Received: 8 February 2022 | Revised: 2 May 2022 | Accepted: 5 May 2022

DOI: 10.1111/cts.13309

ARTICLE

First-in-human development of a pharmacodynamic biomarker for PAC_1 receptor antagonists using intradermal injections of maxadilan

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Funding information
The trial was funded by Amgen Inc.

Abstract

Maxadilan, a potent vasodilator peptide, selectively activates the PAC_1 receptor, a promising target for migraine therapy. Therefore, maxadilan has been suggested as a tool to study the pharmacodynamics (PDs) of PAC_1 receptor antagonists. The objectives of this first-in-human study were to: (1) determine the safety, tolerability, dose response, and time course of the dermal blood flow (DBF) changes after intradermal (i.d.) injections of maxadilan in the human forearm, and (2) assess the inter-arm and inter-period reproducibility of this response. This was a single-center, open-label study in healthy subjects, comprising three parts: (1) dose–response ($n = 25$), (2) response duration ($n = 10$), and (3) reproducibility ($n = 15$). DBF measurements were performed using laser Doppler imaging (LDI) up to 60 min postinjection, or up to 5 days for the response duration assessments. To assess reproducibility, the intraclass correlation coefficient (ICC) and sample sizes were calculated. The i.d. maxadilan (0.001, 0.01, 0.1, 0.9, 3, and 10 ng) produced a well-tolerated, dose-dependent increase in DBF, with a half-maximal effective concentration fitted at 0.0098 ng. The DBF response to 0.9 ng maxadilan was quantifiable with LDI up to 72 h postinjection. The inter-period reproducibility of the DBF response was better upon 0.9 ng (ICC > 0.6) compared to 0.01 ng (ICC < 0.4) maxadilan. However, irrespective of the study design or maxadilan dose, a sample size of 11 subjects is sufficient to detect a 30% difference in DBF response with 80% power. In conclusion, intradermal maxadilan provides a safe, well-tolerated, and reproducible PD biomarker for PAC_1 receptor antagonists in vivo in humans.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Selective target engagement biomarkers have proven to be successful in guiding go/no-go decisions in early clinical drug development. Recently, the PAC_1-receptor

gained interest as novel target for anti-migraine drugs. Intradermal injections of maxadilan, a selective and potent PAC_1 receptor agonist, have been proposed as a pharmacodynamic (PD) biomarker tool to assess PAC_1 receptor activation.

WHAT QUESTION DID THIS STUDY ADDRESS?

This study evaluated the safety and applicability of intradermal maxadilan injection in humans by characterizing the induced vascular changes in terms of safety, dose response, and reproducibility.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

Intradermal injection of maxadilan induces a robust, dose-dependent increase in dermal blood flow with low variability. Hence, it can be used in the early clinical drug development of PAC_1 receptor antagonists.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Implementation of this novel PD biomarker will support the early clinical development of PAC_1 receptor antagonists, thereby reducing the costs and time invested to translate promising results in rodent pain models to human conditions.

INTRODUCTION

Migraine is the most prevalent neurological disorder, affecting more than 100 million people worldwide.[1] It is an episodic or chronic disorder, characterized by incapacitating headache attacks, which are often accompanied by nausea, vomiting, photophobia, and phonophobia.[2] For many years, non-steroidal anti-inflammatory drugs and triptans have been the cornerstone therapies for acute treatment of migraine headache, along with β-blockers which are used as preventive treatment.[3] None of these older drugs were specifically developed to treat migraine. This approach changed as new discoveries identified possible pathophysiological mechanisms involved in migraine. One of the breakthroughs came from the observation that plasma levels of calcitonin gene-related peptide (CGRP) were elevated in the external jugular vein during migraine attacks.[4] Ever since, the CGRP-pathway became an attractive target in the development of novel anti-migraine drugs. Recently, the US Food and Drug Administration (FDA) indeed approved the first drugs targeting the CGRP receptor; the small molecule ubrogepant (Ubrelvy) and the monoclonal antibody erenumab (Aimovig), for acute and preventive treatment of migraine, respectively.[5,6] Additionally, eptinezumab (Vyepti), fremanezumab (Ajovy), and galcanezumab (Emgality), all monoclonal antibodies binding to the CGRP ligand, also became available as preventive treatment.[7] Remarkably, whereas the clinical development of novel drugs takes on average 10 years in the field of neurology, erenumab received its market authorization only 6 years after having entered phase I clinical trials.[8,9] Besides being time-consuming, the overall success rate of clinical development is low in this field, with only 8% of the drug candidates eventually reaching the market.[10] The main pitfall is a lack of efficacy which only becomes apparent during later, more expensive stages of drug development.[11]

