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Title: **Pharmacologic Characterization of ALD403, a Potent Neutralizing Humanized Monoclonal Antibody Against the Calcitonin Gene-Related Peptide**

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Nonstandard abbreviations

ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cell-mediated phagocytosis; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CDC, complement-dependent cytotoxicity; CDR, complementarity-determining region; CLR, calcitonin-like receptor; CTR, calcitonin receptor; CGRP, calcitonin gene-related peptide; CI, confidence interval; ECL, electrochemiluminescence; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FBS: fetal bovine serum; Fc, fragment crystallizable; FDA, Food and Drug Administration; GPCR, G protein-coupled receptor; IC_{50}, half maximal inhibitory concentration; IgG, immunoglobulin G; K_D, equilibrium dissociation constant; k_a, binding constant; k_d, dissociation constant; M, molar; mAb, monoclonal antibody; MEM, minimum essential medium; MSD, Meso Scale Discovery; NHS, N-hydroxysuccinimide; PBS, phosphate buffered saline; PEI, polyethylenimine; RAMP, receptor activity-modifying protein; RU, response unit; SPR, surface plasmon resonance; TK, toxicokinetic; TRPV-1, transient receptor potential vanilloid type 1; VL, variable light; VH variable heavy.

Section

Drug Discovery and Translational Medicine

Running Title: ALD403: A Potent Humanized anti-CGRP mAb

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Abstract (WORD COUNT: 184)
Introduction (WORD COUNT: 703)
Discussion (WORD COUNT: 597)

Abstract

ALD403 is a genetically engineered, humanized immunoglobulin G1 monoclonal antibody that inhibits the action of human calcitonin gene-related peptide (CGRP). Clinical trial data indicate that ALD403 is effective as a preventive therapy for migraine and has an acceptable safety profile. For preclinical characterization of ALD403, rabbit antibodies targeting α-CGRP were humanized and modified to eliminate Fcγ-receptor (FcγR) and complement interactions. The ability of ALD403 to inhibit CGRP-induced cyclic adenosine monophosphate (cAMP) production was assessed using a cAMP bioassay (Meso Scale Discovery). The IC_{50} for inhibition of cAMP release was 434 and 288 pM with the rabbit-human chimera antibody and the humanized ALD403, respectively. ALD403 inhibited α-CGRP binding with an IC_{50} of 4.7×10^{-11} and 1.2×10^{-10} Molar for the α-CGRP and AMY1 receptors, respectively. ALD403 did not induce antibody-dependent cellular cytotoxicity nor complement-dependent cytotoxicity and did not stably interact with any of the FcγR mediating these functions, exhibiting only weak binding to FcγRI. ALD403 significantly lowered capsaicin-induced blood flow responses in rodents at all time points starting at 5 minutes post-application in a dose-dependent manner. In conclusion, ALD403 is a potent functional ligand inhibitor of α-CGRP–driven pharmacology.

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Significance Statement

α-CGRP blockade by ALD403 was assessed via radiolabeled ligand displacement, *in vitro* inhibition of cell signaling, and *in vivo* inhibition of capsaicin-induced vasodilation. Lack of engagement of Fc-mediated immune-effector functions by ALD403 was shown.

Introduction

Migraine is a paroxysmal neurological disorder of moderate to severe headache associated with physiological disruptions of neurological and sensory function (Headache Classification Committee of the International Headache Society (IHS), 2018). Migraines often begin in adolescence or early adulthood and continue over the decades of an individual's life (Bille, 1997). Migraine is the most prevalent neurological disorder for which medical treatment is sought and is considered the sixth leading cause of disability worldwide (Steiner et al., 2013; GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, 2017; Steiner et al., 2018). The debilitating nature of migraine makes preventive treatment an attractive option (Lipton and Silberstein, 2015). It is estimated that up to 40% of adults with frequent episodes of migraine and nearly all with chronic migraine would benefit from preventive therapy (Lipton et al., 2007). In large measure, this is due to poor tolerability, lack of efficacy, and poor adherence to traditional acute migraine treatments (Loder, 2010; Lipton and Silberstein, 2015; Russo, 2015; Obermann and Holle, 2016; Lukacs et al., 2017). These observations suggest an ongoing need for new therapeutic options for migraine prevention that are safe, efficacious, and well tolerated (Silberstein et al., 2012; Lipton and Silberstein, 2015).

While the pathophysiology of migraine is complex and incompletely understood (Noseda and Burstein, 2013; Pietrobon and Moskowitz, 2013), calcitonin gene-related peptide (CGRP) has emerged as an important target for prevention of migraine (Wrobel Goldberg and Silberstein, 2015). CGRP is abundant in trigeminal neurons, which elicit nociceptive signaling pathways involved in migraine (May and Goadsby, 1999; Buzzi et al., 2003). In addition to being implicated in pain transmission and neuronal sensitization in both peripheral and central pain pathways (Durham, 2006), CGRP has been implicated in migraine-related vasodilation and triggering of neurogenic inflammation (Hostetler et al., 2013). A role for CGRP in migraine is further supported by data showing that intravenous infusions of CGRP can induce migraine-like headaches in individuals susceptible to migraine (Lassen et al., 2002; Hansen et al., 2010). Furthermore, many pharmacological agents used as acute or preventive treatment of migraine are known to inhibit the actions of CGRP (Edvinsson et al., 2018).

Small-molecule CGRP-receptor antagonists (gepants) were the first agents to demonstrate CGRP-directed inhibition of migraine, originally targeted as acute therapy (Tepper, 2018). Over the past few years, research efforts in migraine prevention have focused on inhibiting CGRP or its receptor through the use of monoclonal antibodies (mAbs), which have

several advantages over small-molecule antagonists and alleviate safety concerns (Pellesi et al., 2017; Edvinsson, 2018b; Edvinsson et al., 2018). Monoclonal antibodies may be more target-selective, do not easily cross the blood-brain barrier, and are typically degraded by proteolysis rather than by metabolism via cytochrome P450 enzymes and thus are not associated with drug-drug interactions resulting from hepatic metabolism (Ferri et al., 2016).

