose of allergen, without any further improvement in allergen uptake by such cells when increasing the dose in the range tested.

Interestingly, these efficacy results obtained with rBet v 1 are consistent with the ones observed following SLIT with a BP natural extract (2, 9). A similar conclusion was obtained in a SCIT study comparing AIT with a BP extract vs purified natural or recombinant Bet v 1 (10). Thus, although a single allergen (Bet v 1) can be substituted to a whole pollen extract to treat BP-allergic patients, it appears that recombinant allergens *per se* do not exhibit a superior efficacy, when compared with allergen extracts. However, the purity and homogeneity of recombinant allergens can facilitate their formulation with specific vector systems to enhance their capture by antigen-presenting cells, thought to be the limiting factor in AIT efficacy (34). In support of this hypothesis, the formulation of rBet v 1 in carbohydrate-made mucoadhesive microparticles was shown to enhance significantly the efficacy of SLIT in BP-sensitized mice, by further downregulating TH2 responses, decreasing airway inflammation, and fastening the onset of action (35). Consequently, we believe that recombinant allergens such as rBet v 1 formulated with mucoadhesive particles to better target immune cells should be considered as second-generation options for sublingual AIT.

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Conflicts of interest

This work was funded by Stallergenes. EN, JB, MLM, PL, KJ, SH, LM, TB, KA, BR, ODB, and PM are employed by Stallergenes. The other authors declare no competing financial interest.

Authors' contributions

EN contributed to the expression, purification, and characterization of the molecule and wrote the manuscript. JB and MLM performed LC-MS analyses. PL and KJ performed LC-UV, CD, and fluorescence experiments. SH performed the functional characterization. LM supervised the functional characterization. RV, PhL, and AR performed the crystallog-

raphy study. MP and TB contributed to writing the manuscript. KA, BR, and ODB contributed to clinical study design and analysis. SR and MA coordinated the clinical study. PM supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Material and methods.

Figure S1. Clinical study participant flow.

Figure S2. Structural characterization.

Figure S3. Functional characterization of native and oxidized rBet v 1.

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Development and clinical evaluation in birch pollen-allergic patients of a sublingual tablet based on recombinant Bet v 1

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Material and methods

Cloning, expression and purification of rBet v 1

Recombinant Bet v 1.0101 (thereafter named rBet v 1) was expressed within *Escherichia coli* as a soluble protein after transfection with the T7 promoter expression vector pMW172-Bet v 1. Cell culture was performed at the 1,500 L scale and expression was obtained after isopropyl β -D-1-thiogalactopyranoside (IPTG) induction. Clarified bacterial lysates were then submitted to successive ion exchange (IEC), hydrophobic interaction (HIC) and hydroxyapatite chromatographies (HAC) in order to obtain highly pure (i.e. > 98%) Bet v 1.

Capillary IEF

A high performance capillary electrophoresis instrument (PA 800 plus Pharmaceutical Analysis SystemTM, Beckman Coulter) was used in order to determine the pI of rBet v 1 and assess both charged and size variants. Capillary isoelectric focusing (cIEF) separates protein isoforms using high voltage electrophoresis in a pH gradient. Briefly, a neutral coated capillary (Beckman Coulter), cooled at 20°C (dimension 30.2 cm x 50 μ m ID), was mounted into a capillary electrophoresis instrument (PA800plus, Beckman Coulter). 10 μ L of rBet v 1.0101 solution were mixed with 200 μ L cIEF gel, 20 μ L arginine solution (8.72 g%), 2 μ L iminodiacetic acid (2.66 g%), 12 μ L Pharmalyte 3-10 ampholin solution (GE Healthcare) and 1 μ L synthetic pI markers (Beckman Coulter). The sample solution was spiked with 2 pI standards (pI 7.0 and 4.1). Sample injection consisted in applying a 50 psi pressure during 99 secondes. Focusing was carried out by applying a voltage of 25 kV between the anolyte (200 mM H₃PO₄) and the catolyte (300 mM NaOH) for 8 min. Chemical mobilisation with 350 mM acetic acid at 21 kV for 35 min was monitored by UV absorbance at 280 nm.