To predict efficacy early on in the development process, three so-called "pillars of survival" have been identified.[12] In order to exert its anticipated pharmacological effect, the drug has to: (1) reach the target in sufficiently high concentrations, (2) effectively bind the target, and, most importantly, (3) modulate the target in the intended manner. To test whether a drug meets these criteria, implementation of pharmacodynamic (PD) biomarkers, among which target engagement biomarkers, in the earliest stages of clinical development is extremely supportive, as was illustrated by the efficient development of erenumab.[11] By incorporating a PD biomarker in the first phase I trials, erenumab showed dose-dependent inhibition the CGRP-dependent dermal blood flow changes upon topical capsaicin application. Apart from assessing the third pillar, the biomarker also guided the dose selection for phase II trials, as subcutaneous injection of 140 mg erenumab (i.e., the maximal dosage that is currently approved for clinical use), was shown to maximally inhibit the vascular changes.[13]

In addition to CGRP, pituitary adenylate cyclase-activating polypeptide (PACAP) has been suggested as pivotal neuropeptide in the pathophysiology of migraine. It activates three receptors; the designated PAC_1 receptor, and the vasoactive intestinal peptide (VIP)/PACAP receptors $VPAC_1$ and $VPAC_2$. Interestingly, PACAP-binding sites were demonstrated in the trigeminovascular system, and intravenous infusion of PACAP induced dilatation of extracerebral vessels and delayed migraine-like headaches.[14,15] Therefore, the PACAP-pathway gained interest as a target for novel anti-migraine drugs, with two monoclonal antibodies targeting either PACAP as a ligand (ALD1910) or the PAC_1-receptor (AMG301) already

having completed phase I trials (NCT04197349 and NCT03238781, respectively).

To support efficient early clinical development, we aimed to establish a novel PD biomarker for PAC_1-receptor antagonists in humans. Because PACAP activates three different receptors, it is not well-suited as agonist for such studies.[16] However, maxadilan, a potent vasodilator peptide originally isolated from the salivary glands of the sand fly *Lutzomyia longipalpis*, does selectively and potently activate the PAC_1 receptor.[17,18] On this basis, we performed a first-in-human study characterizing the vasodilation following intradermal (i.d.) injections of maxadilan in terms of safety, tolerability, dose response and time course. Additionally, the reproducibility of the vascular response to maxadilan was evaluated to calculate sample sizes for future clinical trials with PAC_1-receptor antagonists incorporating this new PD biomarker.

METHODS

Subjects

Approval of the Ethics Committee of the University Hospitals Leuven, Belgium, and the Federal Agency for Medicines and Health Products, Belgium (EudraCT 2014-001760-36/2014-005373-37) were obtained prior to study initiation. All study procedures were conducted in accordance with the latest versions of the Declaration of Helsinki and International Guidelines on Clinical Trials of Medicinal Products/Good Clinical Practice (ICH/GCP). Written informed consent was obtained from all subjects prior to screening.

All subjects were nonsmoking male volunteers between 18 and 45 years of age. Subjects were considered healthy based on their medical history, physical examination, vital signs, electrocardiogram (ECG), and routine laboratory data. Main exclusion criteria were a medical history of migraine, severe allergies to insect bites, or any skin disorder which could interfere with the study assessments. To standardize the assessments, subjects refrained from caffeinated beverages and alcohol at least 12 and 24 h prior to every study visit, respectively, and fasted for at least 3 h. Except for up to 2000 mg paracetamol per day, medication intake was prohibited during study participation.

Study design

Three characteristics of the dermal blood flow (DBF) changes upon i.d. injection of maxadilan were assessed: (1) dose–response, (2) duration of the vascular response, and (3) inter-arm and inter-period reproducibility. All i.d. injections were open-label.

Dose–response

The dose–response study comprised two parts. Part I was a placebo-controlled trial in 10 subjects, including a screening visit, three study visits, separated by a wash-out period of at least 14 days, and a post-study visit. During every study visit, maxadilan and the corresponding vehicle (placebo) were administered on a proximal and distal site on the volar surface of the forearm, respectively. Three different doses of maxadilan were administered in a fixed order: 3, 10, and 0.9 ng during the first, second, and third study visits, respectively. Doses were initially selected based on preclinical in vitro experiments[17] and animal testing in cynomolgus monkeys (data not published). DBF measurements were performed pre-injection (baseline) and at 15, 30, and 60 min after administration of maxadilan/vehicle. After the final measurement, a picture of the injection site (including a ruler on the skin for calibration) was captured.

Because all doses in part I induced similar DBF changes, the dose–response study was amended to assess the DBF response upon lower doses maxadilan for proper dose-finding. Part II comprised a screening visit, one study visit, and a post-study visit including 15 participants. Four different doses of maxadilan were administered; 0.001, 0.01, 0.1, and 0.9 ng. Group A ($n = 5$) received 0.9 and 0.1 ng, group B ($n = 5$) received 0.1 and 0.01 ng, and group C ($n = 5$) received 0.01 and 0.001 ng; all on the volar surface of the right and left forearm, respectively. DBF measurements were performed at baseline and at 15, 30, and 60 min after administration of maxadilan. After the final DBF measurement, a picture of the injection site was taken.