ALD403 (eptinezumab) is an IgG1 monoclonal antibody that binds human CGRP and was designed to avoid Fcγ receptor and complement interactions via a mutation on the canonical N-linked glycosylation site N297 in its Fc region (Dorokhov et al., 2016; Saxena and Wu, 2016; Baker et al., 2017; Mimura et al., 2018). A randomized, double-blind, placebo-controlled, exploratory, proof-of-concept phase II trial found a single intravenous administration of ALD403 to be generally safe and effective in reducing monthly headache days (mean change from baseline in monthly headache days vs. placebo after 5–8 weeks: –1.0, 95% confidence interval [CI] –2.0 to 0.1; one-sided $P = 0.0306$) in patients with frequent episodic migraines (Dodick et al., 2014). In a phase IIb, parallel-group, double-blind, randomized, placebo-controlled, dose-ranging clinical trial of ALD403 in patients with chronic migraine, where the primary endpoint was ≥75% migraine responder rates, the doses of 300, 100, 30, and 10 mg of ALD403 resulted in 33.3, 31.4, 28.2, and 26.8% reduction, respectively, compared with 20.7% for placebo ($P =$ 0.033, 0.072, 0.201, 0.294 vs. placebo) over weeks 1–12 (Dodick et al., 2019). ALD403 is currently in phase III development for the preventive treatment of migraine for patients with episodic and chronic migraine (ClinicalTrials.gov. National Library of Medicine (U.S.), 2015, September 25-; ClinicalTrials.gov. National Library of Medicine (U.S.), 2016, November 28). The studies summarized here report the characterization of the *in vitro* and *in vivo* pharmacologic properties of the CGRP ligand-directed inhibitor antibody, ALD403.

Materials and Methods

Generation, Humanization, and Engineering of ALD403. Antibodies against human α-CGRP [unless otherwise indicated, "CGRP" refers to α-CGRP] were generated in New Zealand White rabbits immunized with human CGRP (NCBI NP_001029125.1, American Peptides, Sunnyvale, CA, and Bachem, Torrance, CA). Candidate antibodies were initially identified in conditioned B-cell culture supernatants as capable of binding biotinylated CGRP by enzyme-linked immunosorbent assay (ELISA) using conditioned B-cell culture supernatants as previously described (Lightwood et al., 2006). CGRP-binding antibodies were further characterized for their ability to inhibit CGRP-driven intracellular cAMP accumulation in SK-N-MC cells (Van Valen et al., 1990). ALD403 variable regions were cloned onto a human full-length IgG framework and subsequently humanized and engineered to minimize interactions with the immune system. Humanization was performed by grafting the complementarity-determining regions (CDRs) into the closest human variable (V) region light- and heavy-chain framework sequences (V1–27 and V3–66, respectively) as previously described (Jones et al., 1986; Almagro and Fransson, 2008). Inhibition of CGRP-induced cAMP accumulation was used to monitor potency of the humanized variants. To maximize the potency of the humanized sequences of ALD403, a total of 13 amino acids between the light- and heavy-chain human framework sequences were reverted to the rabbit sequence. Canonical asparagine 297 in the heavy chain was specifically mutated to alanine (N297 mutation) to avoid FcγR and complement protein interactions.

Inhibition of CGRP Signaling. To monitor the potency of antibodies, inhibition of CGRP-driven cAMP accumulation was measured using electrochemilumunescence assays (Meso Scale Discovery, MSD). Briefly, antibody preparations to be tested were serially diluted in MSD assay buffer and human CGRP was added (2.6 nM final concentration unless otherwise indicated). This mix was incubated for 1 hour at 37°C. Human neuroepithelioma cells (SK-N-MC, ATCC cat. no. HTB-10) were detached using an ethylenediaminetetraacetic acid (EDTA) solution (5 mM in PBS) and washed using growth media (MEM, 10% FBS, antibiotics) by centrifugation. The cell number was adjusted to 2 million cells per mL in assay buffer, and IBMX (3-Isobutyl-1-methylxanthine, Sigma Aldrich, St. Louis, MO) was added to a final concentration of 0.2 mM. Twenty microliters of the premixed antibody and CGRP were added to the assay plate (MSD); 10 μL of cells were added to this mixture and incubated for 30 minutes with shaking at room

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temperature. The cells were then lysed using 20 μL of lysis buffer containing TAG cAMP (MSD) and incubated for 1 hour while shaken at room temperature. Prior to reading the plate, 100 μL of 1.5x concentrated read buffer (MSD) was added to all wells on the plate and the plate was read using a Sector Imager 2400 (MSD). GraphPad Prism™ software (v8.0) was used for data fit and IC_{50} determination.

Inhibition of CGRP Binding to Human CGRP and AMY1 Receptors. ALD403 preparations were serially diluted in assay buffer [50 nM Hepes-NaOH (pH 7.4), 10 mM $MgCl_2$, 4 mM KCl, 10 mM NaCl, 1 mM EDTA, 1 μM phosphoramidon, 0.3% BSA, and 0.04 bacitracin] to a final antibody concentration ranging from 20 nM to 2 pM and incubated with [^{125}I]-α-CGRP (0.03 nM for CGRP receptor and 0.2 nM for AMY1 receptor, Chelatec, France) for 15 minutes at 22°C. This mixture was then incubated with cell membrane homogenates expressing either the CGRP receptor or the AMY1 receptor (containing 16 or 10 μg of protein, respectively, ChemiScreen, EMD Millipore) and incubated for 90 minutes (CGRP receptor) or 120 minutes (AMY1 receptor) at 22°C. Following this incubation, the samples were filtered under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold rinse buffer (50 mM Tris-HCl, 150 mM NaCl). The filters were dried and counted for radioactivity in a scintillation counter (TopCount, Packard) using a scintillation cocktail (Microscient 0, Packard).

The specific binding signal was determined by subtracting nonspecific binding ([^{125}I]-α-CGRP in the presence of 1 μM unlabeled α-CGRP) from total binding. The standard reference compound was unlabeled α-CGRP, which was tested in each experiment at a range of concentrations to establish a competition curve from which the IC_{50} was calculated.

Characterization of ADCC, ADCP, and CDC Activation by ALD403. To confirm the lack of Fc-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP) by ALD403, the antibody was tested for the ability to activate FcγRIIIa and FcγRIIa, the primary mediators of ADCC and ADCP, respectively (Dorokhov et al., 2016). Reporter bioassays were used according to manufacturer's instructions (Promega Corporation, cat. nos. G7010 and G9991). Briefly, the day before the assay, SK-N-MC cells were harvested using 5 mM EDTA and resuspended in growth media

[EMEM and L-glutamine (ATCC, cat. no. 30-2003), GlutaMAX (Gibco, cat. no. 35050-061), and heat-inactivated FBS (Sigma Aldrich, St. Louis, MO, cat. no. F8067)]. SK-N-MC cells (100 µL per well, 3×10^5 per mL) were plated into 96-well CELLSTAR plates (Greiner Bio-One, cat. no. 655083) and incubated overnight at 37°C. ALD403 preparations were serially diluted in assay buffer (RPMI-1640, 4% low IgG serum, Promega Corporation) for a final assay concentration ranging from 6.1 pM to 40 nM. SK-N-MC cells were washed with assay buffer, after which ALD403 was added to each well. For the assay conditions containing CGRP, a final concentration of 10 nM was added. This amount of CGRP was chosen based on results of separate signaling assays where 10 nM CGRP was found to be in excess of the amount needed for maximum cellular response. CGRP-free assay conditions received an equivalent volume of assay buffer. As a positive control, an anti-CD20 antibody and 1.5×10^4 Raji cells (ATCC, cat. no. CCL-86) expressing CD20 were used. Cells were plated and anti-CD20 IgG1 antibody (Invivogen, cat. no. hcd20-mab1) was serially diluted as described for ALD403 and added to the appropriate wells. After 15 minutes' pre-incubation, 25 µL of Jurkat effector cells expressing the appropriate Fcγ receptor with an NFAT-luciferase reporter gene were diluted in assay buffer per the provider's protocol and were added to each well and incubated for 6 hours at 37°C. Plates were removed from the incubator, placed to ambient temperature for 15 minutes, and 75 µL of Bio-Glo reagent (Promega Corporation) was added to each well and further incubated at room temperature for 15 minutes protected from light. Luminescence was detected using a BioTek Synergy Neo and results were graphed using GraphPad Prism™ software (v8.0).