Size exclusion chromatography

Size exclusion chromatography (SEC) was performed to evaluate both the purity and quaternary structure of rBet v 1 products. SEC allows the separation of analytes in aqueous non-denaturing solution based on their molecular size and shape. Therefore, SEC is able to separate protein monomers from their dimers and polymers without disrupting protein interactions. Briefly, the rBet v 1 was diluted in sodium phosphate buffer (NaP 0.05M, pH 7.2) to 0.25 mg/mL. The column was equilibrated in running buffer (NaP 0.05M, pH 7.2) before sample injection. 100 μ L of diluted product (25 μ g of protein) were injected on a Tricorn SuperdexTM 75 10/300GL column (1 x 30 cm, 24 mL total bed volume, GE Healthcare). The separation was performed at a flow rate of 0.7 mL.min⁻¹ using an EttanLC apparatus (GE Healthcare) controlled by Unicorn 5.10 software (GE Healthcare). The same software was used for chromatogram integration. Multi-angle light scattering detection was performed using a miniDAWN TREOS instrument (Wyatt Technology).

Dynamic light scattering

Dynamic light scattering (DLS) was performed using a Dynapro Nanostar instrument (Wyatt Technology) controlled by Dynamics software (version 7.1.1.16). Ten acquisitions of 5 seconds were averaged. Acquisition was performed at 25°C. The Mw-R model used was globular proteins with a Dn/dc (mL/g) value of 0.185.

Reversed phase UHPLC-UV

RP-UHPLC-UV was performed to quantify rBet v 1. This method also provides information on the sample's homogeneity. Proteins species were separated on a non-polar stationary phase with a gradient of hydrophobicity. Briefly, around 1 μ g of rBet v 1 solution were injected on a C₁₈ Hypersil Gold column (Thermo, 2.1 x 100 mm, 1.9 μ m), thermostated at 65°C. The

elution was performed using a linear gradient of acetonitrile (MeCN) with 0.1% trifluoroacetic acid (TFA, Table IS) and monitored by UV detection at 214 nm (for rBet v 1 and related substances) and at 292 nm (for P303 impurity).

Table IS. Conditions for RP-UHPLC-UV analysis

Time (min)	0	0.5	1.0	4.0	9.0	9.5	10.5	13.0
Flow (μL/min)	750							
H ₂ O (95%) / MeCN (5%) / 0.1% TFA (%)	100	100	60	33	14	0	100	100
H ₂ O (50%) / MeCN (50%) / 0.1% TFA (%)	0	0	40	77	86	100	0	0

LC-MS of the intact protein

Analysis of intact Bet v 1 (*i.e.*, without proteolysis) by LC-MS was carried out using an ESI-QToF MS Maxis (Bruker Daltonics) connected to a RSLC Ultimate 3000 (Thermo, Dionex) system. . Briefly, around 2 μg of rBet v 1 were injected onto a C₁₈ Hypersil Gold column (Thermo, 2.1 x 100 mm, 1.9 μm) at a flow rate of 0.750 mL/min and thermostated at 65°C. The increasing acetonitrile gradient performed is described in table IS.

The TOF analyser was externally calibrated before the analysis using the solution ESI-L low concentration tuning mix (Agilent technologies).

LC-MS/MS peptide mapping

Recombinant Bet v 1.0101 was digested with trypsin (enzyme/substrate ratio, 1:25), which cleaves the protein after Arg (R) and Lys (K) residues, in 50 mM ammonium acetate buffer. After overnight digestion at 37°C, the generated peptides were injected onto a UHPLC-ESIMS system for analysis (Dionex Ultimate 3000 RSLC system connected to Q-TOF MaXis mass spectrometer fitted with an electrospray ion source. The peptides were separated by reversed phase UHPLC chromatography, on a C₁₈ column (Acquity BEH130, 100 x 2.1 mm, 1.7 μm, Waters) at a flow rate of 0.400 mL/min and thermostated at 30 °C, using an

increasing acetonitrile gradient (Table IIS) and detected using an UV detector and the aforementioned mass spectrometer.

Table IIS. Gradient used for peptide mapping

Time (min)	0.0	5.0	45.0	90.0	90.1	92.0	92.1	97.0
H ₂ O, 0.1% TFA (%)	100	100	71	54.5	0	0	100	100
H ₂ O (40%) / MeCN (60%) / 0.1% TFA (%)	0	0	29	45.5	100	100	0	0

The TOF analyser was externally calibrated before the analysis using the solution ESI-L Low concentration tuning mix (Agilent technologies).

Circular dichroism

A Jasco J-815 CD spectrophotometer was used to control the secondary structure of rBet v 1.0101. Spectroscopic profiling of the samples was carried out in the sample buffer, against a buffer blank (NaP buffer 20 mM pH 7.2). The following parameters were used: Band width 1 nm, Response 1 sec, Data pitch 0.2 nm, Scanning speed 100 nm/min, Accumulation 8, Temperature: RT.