Response duration

The duration of the DBF changes upon maxadilan injection was assessed in 10 subjects. All subjects were invited for a screening visit, one study period that lasted for 5 days and a post-study visit. On day 1 of the study period, participants received an injection of 0.9 ng maxadilan and vehicle on the proximal and distal site, respectively, on the volar surface of both forearms. DBF was measured at baseline and at regular timepoints during 5 days postinjection.

Inter-arm and inter-period reproducibility

The reproducibility of the vascular changes upon administration of maxadilan was evaluated in 15 volunteers

who completed a screening visit, five to six study visits, and a post-study visit. All study visits were separated by a period of 7–21 days. The reproducibility of two maxadilan doses was evaluated; a single dose of either 0.9 or 0.01 ng maxadilan was administered in nine and six subjects, respectively. All injections were administered on the volar surface of the proximal forearm, alternating the left and right arm for every study visit. DBF was measured at baseline and at 15, 30, and 60 min after maxadilan administration.

Dermal blood flow measurements

Prior to all DBF measurements, subjects acclimatized in semi-recumbent position in a temperature-controlled room of 23 ± 1°C for 30 min. Subsequently, rubber O-rings (7.66 mm inner diameter, Quad Ring BS011 NBR 70 Shore A; Polymax Ltd., Bordon, UK) were placed on the volar surface of the forearm, avoiding visible veins, to delineate the region of interest (ROI) around the injection site. DBF measurements were performed using Laser Doppler Imaging (LDI, PIMIII, PIMSoft version 1.5, Perimed, Järfälla, Sweden).

Safety and tolerability

Safety evaluation consisted of continuous adverse event monitoring, regular laboratory safety tests, ECG recordings, and vital sign assessments, including blood pressure, heart rate, and temperature. During Part I of the dose–response and the response duration study, the maxadilan-induced pain intensity was assessed via the Numerical Rating Scale-11 (NRS-11), for which subjects were asked to score their pain on a numeric scale from zero to 10; zero meaning "no pain at all" and 10 meaning "the worst pain imaginable." Up until the post-study visit, the forearms were regularly inspected for adverse events.

Maxadilan solutions

Maxadilan acetate, produced by Bachem Americas Inc., was supplied as a sterile, single-use, preservative-free solution at 0.012 mg/ml for clinical trial use. The drug product was diluted with 0.9% sodium chloride and 0.1% human serum albumin, sterile filtered, and aseptically filled into a 6 ml sterile, depyrogenated glass serum vial, with a target fill volume of 3 ml. Both 10 and 3 ng maxadilan were administered in a volume of 50 µl, all other maxadilan doses were administered in a volume of 15 µl. Maxadilan had the following 61 amino acid sequence: H-Cys-Asp-Ala-Thr-Cys-Gln-Phe-Arg-Lys-Ala-Ile-Asp-Asp-Cys-Gln-Lys-Gln-Ala-His-His-Ser-Asn-Val-Leu-Gln-Thr-Ser-Val-Gln-Thr-Thr-Ala-Thr-Phe-Thr-Ser-Met-Asp-Thr-Ser-Gln-Leu-Pro-Gly-Asn-Ser-Val-Phe-Lys-Glu-Cys-Met-Lys-Gln-Lys-Lys-Lys-Glu-Phe-Lys-Ala-NH2. The vehicle consisted of the same, sterile 50:1 v/v solution of 0.9% sodium chloride and 0.1% human serum albumin.

Statistics

Descriptive statistics were used for demographics.

DBF measurements are expressed in arbitrary perfusion units (PUs) and presented both in absolute values as well as percentage change from baseline. The area under the curve from baseline to 60 min postinjection ($AUC_{0-60min}$) was calculated as summary measure.

Histograms were used to evaluate the data distribution. For the dose–response trial and duration of the erythema, a linear mixed model with repeated statement and post hoc Bonferroni correction was performed. A p value <0.05 was considered statistically significant. The within-subject reproducibility of the DBF changes was evaluated between arms (i.e., inter-arm reproducibility) and over time during distinct study visits (i.e., inter-period reproducibility). To that end, the intraclass correlation coefficient (ICC) was calculated using variance components extracted from linear mixed models. An ICC of <0.40 was defined as poor, between 0.40 and 0.60 as reasonable, between 0.60 and 0.80 as good, and between 0.80 and 1.00 as excellent. Sample size calculations (SSCs) for an independent or a paired study design with continuous measures were performed. Sample sizes were calculated to detect a difference of 30% in DBF changes given a type I error probability (α) of 0.05 and a power of 80%. All statistical analyses were performed using SAS University Edition (SAS Institute Inc., Cary, NC).

To describe the dose–response relation between maxadilan and the change in DBF 60 min postinjection, an agonist-response model was fitted through a nonlinear regression analysis of the log-transformed maxadilan doses in GraphPad Prism (version 8.0.2, GraphPad Software, San Diego, CA). The half-maximal effective concentration (EC_{50}), Hill coefficient, basal (bottom) and maximal (top) response of the sigmoidal curve were modeled using the four-parameter equation $y = \text{bottom} + (\text{top}-\text{bottom})/(1+10\text{^}([\log EC_{50}\text{-x}]*\text{Hill slope}))$. For the three-parameter analysis, a Hill coefficient of one was assumed. Ultimately, the most appropriate model was withheld taking into account the goodness-of-fit (R^2) and the precision of the parameter estimates.