To corroborate the lack of ALD403-driven complement-dependent cytotoxicity (CDC), SK-N-MC cells expressing the CGRP receptor were treated with human serum (Corning, cat. no. 35-060-CI) in the presence of ALD403 with or without CGRP. Briefly, the day before the assay, SK-N-MC cells were harvested using 5 mM EDTA and resuspended in growth media. Aliquots of 100 µL per well of SK-N-MC (1×10^5/well) were plated into clear-bottom 96-well black tissue culture plates (Corning, cat. no. 3603). As a positive control, an anti-CD20 antibody and Ramos.2G6.4C10 (Ramos) cells (ATCC, cat. no. CRL-1923) expressing CD20 were used. On the day of the assay, media was aspirated and rinsed with serum-free SK-N-MC growth media. Ramos cells were pelleted, washed, and resuspended in serum-free Ramos growth media [RPMI-1640 (ATCC cat. no. 30-2001), GlutaMAX]. Ramos cells (100 µL, 1×10^5/well) were plated into a V-bottom 96-well plate (Corning, cat. no. 3894), pelleted, and the supernatant was removed. ALD403 and anti-CD20 IgG1 antibodies were serially diluted in cell-specific media

supplemented with 20% human serum with or without CGRP (10 nM final concentration) and added to cells. Control wells received heat-inactivated human serum. Final antibody concentrations ranged from 93.3 pM to 66.7 nM (final volume of 100 µL). Samples were incubated at 37°C for 2 hours, after which the supernatants were aspirated (SK-N-MC cells) or pelleted and aspirated (Ramos cells), and the cells washed with 200 µL DPBS. Cells were resuspended in 150 µL per well of DPBS containing 100 nM Calcein AM (Invitrogen, cat. no. C3099) and further incubated for 30 minutes. Ramos cells were then pelleted and washed using DPBS, and SK-N-MC cells were harvested using 25 µL of Trypsin (HyClone, cat. no. SH30042.01) followed by the addition of 125 µL of growth media to inactivate the trypsin. Cells were then pelleted and washed in DPBS followed by a final resuspension in 150 µL of DPBS. Cell viability was assessed by Calcein AM uptake as determined by flow cytometry (BD Accuri C6) and results were analyzed using GraphPad Prism™ software (v8.0).

ALD403 Interactions with Fcγ Receptors. The interaction of ALD403 with human Fcγ (RI, RIIA, RIIB/C, RIIIA, and RIIIB) receptors was assessed using surface plasmon resonance. A GLC primary amine-coupling sensor (Bio-Rad, Hercules, CA, cat. no. 176-2240) was hydrated and preconditioned using 0.5% SDS, 50 mM NaOH, and 100 mM HCl per manufacturer protocol. The sensor surface was activated by injecting a mixture of 50% EDC / 50% NHS solution at final 1× concentration (Bio-Rad), with a 30 µL/min for 300 seconds' flow. Prior to immobilization, the Fcγ receptors (R&D Systems, Minneapolis, MN, FcγRI/CD64, cat. no. 1257-FC-050; FcγRIIA/CD32a, cat. no. 1330-CD-050; FcγRIIB/C (CD32b/c), cat. no. 1875-CD-050; FcγRIIIA/CD16a, cat. no. 4325-FC-050; FcγRIIIB/CD16b, cat. no. 1597-fc-050/CF) were diluted to 7 µg/mL in 20 mM sodium acetate pH 5.5 and coupled to the EDC/NHS-activated GLC sensor prior to ethanolamine (0.33 M, Bio-Rad) deactivation per default settings. Prior to test article injection, running buffer was allowed to equilibrate to achieve a stable baseline signal. To determine the association rate of test articles onto immobilized FcγR surface, five 3-fold serial dilutions of antibodies were made from 10,000 nM down to 123 nM in running buffer [PBS containing 1% Tween-20 (Teknova, cat. no. P1192)]. The antibody dilutions were allowed to interact with the FcγR using a 100 µL/min flow rate for a 30-second injection followed by a 90-second dissociation phase using running buffer. Experiments for multiple test articles and the positive control were performed in sequence and bracketed by 1000-second blank injections.

Data were referenced against interspots and fit using a 1:1 Langmuir model (Bio-Rad ProteOn Manager Software, v3.1.0.6) with k_a and k_d grouped by test article, and R_{max}, Chi^2 reported as averages for each test article. Subsequently, K_D was calculated from the k_d/k_a values. Samples with <10 RU of signal above blank injection or <5% of positive control visual R_{max} were assigned "no measurable binding."

Binding Kinetics and Specificity of ALD403. Surface plasmon resonance was used to characterize the binding of ALD403 to human and rat CGRP peptides using the ProteOn XPR36 instrument (Bio-Rad). For analysis of human CGRP peptides, ProteOn Neutravidin sensors (Bio-Rad, Hercules, CA, cat. no. 176-5021) were hydrated and conditioned using 1 M NaCl and 50 mM NaOH per the manufacturer's protocol. ALD403 was biotinylated [1:20 molar ratio of antibody: Sulfo-NHS-LC-LC-Biotin (Thermo Fisher Scientific, Waltham, MA, cat. no. 21338)] and exchanged to DPBS using a 0.5 mL 7 k MWCO Zeba Spin Column (Thermo, cat. no. 89882). Biotinylated ALD403 was diluted in running buffer (1× HBS-EP, 0.2 M arginine, 0.2 mg/mL BSA, and 0.005% Na azide) to a final concentration of 3–5 μg/mL and allowed to interact with the biosensor (300 sec at 30 uL/min) until an average surface density of 1000–2300 response units (RU) were reached. For analysis of rat CGRP peptides, the composition of the running buffer used was (1× HBS-EP$^+$ + 0.2 mg/mL BSA + 0.005% Na azide). GLM sensors (Bio-Rad, cat. no. 176-5012) were hydrated and conditioned using 0.5% SDS solution, 50 mM NaOH, and 100 mM HCl, and each sensor was activated using a mixture of EDC (1x) and NHS (1x) according to manufacturer recommendations. ALD403 antibody preparation was diluted to 15 and 2 μg/mL using a 10 mM sodium acetate pH 5.0 buffer and allowed to couple onto the EDC/NHS-activated GLM sensor using a 30 μL/min flow rate for a total of 300 seconds. Ethanolamine (1 M) was then used for quenching according to manufacturer's recommendations. This resulted in surface densities of ALD403 of 7000–10000 RU and 2000–3000 RU, respectively, after coupling to the activated sensor.