Fluorescence spectroscopy

Characterization of tertiary structure was performed using fluorescent 8-anilino-1-naphthalenesulfonic acid (ANS) ligand, which binds to Bet v 1 hydrophobic cavity. Experiments were performed using 5 μ M of rBet v 1 and increasing concentrations of ANS in independent aliquots, in NaP buffer 10 mM pH 8. ANS was excited at 350 nm with a 5 nm slit width and the emission was measured at 480 nm in single point method using a Fluoromax-4 spectrofluorometer (Horiba Jobin-Yvon).

Blood samples

For moDC analysis, buffy coats were obtained from Etablissement Français du Sang (Rungis, France). The blood-bag was stored at 4°C overnight before the experiment. For basophil activation test and PBMC analysis, peripheral blood samples were obtained from birch pollen (BP) allergic donors recruited at Hospital of Antwerp (Antwerpen, Belgium). Informed consent was obtained from all participants in this study. BP allergic donors (male or female from 18-60 years of age) were selected based upon their clinical record, a positive specific skin prick test (wheal diameter greater than 3 mm) and a specific IgE (extract birch pollen) dosage level of at least 0.35 kUA/L. Peripheral blood samples were collected aseptically into sterile blood collection tubes, using citrate dextrose as an anticoagulant. Samples were directly sent to Stallergenes at room temperature (18-25°C) for subsequent processing, within 24 hours after blood collection.

Basophil activation test

Basophil activation was measured using Beckman's allergenicity kit according to the manufacturer's instructions. The reagents consisted of a three-color combination of fluorescent monoclonal antibodies (anti-CD203c-PE, CD3-PC7 and CRTH2-FITC), an activation solution, a positive control for IgE-mediated basophils activation, a stop solution, a lysing and a fixative solution. Briefly, blood samples from birch pollen allergic patients were stained with the aforementioned monoclonal antibodies in the presence of rBet v 1 at various dilutions. Erythrocytes were then lysed prior to flow cytometry analysis. Once T lymphocytes (CD3 positive cells) were excluded, basophils were analysed by flow cytometry using CRTH2 and CD203c expression. Non activated and resting basophils were identified as CRTH2^{pos}CD203c^{dim}CD3^{neg}, whereas *in vitro* activated basophils were identified as

CRTH2^{pos}CD203c^{bright}CD3^{neg}. Samples were run on a Beckman Coulter FC500 flow cytometer, using CXP Cytometer 2.2 and CXP Analysis 2.1 software.

MoDC generation

Human PBMCs were separated out of buffy coats obtained from healthy volunteers Etablissement Français du Sang (Rungis, France) by centrifugation over a Ficoll-Paque plus gradient (PAA, Les Mureaux, France). To generate monocyte-derived DCs (DCs), $5 \cdot 10^8$ to $8 \cdot 10^8$ cells were cultured at 37°C, 5% CO₂ in RPMI medium with stable glutamine supplemented with 10 µg/ml gentamycin, 50 µM 2-ME, 1% nonessential amino acids (all obtained from Invitrogen, Carlsbad, CA, USA) and 10% FCS (Gentaur, Brussels, Belgium). After 1 h, nonadherent cells were removed, and adherent cells were further cultivated for 6 days in presence of human rGM-CSF and rIL-4 (Gentaur), using 100 and 50 ng/ml concentrations, respectively. After 6 days, a pure population of DCs was obtained, with more than 95% CD14⁻CD1a⁺ cells detected by flow cytometry using a FC500 cytometer and the CXP analysis software (Beckman Coulter, Villepinte, France).

MoDC or PBMCs in vitro stimulation

Up to 10^6 moDCs or PBMCs were plated in a 24-well plate in presence of medium, native or oxidized rBet v 1 (1–10 µg/ml). PBMCs or moDCs incubated with LPS or PPD (1 µg/ml) served as positive controls. 24 h or 96 h after the start of the culture, supernatants were removed for cytokine analyses.

Analysis of cell surface markers and cytokine production

For immuno-fluorescence staining, cells were harvested, washed in PBS and incubated for 20 min at 4°C with the following mAbs: FITC anti-CD80, PE anti-CD86, and PC5 anti-CD83 (Beckman coulter). Cells were stained with corresponding isotype-matched control mAbs. Samples were analyzed by flow cytometry with results expressed as mean variations in percentages of positive cells.