RESULTS

Demographics

Demographics are presented in Table 1.

Safety and tolerability

There were no clinically relevant changes in vital signs, laboratory tests, physical examinations, or ECG recordings. No maxadilan-related adverse events were reported. During the dose–response assessments, a maximal NRS-11 pain score of one was reported by two subjects (2/10, 20%) upon injection of 10 ng maxadilan, and by one subject (1/10, 10%) upon injection of 3 and 0.9 ng maxadilan. During the response duration assessments, an NRS-11 pain score of zero was reported by all subjects (10/10, 100%) up until 5 days after injection of 0.9 ng maxadilan.

Dose–response

Based on histograms (not displayed), a normality assumption of the data was made. In Part I of the dose–response assessments (Figure 1), all three doses (0.9, 3 and 10 ng) of maxadilan induced a robust increase in DBF compared to vehicle ($p < 0.0001$) and baseline ($p < 0.0001$) that lasted for at least 60 min. At individual points in time (15, 30, and 60 min postinjection), the three maxadilan doses induced similar DBF changes ($p > 0.05$). Expressed as $AUC_{0–60min} \pm SEM$, 0.9 ng maxadilan induced a statistically significant higher increase in DBF compared to 3 ng (24,854 ± 3485 PUs*min and 20,813 ± 3370 PUs*min, respectively, $p = 0.0345$), but not to 10 ng (23,950 ± 6275 PUs*min, $p = 1.00$). Maximal changes in DBF (time to maximum concentration [T_{max}]) varied from 30 to 60 min postinjection. Maximal mean DBF ± SEM for 0.9, 3 and 10 ng maxadilan were 471 ± 31 PUs (T_{max} = 30 min), 413 ± 26 PUs (T_{max} = 60 min), and 464 ± 43 PUs (T_{max} = 60 min), respectively. Vehicle injection also induced a transient increase in DBF, which was significant compared to baseline at 15 min (38 ± 3 PUs and 105 ± 10 PUs, respectively, $p = 0.0004$) and disappeared at 30 min postinjection (73 ± 7 PUs, $p = 1.00$ compared to baseline).

In part II, injection of lower maxadilan doses (0.001, 0.01, 0.1, and 0.9 ng) induced significant, dose-dependent DBF changes when compared to each other ($p < 0.05$) at individual points in time or when expressed as $AUC_{0–60min}$, with the exception of 0.1 ng versus 0.9 ng maxadilan. The T_{max} was observed at 15 or 30 min postinjection. Maximal mean DBF ± SEM for 0.001, 0.01, 0.1, and 0.9 ng were 242 ± 28 PUs (T_{max} = 15 min), 330 ± 24 PUs (T_{max} = 30 min), 455 ± 34 PUs (T_{max} = 30 min), and 491 ± 41 PUs (T_{max} = 30 min), respectively. DBF changes induced by 0.001 ng returned to baseline values at 60 min postinjection ($p = 1.00$), whereas the vascular response induced by the other doses lasted for at least 60 min ($p < 0.05$ at 60 min compared to baseline).

A total of 70 matched maxadilan-DBF data points from parts I and II were included in the dose–response model. A three-parameter, least squares fit described the data best ($R^2 = 0.8125$). The EC_{50} (95% confidence interval [CI]) was fitted at 0.0098 ng (0.0057–0.0171) maxadilan, with a basal and maximal response of 39 (−7 to 83) and 454 (429–479) PUs, respectively. Noteworthy, the four-parameter, nonlinear regression analysis (R^2 = 0.8145) estimated a Hill coefficient of 1.34, but failed to compute the 95% CI of two (Hill slope and top) of the four parameters.

Response duration

After bilateral injection of 0.9 ng maxadilan, a significant increase in DBF was observed with LDI from 5 min until 48 h postinjection compared to baseline ($p < 0.05$, Figure 2). At 24 h postdose, a peak in DBF was observed bilaterally at the injection site. Afterward, the DBF decreased gradually to reach baseline again at 72 h postinjection. Although not measurable with LDI, a flare reaction was still visible from 72 to 120 h after maxadilan administration. DBF changes upon 0.9 ng maxadilan and vehicle were similar on both arms ($p = 1.00$).