To determine k_a and k_d and to calculate the K_D of the CGRP peptide analytes, five 3-fold serial dilutions of the different CGRP peptides [human α-CGRP (American Peptides, Sunnyvale, CA, cat. no. 22-1-12); human α-CGRP (Bachem, Torrance, CA, cat. no. 1057151)] were made with a starting concentration of 100 nM and the lowest concentration being 1.2 nM in running buffer. Dissociation constant experiments for both forms of CGRP were performed using the highest analyte concentration and dissociation times of 80,000–86,000 seconds.

To establish the specificity of ALD403 for CGRP compared to closely related peptides, a similar method to that described above to determine the binding kinetics was used with the following modifications. ALD403 was immobilized via amine coupling using a ProteOn GLC Chip (Bio-Rad, Hercules, CA, cat. no. 176-5011) per manufacturer's recommendations, targeting average immobilization of 2000–5000 RU. As control, 30 nM CGRP was allowed to interact with immobilized ALD403 using a 100 µL/min flow rate for 90 seconds. Buffer used was 1× HBS-EP$^+$ (Teknova), 0.2 M arginine, 0.2 mg/mL BSA, and 0.005% Na azide. In addition to CGRP, the following peptides were tested using a 25-fold higher concentration than that used for CGRP (1000 nM): amylin (AnaSpec, Fremont, CA, cat. no. AS-60254-1) and rat amylin (cat. no. AS-60253-1), calcitonin (cat. no. AS-20673), intermedin (cat. no. 65334-05), and adrenomedullin (cat. no. AS-60447).

Inhibition of Capsaicin-driven Blood Flow in Rodents. The day prior to testing, male Sprague-Dawley rats (Harlan) were randomly assigned the following groups for dosing: Group 1 received 30 mg/kg of a nonspecific control antibody, and Groups 2–6 received ALD403 at 30, 10, 3, 1, or 0.3 mg/kg, respectively. Antibodies were diluted in phosphate-buffered saline and administered intraperitoneally. An area approximately 2×6 cm on the dorsal lower back was shaved and depilated for Doppler imaging.

On the day of the test, rats were anesthetized with isoflurane gas and placed on a temperature-controlled heating pad and fitted with a nose cone to provide continuous isoflurane delivery. A Moor LDI2 IR Laser Doppler imaging system (Moor Instruments, UK) was used to monitor vasodilation. A baseline Doppler scan was conducted to identify areas of low blood flow for placement of two rubber O-rings (<1 cm in diameter). After O-ring placement, a baseline Doppler scan was performed. Immediately after completion of the scan, 1 mg capsaicin (Sigma Aldrich, St. Louis, MO, cat. no. M2028) in 5 µL 1:1 ethanol:acetone was applied to the skin within each O-ring. Doppler scans were repeated at 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, and 30 minutes after capsaicin application. Percent change from baseline mean flux within each of the two O-rings was plotted as the results of vasodilation due to capsaicin. Means and standard deviations for each group, at each time, were calculated. GraphPad Prism™ software (v8.0) was used for statistical analysis.

Toxicokinetic Parameters for ALD403 in Sprague-Dawley Rats Following a Single Intravenous Bolus Injection. The toxicokinetic (TK) characteristics of ALD403 were evaluated following a single intravenous injection to Sprague-Dawley rats at dose levels of 0, 10, 30, or 100 mg/kg ALD403 and 14 days' observation. A total of 12 rats (6 male and 6 female) were dosed. Blood samples were collected prior to dosing, at 1 minute, and at 24, 96, 168, and 336 hours post-dosing. The blood samples were processed for plasma and analyzed for ALD403 concentration by Charles River Laboratories, Reno, NV, using a validated indirect electrochemiluminescence (ECL) method. Plasma samples, standards, and quality control samples containing ALD403 at various concentrations were added to goat anti-human IgG–coated microtiter plates, 96-well format, and incubated for 60 ± 5 minutes at room temperature. After washing, a detection antibody (Biotinylated Human IgG) was added and incubated for an additional 60 ± 5 minutes at room temperature. The plates were washed again, 1x MSD Read Buffer was added to the wells, and an MSD Sector imager was used to evaluate light intensity, yielding quantitative measures (ECL signals) for ALD403 present in the samples. The ECL measurements were transferred to SoftMax® Pro GxP, where the ALD403 concentrations were calculated using a 4-parameter standard calibration curve.

The TK parameters were estimated using WinNonlin® pharmacokinetic software (Pharsight Corp., Mountain View, CA). A non-compartmental approach consistent with the intravenous route of administration was used for parameter estimation. All parameters were generated from mean ALD403 concentrations in plasma from day 1–14 using sampling times relative to the start of dose administration.

Results

Identification of ALD403. A panel of anti-CGRP antibodies was generated by single B-cell antibody cloning from rabbits that had been immunized with human CGRP. The candidate anti-CGRP antibodies were identified by their ability to bind to CGRP and by their ability to inhibit CGRP-driven intracellular cAMP accumulation in SK-N-MC cells (not shown). These recombinant antibodies were initially chimeric, with the variable domains from rabbits and the constant heavy- and light-chain domains from human IgG1 and human kappa, respectively. ALD403 was humanized by CDR grafting into compatible human immunoglobulin germline variable genes (see Materials and Methods). Several additional non-CDR rabbit residues in the framework regions of the variable domains were determined to be necessary to retain desired binding and functional characteristics in the final humanized antibody. Figure 1 shows the original rabbit variable sequences of ALD403 along with the human germline genes used and the final humanized variable sequences of ALD403. Humanized ALD403 retained the capacity to inhibit CGRP-induced cAMP production; the chimeric antibody exhibited an IC_{50} of 434 pM compared to an IC_{50} of 288 pM for the humanized ALD403 (See Supplemental Figures 1 and 2).

ALD403 Does Not Support ADCC, ADCP, CDC, nor Stable Fcγ Receptor Interactions. Activation of cell-mediated and/or complement-mediated immune processes by IgG antibodies heavily depend on the N-linked glycan at amino acid N297 of the Fc domain within the IgG molecule (Dorokhov et al., 2016; Saxena and Wu, 2016; Mimura et al., 2018). The amino acid N297 of ALD403 was mutated to alanine to prevent N-linked glycosylation and, as expected, no N-linked glycosylation or N-linked carbohydrate was observed in ALD403 (data not shown).