Cytokine measurement was performed in supernatants using the cytometric bead array technology. IFN- γ , IL-1 β , IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A and TNF- α were measured using the human inflammatory CBA kit or CBA Flex sets (BD Biosciences, Le Pont de Claix, France) and analyzed by flow cytometry using an FACSArray instrument and the FCAP Software (BD Biosciences).

Assessment of the biological activity of native and oxidized rBet v 1

We investigated the capacity of native and fully oxidized rBet v 1 to induce the expression of costimulatory molecules (i.e., CD80, CD83, CD86) by moDCs (Fig. S3A), using lipopolysaccharide as a positive control. Neither the native nor oxidized rBet v 1 had any impact on the expression of co-stimulatory molecules. In addition, none of those two molecules induced the secretion of pro-inflammatory cytokines (i.e., IL-1 β , IL-6, IL-8, IL-10, IL-12p70) by moDCs from healthy individuals (data not shown), or PBMCs from BP allergic patients (Fig. S3B). In contrast, lipopolysaccharide and the tuberculin purified protein derivative from *Mycobacterium tuberculosis*, included as positive controls, induced the secretion of IL-1 β , IL-6, TNF- α (Fig. S3B) as well as IFN- γ and IL-10 or IL-13 by PBMCs (Fig. 3B). Lastly, both native and oxidized rBet v 1 exhibited a similar capacity to upregulate in a dose dependent manner the expression of the CD203c activation marker, when incubated

with basophils from BP allergic donors (Fig. S3D). Taken together, these data demonstrate that highly purified native (or oxidized) rBet v 1 do not exhibit any detectable immunological activity beyond IgE engagement.

X-ray crystallography

Initial crystallization trials were performed by the sitting-drop vapour-diffusion method at 293 K in 96-well Greiner plates with the Wizard I and II (Emerald BioSystems), PEGs Suite (Qiagen) and Crystal Screen and Crystal Screen 2 (Hampton Research) screens using a Cartesian HoneyBee robot (Genomic Solutions). Drops were prepared by mixing different volumes (100, 200 and 300 nL) of protein solution (10 mg/mL) and 100 nL precipitant solution and were equilibrated against a 150 nL reservoir volume. Crystallization hits occurred in several conditions, especially MDL Classic No. 83 [0.1 M sodium acetate pH 4.6, 0.2 M ammonium sulfate, 30% (w/v) PEG MME 2000]. After optimization, the final crystallization conditions were 0.1 M sodium acetate pH 4.6, 20 mM ammonium sulfate and 28%(w/v) PEG MME 2000, with a protein:precipitant ratio of 3:1(v:v) in 4 µL hanging drops.

Crystals appeared within a few days and were briefly soaked in crystallization solution supplemented with 20% (v/v) glycerol before being flash-cooled in a nitrogen-gas stream at 100 K. Crystals of rBet v 1 in complex with P303 were collected to 1.2 Å resolution on beamline Proxima 1 at the french synchrotron Soleil. The data set was integrated with XDS¹ and scaled with SCALA² from CCP4 Suite v6.3.0³. Data collection statistics are reported in Table IIS.

The structure of rBet v 1 in complex with P303 was solved by molecular replacement using the program auto-AMoRe⁴ and, as a template, the structure of Bet v 1-A (PDB entry:

207 1FSK-A). A first round of refinement was performed with Refmac⁵ and modification of the
208 model was made manually with the program Turbo-Frodo 6.

209 The P303 molecule was constructed so as to satisfy MS results and to fit into the residual
210 electron density clearly visible in the hydrophobic cavity of rBet v 1, known to accommodate
211 ligands. The SMILES (Simplified Molecular-Input Line-Entry System) string representing
212 P303 was generated by Cactus software (<http://cactus.nci.nih.gov/translate/>) and used to
213 generate the corresponding pdb and library files with the program Grade (Global Phasing
214 Ltd). P303 was then placed manually into the electron density map with COOT⁶. The model
215 of the complex was first refined with autoBUSTER⁷, and further refinement with anisotropic
216 B factors was performed with Refmac. The structure was corrected with COOT and model
217 validation was done with Molprobity⁸. Refinement statistics are reported in Table IIS.