TABLE 1 Demographics

Demographics	Age (years)	BMI (kg/m^2)	SBP (mm Hg)	DBP (mm Hg)	HR (bpm)
Dose–response					
Part I ($n = 10$)	27 ± 8 (20–39)	23.1 ± 2.2 (20.6–27.5)	128 ± 10 (107–139)	73 ± 6 (65–86)	53 ± 11 (38–76)
Part II ($n = 15$)	24 ± 3 (19–34)	22.9 ± 2.5 (19.0–27.9)	128 ± 8 (109–138)	73 ± 6 (66–85)	62 ± 9 (47–75)
Response duration ($n = 10$)	21 ± 3 (19–24)	21.1 ± 2.6 (18.1–25.7)	128 ± 9 (114–138)	71 ± 4 (66–78)	65 ± 11 (43–89)
Reproducibility ($n = 15$)	26 ± 5 (19–41)	23.2 ± 1.2 (20.7–25.1)	135 ± 30 (105–139)	73 ± 9 (56–86)	56 ± 13 (43–87)

Abbreviations: BMI, body mass index; bpm, beats per minute; DBP, diastolic blood pressure; HR, heart rate; SBP, systolic blood pressure.

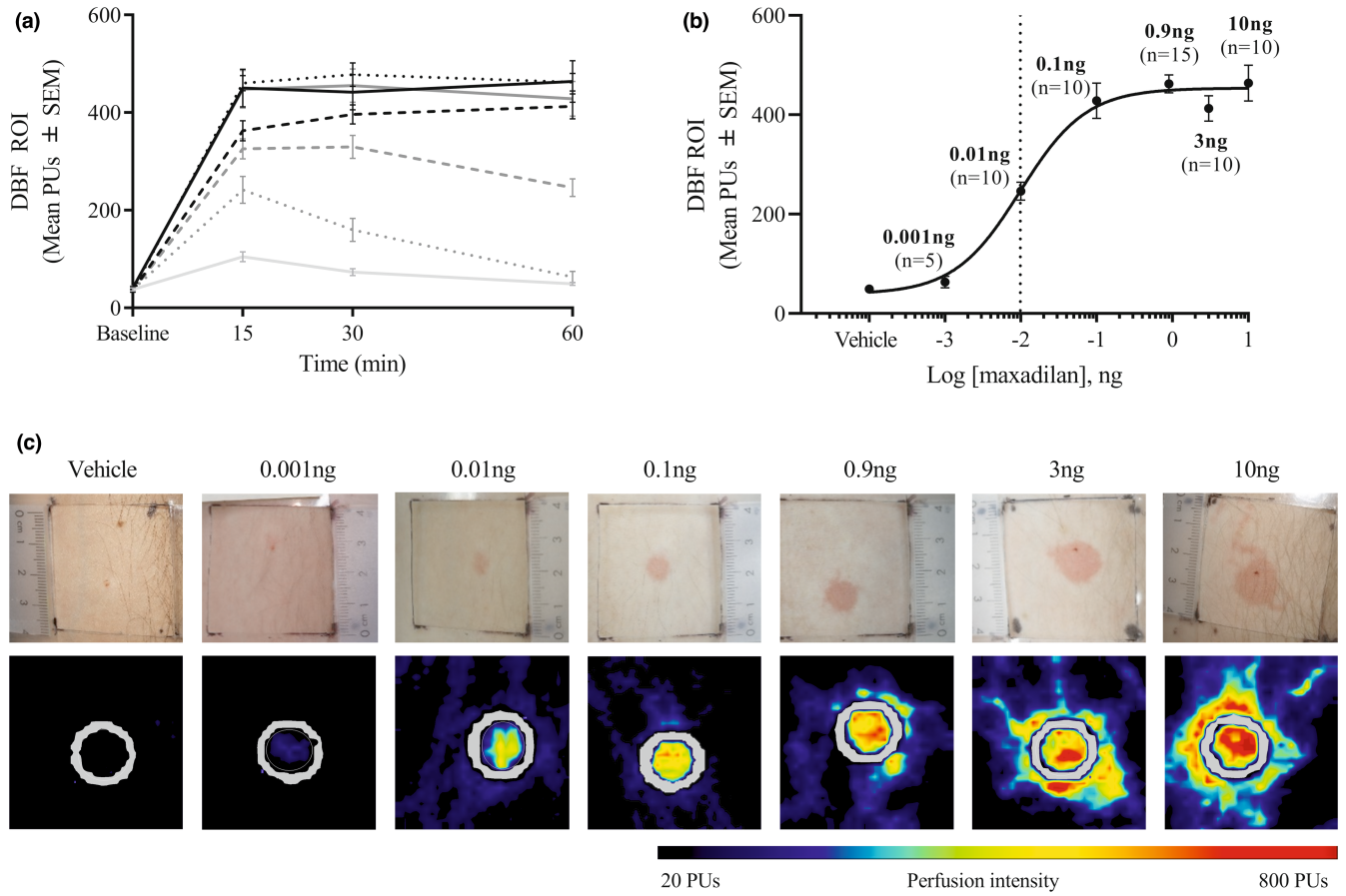

FIGURE 1 Dermal blood flow (DBF) response upon intradermal injection of six different maxadilan doses together with vehicle in the region of interest (ROI) on the forearm in 5 to 15 healthy volunteers. (a) DBF response over time during 60 min, expressed as mean perfusion units (PUs) ± SEM, (b) dose response of the DBF changes at 60 min postinjection with fitted half-maximal effective concentration (EC_{50}; dotted line) and (c) images of the vasodilation in a single volunteer, obtained 60 min after i.d. injection using Laser Doppler Imaging (LDI) Maxadilan doses: 10 ng (black full), 3 ng (black striped), 0.9 ng (black dotted), 0.1 ng (gray), 0.01 ng (gray striped), 0.001 ng (gray dotted), and vehicle (light gray).