As engineered, ALD403 did not activate FcγRIIIa (primary ADCC receptor (Koene et al., 1997) nor FcγRIIa (primary ADCP receptor (Richards et al., 2008)) alone or in the presence of CGRP or CGRP plus SK-N-MC cells (**Fig. 2A**). The positive control demonstrated robust signaling through both receptors. To monitor for complement-mediated immune functions, ALD403 was incubated with SK-N-MC cells in the presence of complement-containing human serum with or without CGRP. ALD403 did not support complement-mediated cell lysis (**Fig. 2B**). The positive control exhibited significant levels of cytotoxicity. Collectively, these data support that the Fc engineering performed in the construction of ALD403 successfully minimized/eliminated antibody effector functions.

To further confirm the profile of ALD403, in particular its interactions with the receptors that mediate the major immune processes mentioned above, the binding of ALD403 to the human Fcγ receptors RI, RIIA, RIIB/C, RIIIA, and RIIIB was assessed by surface plasmon resonance and compared to a wild-type, glycosylated control IgG1 antibody. These receptors mediate immune functions such as ADCC, ADCP, maturation of dendritic cells, etc. ALD403 had no measurable binding to 4 of the 5 Fcγ receptors and only weak binding to FcγRI (**Fig. 3** and **Table 1**). The affinity (K_D) of ALD403 for FcγRI was reduced greater than 40-fold versus wild type—non-mutated—IgG control (**Table 1**). This pattern of Fcγ receptor binding is consistent with presence of the N297A mutation in the heavy chain of ALD403 (Mimura et al., 2018).

Binding Kinetics and Specificity of ALD403. ALD403 binds with high affinity to both forms (α and β) of human and rat CGRP with a fast on-rate and a slow off-rate (**Table 2**). CGRP sequences are most homologous with the peptides amylin, calcitonin, adrenomedullin, and intermedin (Wimalawansa, 1996), which have been proposed to be part of a superfamily of peptides (Wimalawansa, 1997). Surface plasmon resonance (ProteOn) was used to address the specificity of ALD403 binding to CGRP compared to the above-mentioned peptides. ALD403 was immobilized via amine coupling onto a GLC ProteOn chip. Subsequently, each peptide was presented to the immobilized antibody and their binding was monitored. As expected, CGRP was able to efficiently bind and be retained by the immobilized ALD403. In contrast, none of the other peptides tested were able to stably interact with the immobilized antibody (**Fig. 4**).

ALD403 Inhibits Binding of CGRP to Both the CGRP and AMY1 Receptors. CGRP binds two receptors with high affinity: the CGRP receptor (which is composed of a complex of CLR and RAMP1) and the AMY1 receptor (composed of CTR and RAMP1) (Tilakaratne et al., 2000; Hay et al., 2006; Moore and Salvatore, 2012; Walker et al., 2015; Hay and Walker, 2017; Hay et al., 2018) The ability of ALD403 to inhibit binding of CGRP to the CGRP receptor and AMY1 receptor was evaluated in competitive binding experiments using cell-membrane preparations expressing each of these receptors. Pre-incubation of ALD403 with [^{125}I]-labeled α-CGRP was able to inhibit CGRP binding in a dose-dependent manner exhibiting an IC_{50} of 4.7×10^{-11} and 1.2×10^{-10} M for the CGRP and AMY1 receptors, respectively (**Fig. 5**). Unlabeled α-CGRP was

used as a positive control in these assays and displayed an IC_{50} of 4.5×10^{-11} for the CGRP receptor and 3.7×10^{-10} M for the AMY1 receptor.

Inhibition of Capsaicin-driven Increased Blood Flow and TK Parameters of ALD403

Dermal application of capsaicin produces a localized increase in blood flow that can be measured via laser doppler imaging. Capsaicin activates the transient receptor potential vanilloid type 1 receptor (TRPV1), producing local release of vasoactive mediators, including CGRP which results in localized dermal vasodilation. (Hershey et al., 2005; Van der Schueren et al., 2007; Sinclair et al., 2010) The ability of ALD403 to inhibit this activity was evaluated in rats. Animals previously treated with an IgG control antibody responded to capsaicin treatment with elevations of blood flow starting at 2.5 minutes after capsaicin administration. The response plateaued at around 12.5 minutes and was sustained for at least 15 minutes. Administration of ALD403 24 hours prior to capsaicin treatment resulted in a dose-dependent inhibition of capsaicin-driven blood flow relative to animals treated with the negative control antibody. Animals that had received ≥0.3 mg/kg doses of ALD403 exhibited significantly lower responses at 5, 10, 12.5, and 15 minutes post capsaicin administration (**Fig. 6A**).

To characterize the TK parameters of ALD403 in rats, both male and female rats were dosed with increasing amounts of ALD403, and blood samples were collected prior to dosing and at 1 minute, and at 24, 96, 168, and 336 hours post-dosing for analysis of ALD403 in plasma. The mean plasma concentration-time profiles for ALD403 in rats were characterized by an apparent mono-exponential decline (**Fig. 6B**). The TK parameters are summarized in **Table 3** and revealed a half-life ($T_{1/2}$) of 124 hours.

Discussion

CGRP has been established as an important signal molecule in the pathophysiology of migraine, and inhibition of its function has demonstrated efficacy in the clinic for the treatment and prevention of migraines (Russo, 2015; Edvinsson, 2018a; Agostoni et al., 2019; Ceriani et al., 2019; Charles and Pozo-Rosich, 2019; Parikh and Silberstein, 2019; Tepper, 2019; Yuan et al., 2019). This report summarizes the discovery, engineering, and preclinical characterization of the humanized monoclonal antibody ALD403 targeting CGRP for the prevention of migraine. Advances in molecular engineering have facilitated the development of monoclonal antibodies with characteristics that maximize target interaction and minimize unwanted activities. Potential

therapeutic antibodies are commonly humanized in an attempt to minimize the potential for an undesirable immune response (Kuramochi et al., 2019; Mayrhofer and Kunert, 2019; Waldmann, 2019). Humanization of ALD403 maintained its high affinity for both human and rat CGRP isoforms as well as its specificity for binding to CGRP over related peptides. The potency of inhibition in cell-based assays was also conserved after humanization. The Fc domain of ALD403 was successfully modified to minimize immune-functions via either Fcγ receptors or complement. ALD403 exhibits a very high affinity for both α and β forms of human and rat CGRP, and its mechanism of action was demonstrated through the complete and potent blocking of ^{125}I-CGRP binding to both of its primary receptors, the CGRP receptor and the AMY1 receptor. This inhibition of receptor binding results in the subsequent inhibition of CGRP-induced cAMP production *in vitro* and *in*-vivo translates into inhibition of capsaicin-induced increases in dermal blood flow. These findings are consistent with clinical data demonstrating significant reduction in migraine headache days with favorable safety and tolerability (Dodick et al., 2014; Dodick et al., 2019).