218

219

Table IIIS. Data collection and refinement statistics

<i>Data collection</i>	
Wavelength (Å)	0.9334
Space group	P2 ₁ 2 ₁ 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	39.93, 58.58, 58.77
Resolution (Å) ^a	40-1.2 (1.26-1.2)
Unique reflections ^a	41,699 (4,782)
Multiplicity ^a	3.5 (1.8)
Completeness (%) ^a	95.4 (76.4)
<i>I</i> /σ ^a	20.3 (2.6)
R _{meas} (%) ^{a,b}	0.043 (0.402)
R _{p.i.m.} (%) ^{a,c}	0.022 (0.268)
Mosaicity	0.19
<i>Refinement</i>	
<i>and model quality</i>	
Resolution (Å)	30-1.2
Reflections	39520
R _{cryst} /R _{free} (%) ^d	13.3/16.0
Average B-factors (Å ²)	
protein/ligand/water	9.7/13.8/25.1
Rmsd ^e	
Bond (Å)	0.010
Angle (°)	1.47
Ramachandran plot (%)	
Most favored regions	98.1
Additionally allowed regions	1.9
PDB accession code	4MNS
^a Values in parentheses are for the highest-resolution shell. ^b $R_{meas} = \sum \sqrt{(n/n-1)} I_{hl} - \langle I_h \rangle / \sum \langle I_h \rangle $, where <i>I</i> _{hl} is the observed intensity and <i><I_h></i> the average intensity from <i>n</i> observations (symmetry-related and duplicate measurements of a unique reflection). ^c $R_{p.i.m.} = \sum \sqrt{(1/n-1)} I_{hl} - \langle I_h \rangle / \sum \langle I_h \rangle $. ^d $R_{cryst} = \sum_{hkl} F_o - F_c / \sum_{hkl} F_o $; R _{free} is calculated for 5% of	

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Design of the clinical trial

The study consisted in a screening phase and a treatment phase of approximately 5.5 months followed by a treatment-free phase of 2 weeks (Fig 4A). Eligible patients were randomized 1:1:1:1, using a computer-generated list (block-size of 8), to receive sublingual tablets of Placebo or active treatment at a dose of 12.5 µg, 25 µg or 50 µg once daily. All participants, investigators and other trial personnel remained blinded for the entire study.

Subjects enrolled in the clinical study

Men and women enrolled were aged 18 to 65, with symptomatic birch pollen-related allergic rhinoconjunctivitis for at least the last 2 years, confirmed with positive skin prick test to birch pollen (wheal diameter > 3 mm) and birch- and rBet v 1-specific serum IgE levels > 0.70 kU/L, with a Retrospective Rhinoconjunctivitis Total Symptom Score of at least 12 (on a 0-18 scale), and a forced expiratory volume in 1 sec (FEV₁) > 80%.

Study treatment and rescue medications

Active treatment consisted of 50 mg sublingual tablets containing 12.5 µg, 25 µg or 50 µg of freeze-dried cGMP-grade rBet v 1. Active and placebo tablets were identical in appearance, taste and packaging to ensure blinding. Treatment was initiated approximately 4 months before the expected start of the birch pollen season, as determined at each study center using historical pollen count data, and continued until about 1 week after its end. The first dose of treatment was administered under the investigators' supervision and participants were monitored for 30 min. The remainder of the treatment was taken at home. Participants were instructed take 1 sublingual tablet every day at the same time and to leave it under the tongue until completely dissolved before swallowing.

Birch pollen period

At each study centre, birch pollen counts were collected daily. For each site, start and end dates of the 2009 birch pollen period were defined so that the corresponding period covered 90% of the total annual pollen count at that site. All decisions regarding each centre-specific start and end dates of the birch pollen period were made before data unblinding. For the analysis, two evaluation periods were considered for each study center: the pollen period and the peak pollen period. For each study center, the average of pollen counts over the pollen period ranged from 47.9 to 1126.7 grains/m³ and over the peak pollen period from 100.8 to 2080.2 grains/m³.

Assessments

Starting about 3 weeks before the expected start of the pollen season, participants received diaries to self-score daily each of their six rhinoconjunctivitis symptoms (rhinorrhea, sneezing, nasal pruritus, nasal congestion, ocular pruritus and watery eyes) on a 0-3 scale (absent to severe), and recorded their use of rescue medications. The daily rhinoconjunctivitis total symptom score (RTSS) was the sum of the six rhinoconjunctivitis symptom scores (scale 0-18). For each participant, the daily adjusted symptom score (AdSS, scale 0-18) was equal to the daily RTSS adjusted for their use of rescue medication, as described by Grouin and colleagues.⁹ The Average AdSS (AAdSS, scale 0-18) is an average of the AdSSs over the birch pollen period. The primary efficacy endpoint was the AAdSS while on treatment during the birch pollen period in the Intent-to-Treat (ITT) population.