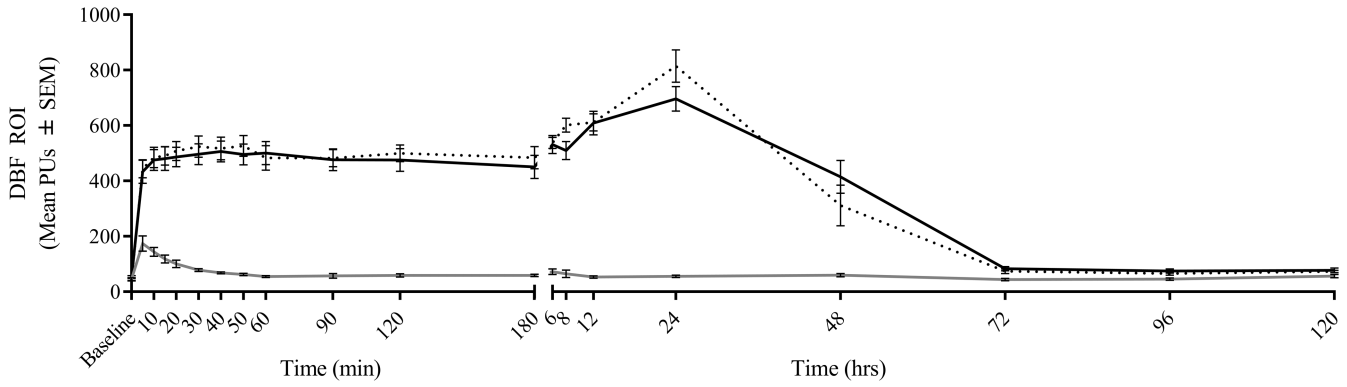

FIGURE 2 Duration of the dermal blood flow (DBF) response after 0.9 ng/15 µl maxadilan and vehicle injection in 10 healthy volunteers (except for $n = 5$ at 6, 8 and 12 h postinjection). DBF was measured using Laser Doppler Imaging (LDI) in the region of interest (ROI) and is expressed as mean perfusion units (PUs) ± SEM. The 0.9 ng/15 µl maxadilan right (black full), 0.9 ng/15 µl maxadilan left (black dotted), vehicle right (gray full), and vehicle left (gray dotted).

Reproducibility

Both the inter-arm and the inter-period reproducibility of the DBF changes, expressed in absolute values and percentage change from baseline, upon injection of 0.9 and 0.01 ng maxadilan were assessed (Table 2). Expressed in absolute values, the DBF increase induced by 0.9 ng maxadilan at 60 min postinjection as well as $AUC_{0–60min}$

TABLE 2 ICC together with the 95% CI of dermal blood flow measurements expressed in absolute values or percentage change from baseline (% Change) in the ROI performed on the left or right arm (i.e., inter-arm ICC) or during different study visits (i.e., inter-period ICC) at 60 min after i.d. injection of 0.01 ng/15 µl or 0.9 ng/15 µl maxadilan or as calculated $AUC_{0–60min}$, postinjection

	0.01 ng/15 µl Maxadilan		0.9 ng/15 µl Maxadilan	
ICC (95% CI)	Inter-arm	Inter-period	Inter-arm	Inter-period
T_{60min}				
Absolute values	0.182 (−0.182; 0.501)	0.143 (−0.206; 0.460)	0.721 (0.402; 0.884)	0.694 (0.368; 0.868)
% Change	0.190 (−0.188; 0.518)	0.260 (−0.450; 0.769)	0.381 (0.016; 0.656)	0.437 (0.089; 0.690)
$AUC_{0–60min}$				
Absolute values	0.272 (−0.218; 0.652)	0.026 (−0.315; 0.361)	0.780 (0.474; 0.918)	0.741 (0.423; 0.897)
% Change	0.337 (−0.156; 0.695)	0.279 (−0.353; 0.736)	0.284 (−0.081; 0.582)	0.445 (0.038; 0.725)

Abbreviations: $AUC_{0–60min}$, area under the curve from baseline until 60 min postinjection; CI, confidence interval; ICC, Intraclass Correlation Coefficient; ROI, region of interest.