Scaffold glycoengineering such as that used to construct ALD403 (Tao and Morrison, 1989; Simmons et al., 2002) has allowed researchers to design antibodies that do not engage into unnecessary immune functions for their mode of action. Elimination of N-linked glycosylation on the canonical N297 residue, reduces FcγR and complement interactions—minimizing the potential for activation of immune effector functions without adversely impacting FcRn binding and plasma half-life (Tao and Morrison, 1989; Simmons et al., 2002; Pyzik et al., 2015; Rath et al., 2015; Jacobsen et al., 2017) nor the ability of ALD403 to inhibit binding to CGRP and subsequent inhibition of CGRP signaling. Experiments performed *in vitro* using both FcγR binding and activation assays confirmed the lack of interaction of ALD403 with Fcγ receptors. In addition, there was no stimulation of complement-mediated cell cytotoxicity with ALD403.

Capsaicin-induced dermal blood flow is a common, valuable tool for measuring in vivo CGRP-driven neurogenic vasodilation (Hershey et al., 2005; Van der Schueren et al., 2007; Sinclair et al., 2010). Capsaicin activates the transient receptor potential vanilloid type 1 receptor in the blood vessels of the skin, producing neurogenic inflammation and vasodilation via the local release of vasoactive mediators, including CGRP. Capsaicin is applied topically, and changes in the dermal blood flow are measured using a laser Doppler imaging system. The ability of ALD403 to inhibit CGRP-mediated neurogenic vasodilation in the skin of the rat was

demonstrated. This finding is consistent with data from approved anti-CGRP mAbs, all of which have demonstrated the ability to inhibit capsaicin-induced vasodilation (Vermeersch et al., 2015; Monteith et al., 2017; Vu et al., 2017).

In conclusion, ALD403 potently binds to the human CGRP peptide, showing species specificity and strong pharmacodynamic activity. These results confirm the utility of the ALD403 mAb as a competitive inhibitor to the CGRP ligand, effectively blocking the CGRP signaling pathway, and exhibiting clinical utility in migraine.

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Authorship Contributions.

Participated in research design: LFGM, BK, JAL, BB, CJR, DA, BD

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Wrote or contributed to the writing of the manuscript: CJR, EO, BD, KA, BB, JB, MSK, CK, DA, JAL, BK, ES, LFGM

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TABLES AND FIGURES

TABLE 1

Affinity of ALD403 for Fcγ receptors

TABLE 2

Binding constants of ALD403 to different CGRP molecules

TABLE 3

Single-dose toxicokinetic parameters for ALD403 in rodents.

Fig. 1. Humanization of ALD403 variable region. The original rabbit-variable light-chain and variable heavy-chain amino acid sequences ("Rabbit 403VL" and "Rabbit 403VH") are aligned with the obtained homologous human variable light-chain and variable heavy-chain amino acid sequences ("Human V1–27" and "Human V3–66," respectively). The final humanized sequences are shown; red amino acids are identical to the human sequences and black amino acids are different than the human sequences and were derived from the original rabbit antibody sequences.

Fig. 2. Lack of ADCC (A), and ADCP (B) activation, and CDC (C) stimulation by ALD403. ADCC, ADCP, and CDC activation by ALD403 were analyzed *in vitro* and results were graphed compared to a positive control antibody. ADCC and ADCP activities were determined by activation of the relevant Fcγ receptors (FcγRIII and FcγRII, respectively) in reporter cell lines.

Results show averages ± SD of triplicate samples. CDC was measured by ability of target cells to absorb a fluorescent vital dye in the presence of antibody and active complement components. Results are single samples in an assay representative of 3 independent analyses.

Fig. 3. Characterization of ALD403 interactions with Fcγ receptors (left column). ALD403 displays limited binding to FcγRI and does not interact with RIIA, RIIB/C, RIIIA, and RIIIB immobilized via amine coupling onto a GLC Biosensor (ProteOn XPR36). Antibody concentrations used 10 μM (red), 3.3 μM (light blue), 1.1 μM (dark blue), 0.37 μM (green), 0.12 μM (magenta), and buffer (orange). Data are referenced to interspots and buffer blank. Also shown (right column) binding by positive control whole human IgG1.

Fig. 4. Binding specificity of ALD403. Binding by α-CGRP and non-interaction of superfamily of peptides (amylin, calcitonin, intermedin, adrenomedullin) to amine-coupled immobilized antibody (ALD403) on a GLC Biosensor (ProteOn XPR36). Peptides were allowed to interact with the immobilized ALD403 at a 1000 nM concentration for 90 seconds. Data were referenced to interspots.

Fig. 5. Inhibition of CGRP binding to CGRP receptor and AMY1 receptor with ALD403. Displacement of ^{125}I-CGRP binding to CGRP and AMY1 receptors by increasing amounts of ALD403 and unlabeled CGRP were measured and IC_{50} values were obtained. GraphPad Prism™ software (v8.0) was used to generate the fit curves and calculate IC_{50} values. Results show averages of duplicate samples.

Fig. 6. Inhibition of capsaicin-driven increased blood flow (A) and mean plasma concentration-time profiles of ALD403 in rodents (B). Changes in dermal blood flow were measured using laser Doppler imaging following topical application of capsaicin solution. ALD403 at different dose levels or a nonspecific isotype control Ab (AD26-10) were injected intraperitoneally 24 hrs prior to capsaicin challenge. Doppler scans were repeated every 2.5 min for 15 min after application. The percent change from baseline was calculated from the mean flux values for each animal (n = 6). ALD403 plasma concentration levels were measured for all doses and shown in (B).

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TABLE 1

Affinity of ALD403 for Fcγ receptors

Receptor	Antibody	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)
FcγRI	ALD403	8.19 E+04	5.95 E-02	7.26 E-07
	Human IgG	1.17 E+05	2.03 E-03	1.74 E-08
FcγRIIA	ALD403	NMB	NMB	NMB
	Human IgG	2.38 E+05	4.46 E-01	1.87 E-06
FcγRIIB/C	ALD403	NMB	NMB	NMB
	Human IgG	1.36 E+05	5.30 E-01	3.88 E-06
FcγRIIIA	ALD403	NMB	NMB	NMB
	Human IgG	5.29 E+04	8.79 E-02	1.66 E-06
FcγRIIIB	ALD403	NMB	NMB	NMB
	Human IgG	7.46 E+04	5.10 E-01	6.84 E-06

IgG = immunoglobulin; NMB = no measurable binding.