Treatment compliance was calculated from the actual use of the investigational products and checked by recording the number of tablets dispensed at Visit 2 and Visit 4 and returned at Visit 4 and Visit 6. At each visit the patients must return all unused investigational products as well as any empty boxes and blister packs. A patient was considered compliant when their

272 intake was $\geq 80\%$ and $\leq 120\%$ of theoretical number of tablets over the treatment period. If
273 the patient's intake was below 80% and above 120% or their compliance data were missing,
274 the patient was considered non-compliant.
275 Safety was assessed by means of adverse event monitoring and safety laboratory testing.

Statistics

Sample size calculation was based on the general knowledge of birch extract immunotherapy and sublingual immunotherapy. The following assumptions were made: given an α of 0.05 and a common SD of 3.4, a sample size of 105 participants per treatment groups would provide at least 80% power to detect a mean difference of 1.35 between active and placebo groups in the AAdSS during the pollen period while on treatment. This mean reduction of 1.35 should correspond to a more than 20% improvement, which is generally considered clinically significant.¹⁰ Assuming a 20% screening failure rate and a 15% drop-out rate, it was decided to screen approximately 616 patients and randomize 492 patients (123 per treatment group).

Statistical analyses were performed with SAS software (SAS Institute, Cary, NC). The ITT population included all participants who received at least one dose of treatment and had at least 1 AdSS while on treatment during the pollen period. The safety Set included all participants who received at least one dose of treatment.

The primary efficacy endpoint, (AAdSS) was analyzed using an analysis of covariance (ANCOVA) with treatment and pooled study centers as main effects, and age, gender, birch seasonal asthma status and sensitization status (mono- vs. poly-sensitized at baseline) as covariates. To control the overall type I error rate at 5%, treatment comparison was done using a step-down approach (50 μ g vs. Placebo, then 25 μ g vs. Placebo, then 12.5 μ g vs. Placebo); the next step was performed only if statistical significance was declared in the previous step. All inferential tests were two sided.

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FIGURES

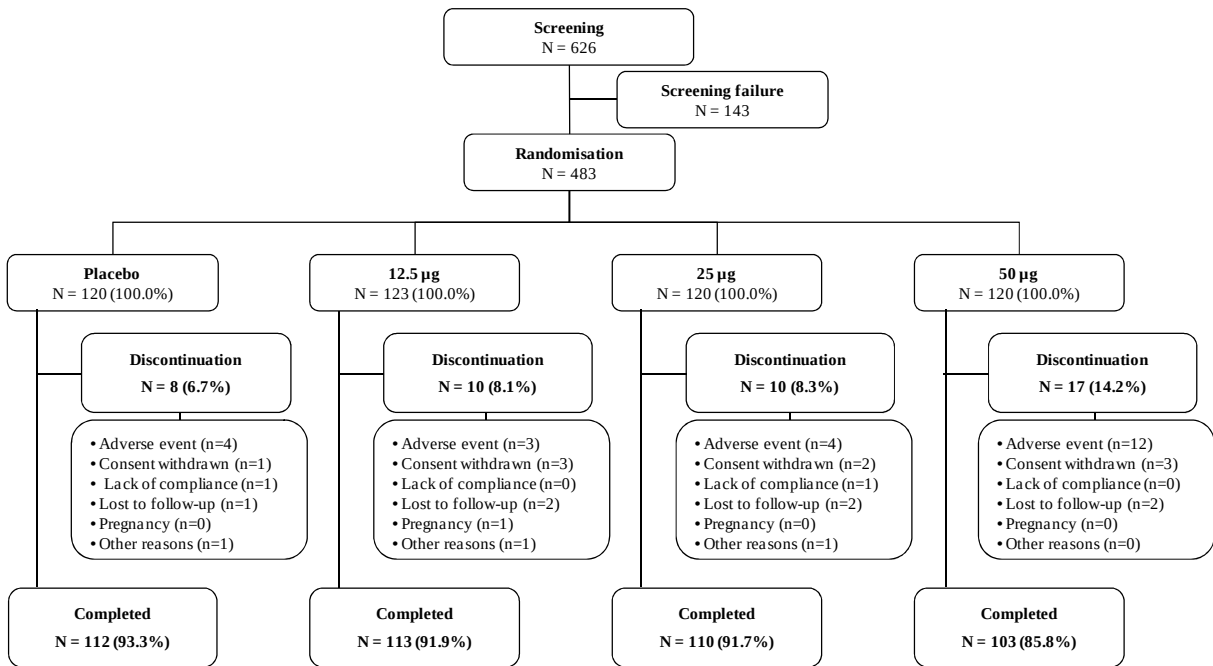


Figure S1. Clinical study participant flow.