TABLE 3 SSCs to detect a significant difference of 30% in a paired or independent study design with significance level of 0.05 and power of 0.8

	0.01 ng/15 µl Maxadilan		0.9 ng/15 µl Maxadilan	
SSC	Independent	Paired	Independent	Paired
T_{60min}				
Absolute values	11	11	6	4
% Change	10	9	13	9
$AUC_{0–60min}$				
Absolute values	9	10	5	4
% Change	15	12	18	4

Abbreviations: $AUC_{0–60min}$, area under the curve from baseline until 60 min postinjection; SSC, sample size calculation.

calculations were well reproducible (ICC > 0.60) both between arms and during distinct study visits. Absolute DBF changes induced by 0.01 ng maxadilan were less reproducible (ICC < 0.40) between arms as well as between distinct study visits, when measured at 60 min postinjection or when calculated as $AUC_{0–60min}$. Both for 0.9 and 0.01 ng maxadilan, SSC performed for an independent as well as paired study design and are presented in Table 3.

DISCUSSION

To support the early clinical development of PAC_1-receptor antagonists, we successfully developed a novel PD biomarker by using intradermal injections of the selective PAC_1-receptor agonist maxadilan. Intradermal injections from 0.001 to 10 ng maxadilan were shown to be safe and well-tolerated by all trial participants. Except for 0.001 ng maxadilan, all doses induced a robust, rapid-onset DBF increase which lasted for at least 60 min. The DBF increase was dose-dependent with the EC_{50} (95% CI) fitted at 0.0098 ng (0.0057–0.0171) maxadilan. Maximal DBF changes induced by the highest doses of maxadilan (i.e., 0.1, 0.9, 3, and 10 ng), were not significantly different, suggesting receptor saturation at doses exceeding 0.1 ng. The small, transient DBF increase observed after vehicle administration was most likely due to a local inflammatory reaction in response to the injection procedure itself because it rapidly returned to baseline. To further evaluate its potential use as a biomarker, the reproducibility of the increase in DBF upon intradermal injection of both 0.01 ng (i.e., the EC_{50}) and 0.9 ng maxadilan was assessed. Both the inter-arm and inter-period reproducibility of the DBF changes were improved after injection of 0.9 ng compared to 0.01 ng maxadilan, which could be expected due to receptor saturation. However, in spite of the small injection volumes, the variability of the DBF responses upon both 0.01 and 0.9 ng maxadilan injection was low. Hence, irrespective of the study design (i.e., paired or independent), a sample size of 11 subjects is sufficient to detect a difference of 30% in DBF increase. Sample sizes of this order are small enough to be included in phase I clinical trials. Taken together, the excellent safety profile and robust vascular changes with low variability support the use of intradermal maxadilan injections, preferably at doses of 0.01 ng to avoid receptor saturation, as a PD biomarker in the early clinical development of PAC_1-receptor antagonists.

Ideally, to be useful as such, the present biomarker should be able to address the three "pillars of survival": does the drug (1) reach the target, (2) bind the target, and (3) have an effect on the target?[11,12] In the context of migraine, PACAP-binding sites were found in the trigeminal system and on nerve fibers innervating the dura mater and cerebral arteries.[14,19] In contrast, this biomarker model evaluates maxadilan-induced DBF changes on the forearm. Nevertheless, as both regions are located in the peripheral nervous system without any particular barrier, there is no reason to expect major deviations from general plasma levels in the trigeminal system. Moreover, the capsaicin model, with which PDs of CGRP-(receptor) antagonists can be evaluated, also includes vascular assessments on the forearm whereas it has proven its value in the development of anti-migraine drugs.[20] Thus, pharmacokinetic (PK)/PD models based on this biomarker are useful to assess the first pillar in the context of migraine and PAC_1-antagonism. Although our model does not directly prove receptor occupancy, it is able to assess an anticipated pharmacological effect. Thereby, addressing the third pillar, the biomarker also indirectly demonstrates receptor binding. Namely, maxadilan is a well-known selective and potent PAC_1-agonist.[17,18] As this trial showed dose-dependent vascular changes upon intradermal injection of maxadilan compared to vehicle, inhibition of the induced vascular changes confidently implies receptor occupancy and PAC_1-antagonism. Moreover, the biomarker might also facilitate dose-decisions as the effect of different doses can be evaluated to identify the optimal dose with maximal effect on the vascular changes and minimal adverse reactions.

As with any peripheral biomarker developed for central nervous system CNS indications, this biomarker has limitations. First, if the target is localized in the CNS, a peripheral biomarker does not take into account blood–brain barrier penetration. Thus, if a dose shows maximal inhibition of the anticipated pharmacological effect by using the biomarker but lacks efficacy, a pathophysiological role of the target cannot be excluded, especially if it concerns a monoclonal antibody with poor CNS penetration. Accordingly, proven target engagement does not necessarily imply clinical efficacy. Hence, irrespective of PAC_1-antagonism, actual proof-of-concept still requires anti-migraine assessments in phase II/III trials. Unfortunately, recent data from a phase II trial in patients with migraine failed to demonstrate efficacy after subcutaneous AMG301 administration.[21] Efficacy assessments of ALD1910, targeting the PACAP ligand itself, have not yet been published. Second, the PDs of monoclonal antibodies scavenging the PACAP ligand cannot be assessed with the current biomarker because the PAC_1-receptor is activated by an exogenous stimulus. Furthermore, the biomarker remains to be validated with a selective PAC_1-receptor antagonist in humans. Yet, $PACAP_{38}$, a PACAP fragment and PAC_1 agonist, as well as maxadilan-induced DBF changes could be reversed by a PAC_1-receptor antagonist (Peptide 18) in rats.[16] As interspecies differences have not been described, there is little reason to doubt the validity of the model in humans.[22] Finally, little is known about the physiology upon activation of the PAC_1 receptor. Unexpected inhibition of downstream pathways may also lead to incorrect assumptions of PAC_1-antagonism.