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TABLE 2

Binding constants of ALD403 to different CGRP molecules

CGRP Molecule	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)
Human Alpha	2.6E+05	1.0E-06	4E-12
Human Beta	2.9E+05	1.0E-06	3E-12
Rat Alpha	3.9E+05	6.4E-05	1.7E-10
Rat Beta	5.8E+05	5.1E-06	8.4E-12

CGRP = calcitonin gene-related peptide; k_a = on-rates for binding affinity; k_d = off-rates for binding affinity; K_D = binding affinity constant (k_d/k_a).

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TABLE 3

Single-dose toxicokinetic parameters for ALD403 in rats

Dosage (mg/kg)	Sex	t_{max} (h)	C_{max} (µg/mL)	$AUC_{0\text{-}tlast}$ (µg·hr/mL)	$AUC_{0\text{-}inf}$ (µg·hr/mL)	$t_{1/2}$ (hr)	CL (mL/hr·kg)	Vz (mL/kg)
10	M	0.017	302	24998	28205	120	0.355	61.5
	F	0.017	301	21102	26351	155	0.380	84.8
30	M	0.017	774	67822	81448	138	0.368	73.4
	F	0.017	770	72479	RNR	RNR	RNR	RNR
100	M	0.017	2629	203424	226313	105	0.442	67.2
	F	0.017	2313	164289	180871	102	0.553	81.4

$AUC_{0\text{-}inf}$ = area under the plasma concentration-time curve from time zero extrapolated to infinity; $AUC_{0\text{-}tlast}$ = area under the plasma concentration-time curve from time zero to the time of the last quantifiable ALD403 concentration; CL = total body clearance; C_{max} = maximum observed concentration; F = female; hr = hour; kg = kilogram M = male; mL = milliliter; RNR = result not reported as the $AUC_{0\text{-}inf}$ was extrapolated by more than 20% or the Rsq was <0.800; T_{max} = time at maximal concentration; $t_{1/2}$ = terminal half-life; µg = microgram; V_Z = apparent volume of distribution during terminal elimination phase.

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Fig. 1. Humanization of ALD403 variable region