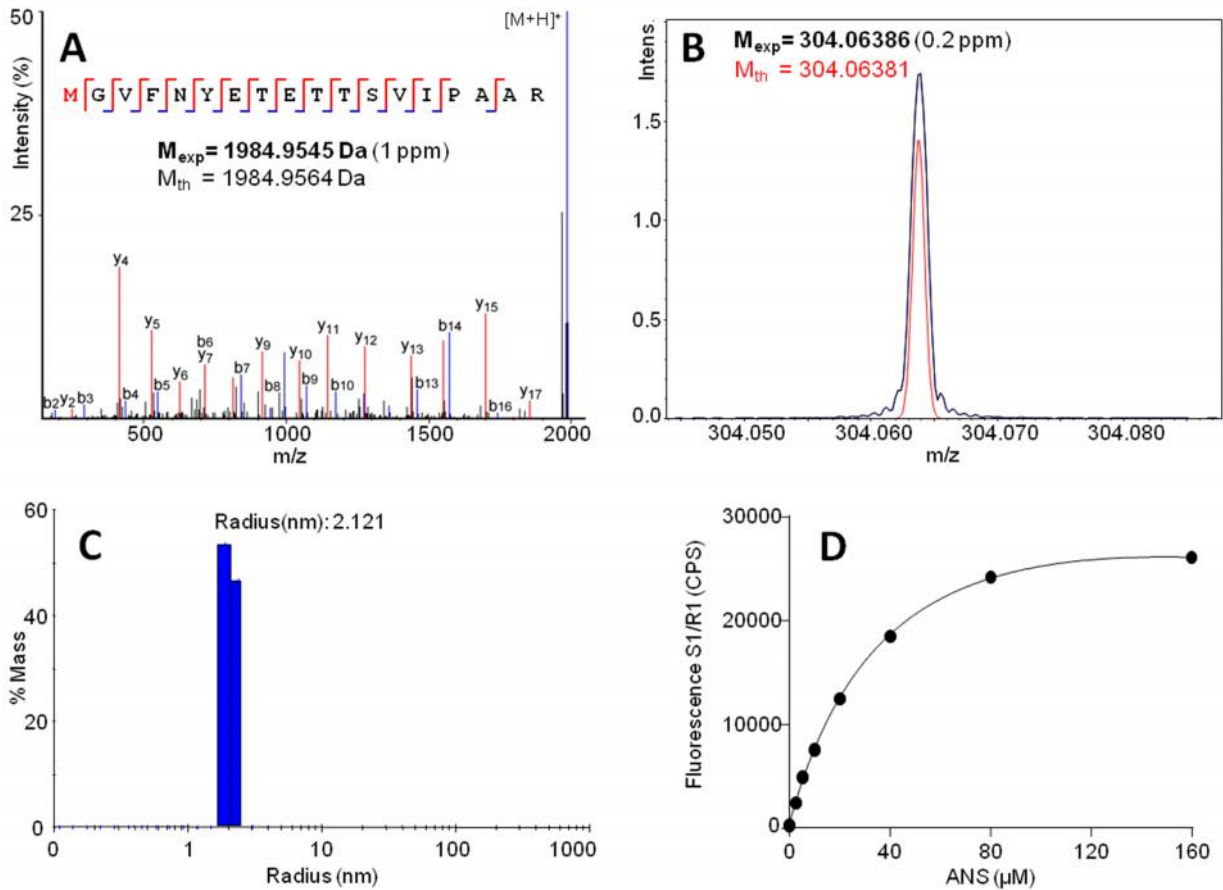
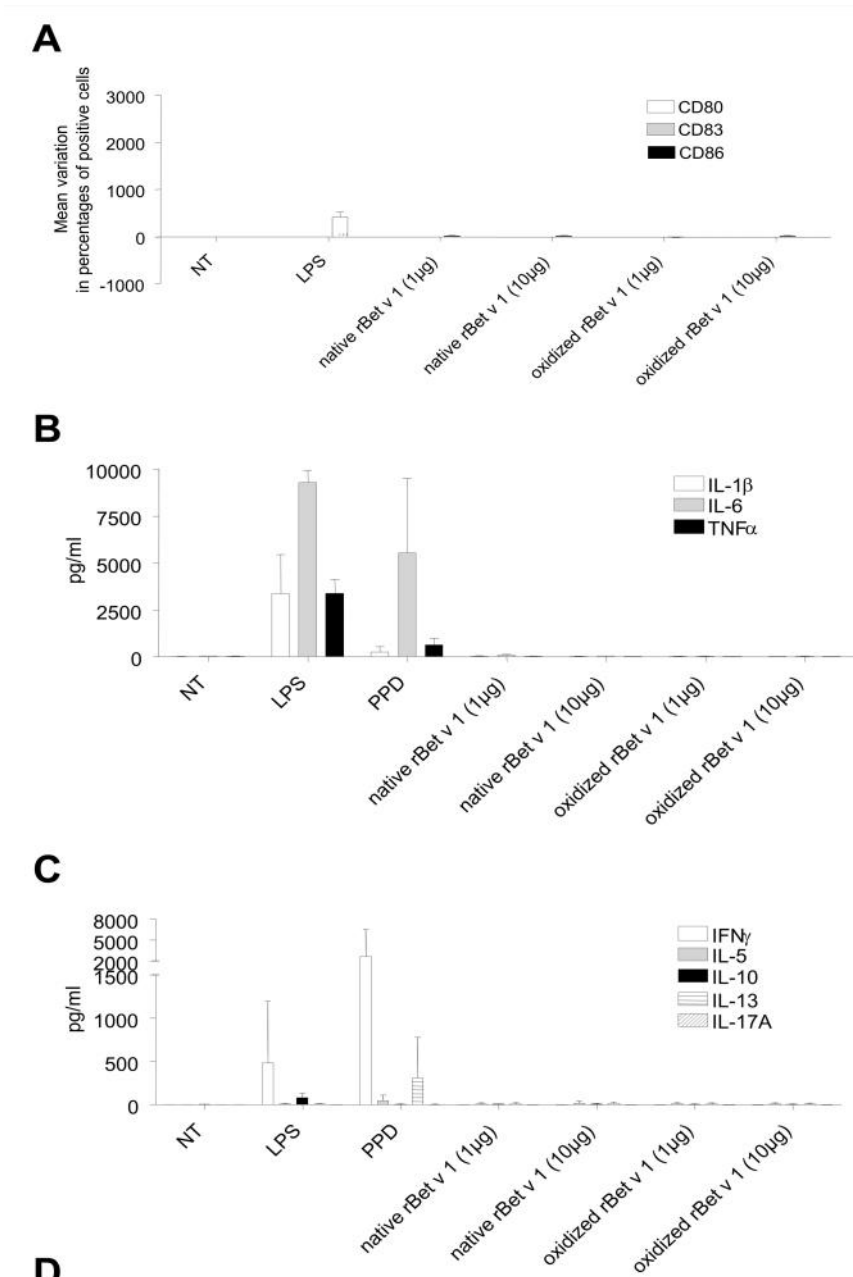