Regarding the downstream mechanisms upon activation of the PAC_1 receptor, various transduction pathways are involved. First, PAC_1 is a G protein-coupled receptor (GPCR), activating the adenylyl cyclase pathway, which stimulates the production of cAMP, and protein kinase A (PKA).[23,24] PKA-mediated phosphorylation opens ATP-sensitive potassium channels (K_{ATP}) on vascular smooth muscle cells in cranial arteries, leading to vasodilation.[19,25] Subsequently, it is hypothesized that this vasodilation in turn activates trigeminal afferents, initiating migraine attacks.[19] The cAMP-PKA-K_{ATP}-pathway is presumed to be a common pathway downstream CGRP- and PAC_1-receptor activation, which might explain why CGRP-(receptor) antagonists only partially abort migraine attacks.[3,19] Second, the PAC_1 receptor can be subject to internalization and endosomal activation of extracellular signal-regulated kinase (ERK).[26] The latter activates glial cells, which in turn stimulate the production of inflammatory mediators and neuronal activity, thereby enhancing and maintaining nociception. However, the exact inflammatory mediators involved still need to be uncovered and the PAC_1-ERK pathway has thus far most profoundly been studied in the CNS.[27] Thus, many aspects of the downstream pathways upon PAC_1 receptor activation require further investigation. Importantly, this may also provide additional applications of the biomarker to evaluate PDs of drugs interfering with downstream pathways (e.g., K_{ATP} blockers).

The long-lasting vascular response (i.e., up to 48 h after maxadilan injection), supports the hypothesis of a common pathway downstream CGRP- and PAC_1-receptor activation. Although the half-life of maxadilan has not yet been described in humans, CGRP is known to have a plasma half-life of roughly 10 min whereas the vascular changes induced by a 10 min local CGRP infusion lasted up to 90 min.[28–30] Unfortunately, further long-term duration of the vascular changes was not assessed. Interestingly, $PACAP_{38}$-induced migraine-like attacks were reported to occur within 4 h but also as late as 12 h following $PACAP_{38}$ infusion, whereas $PACAP_{38}$ itself has an even shorter half-life of only 3.5 min.[22,31,32] In line with our results, prolonged extracerebral vasodilation was also seen after systematic infusion of $PACAP_{38}$, which might explain the delayed attacks.[22,31] Another mechanism by

which $PACAP_{38}$ induces migraine-like attacks, is induction of neurogenic inflammation characterized by an influx of inflammatory cells and activation of mast cells. Upon degranulation, the released mediators stimulate sensory trigeminal fibers and activate pain receptors.[31] Yet, although the marker might not have been ideal, Amin et al.[22] failed to prove the involvement of mast cell degranulation in $PACAP_{38}$-induced migraine-like attacks by measuring serum levels of tryptase. Remarkably, in the current study, an unexpected peak in DBF occurred at 24 h postinjection of 0.9 ng maxadilan, which was unlikely due to a direct effect of maxadilan itself. The "secondary" increase in DBF might indicate such delayed inflammatory reaction related to the release of CGRP, substance P, prostaglandins, and/or nitric oxide.[33,34] However, further investigation is warranted to confirm this hypothesis and to identify the exact mediators involved.

In summary, a safe, well-tolerated and reproducible PD biomarker was developed to test PAC_1-receptor antagonists in humans. The maxadilan-based biomarker provides an objective PD readout which is easy to incorporate in early clinical drug development. Using this biomarker model in exploratory clinical trials with PAC_1-receptor antagonists and/or drugs interfering with PAC_1-receptor downstream pathways is an extra asset to facilitate dose-selection and guide go/no-go decisions in early clinical drug development.

AUTHOR CONTRIBUTIONS

H.M., L.B., D.B., and J.d.H. wrote the manuscript. L.B., M.D., A.V.H., K.G., S.S., G.V., and J.d.H. designed the research. L.B. and E.A. performed the research. H.M., L.B., and D.B. analyzed the data.

ACKNOWLEDGMENTS

The authors thank the staff at the Center for Clinical Pharmacology for the careful data collection and professional conduct of this clinical trial.

CONFLICT OF INTEREST

The authors declared no competing interests for this work.

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How to cite this article: Marynissen H, Buntinx L, Bamps D, et al. First-in-human development of a pharmacodynamic biomarker for PAC_1 receptor antagonists using intradermal injections of maxadilan. *Clin Transl Sci*. 2022;15:1968-1977. doi:10.1111/cts.13309