Figure S2. Structural characterization. (A) MS/MS spectrum of N-terminus peptide of rBet v 1 produced with complex growth medium, confirming the presence of N-terminus methionine. (B) Overlay of theoretical (red) and experimental (black) MS spectra of P303 compound indicated by an arrow in Fig 2,B, confirming its unique $C_{15}H_{13}O_4NS$ formula. (C). Dynamic light scattering profile of purified rBet v 1 showing a molecular size matching with a monomeric structure. (D) ANS titration of rBet v 1 recorded in 10 mM NaP (pH 8) using excitation and emission wavelengths at 350 and 480 nm, respectively, confirming ANS uptake.

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Figure S3. Functional characterization of native and oxidized rBet v 1. (A) Expression of co-stimulatory markers by moDCs after incubation with native and oxidized rBet v 1. Results are expressed as the mean variation $\pm$ SEM in percentages of positive cells when compared with non-treated DCs (n=5). (B) Pro-inflammatory cytokine secretion in PBMCs, 24h after incubation with native and oxidized rBet v 1. Results are expressed as the mean cytokine concentrations $\pm$ SEM (n=3). (C) Effector cytokine secretion in PBMCs, 6 days after incubation with native and oxidized rBet v 1. Results are expressed as the mean cytokine concentrations $\pm$ SEM (n=3). (D) Percentages of activated basophils (CD203c bright) upon stimulation with various doses of native and oxidized rBet v 1 (mean percentage $\pm$ SD of triplicates). Data obtained with basophils from a representative patient (out of 5 tested) are shown.