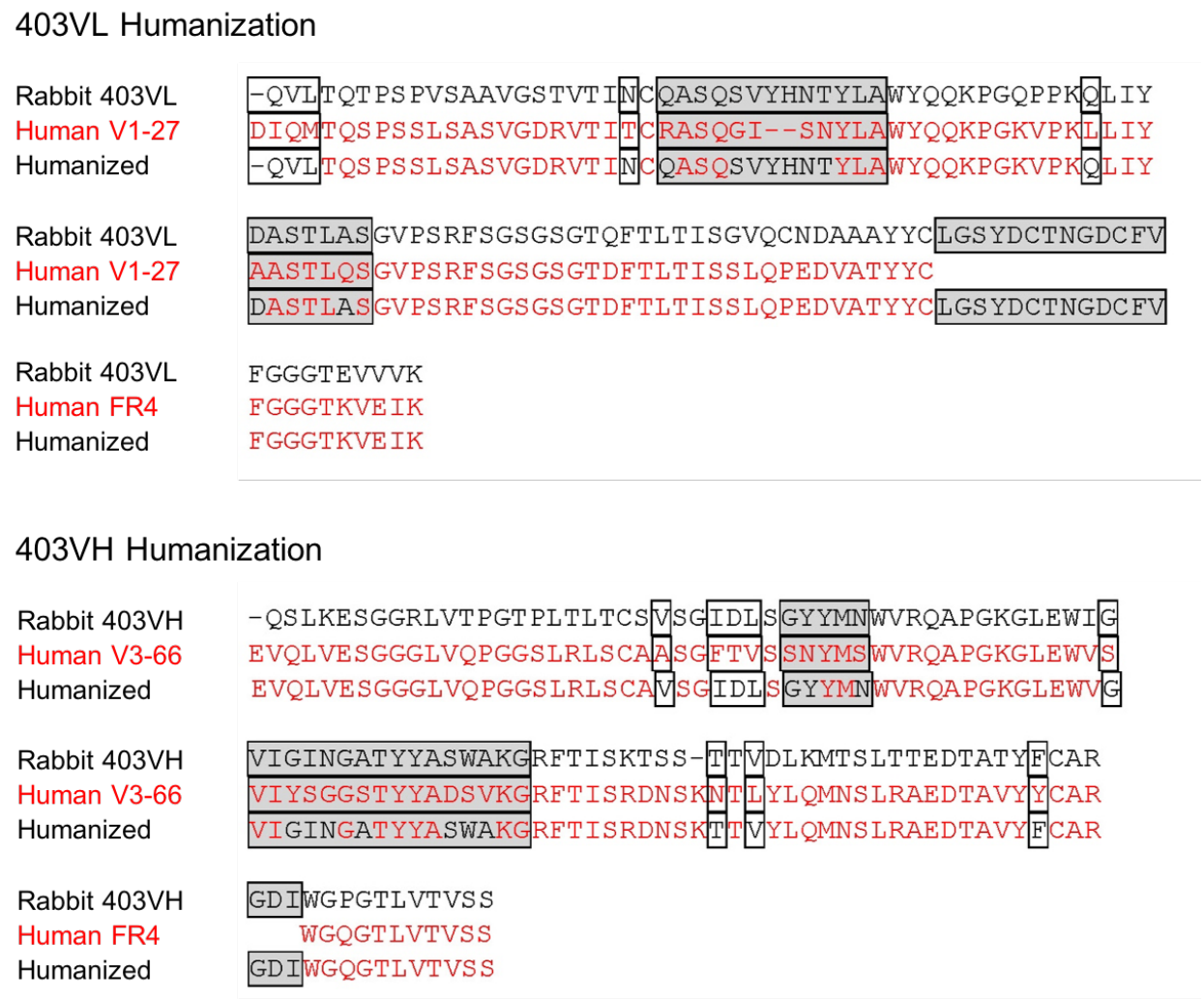

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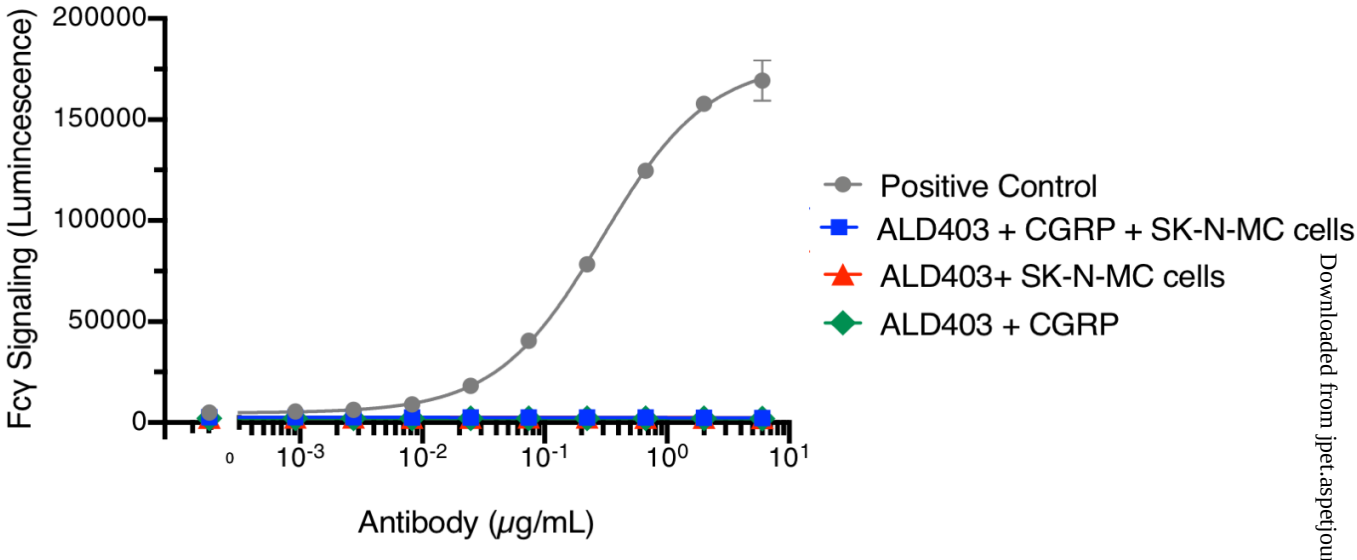
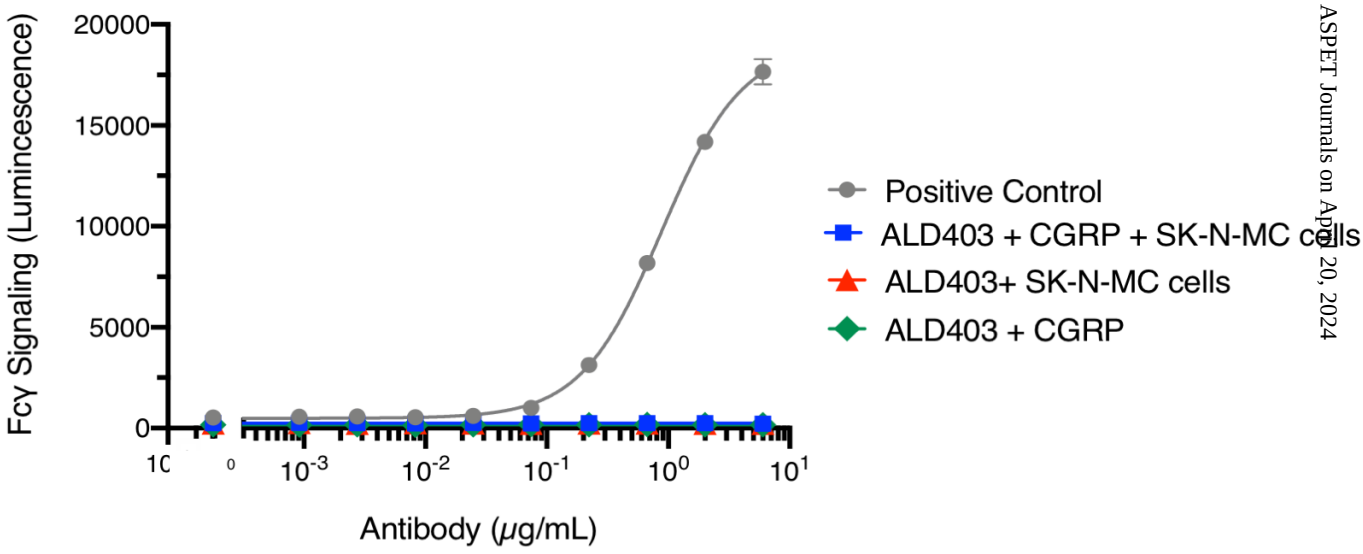
Fig. 2. Lack of activation of A) ADCC, B) ADCP, and C) CDC by ALD403

A.



B.



C.

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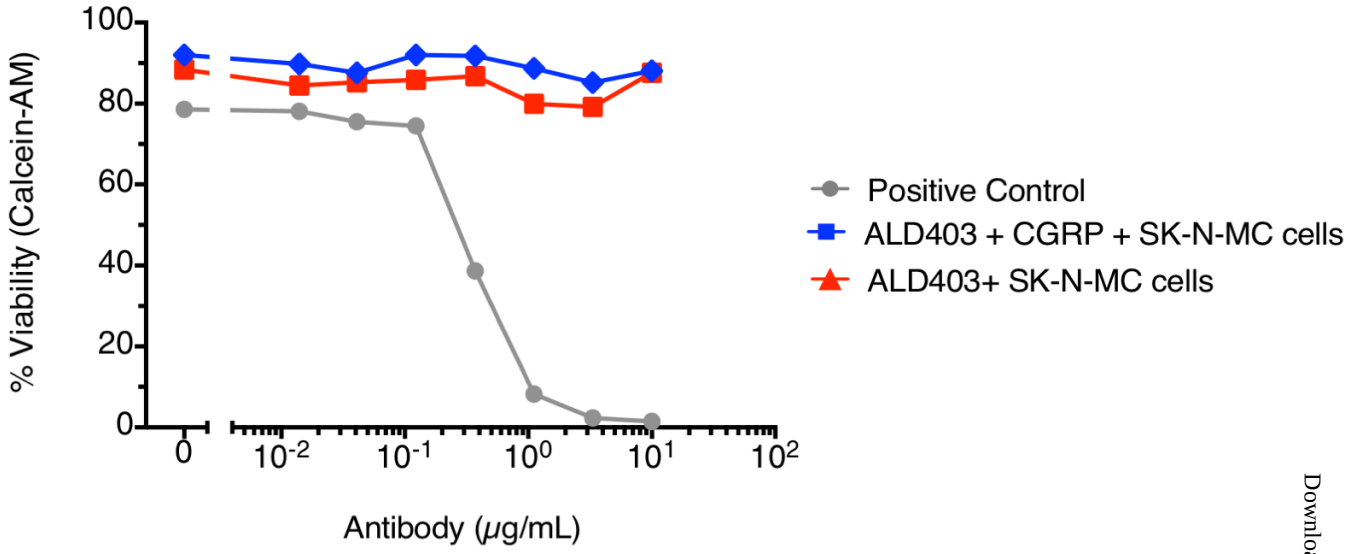

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Fig. 3. Characterization of ALD403 interactions with Fcγ receptors

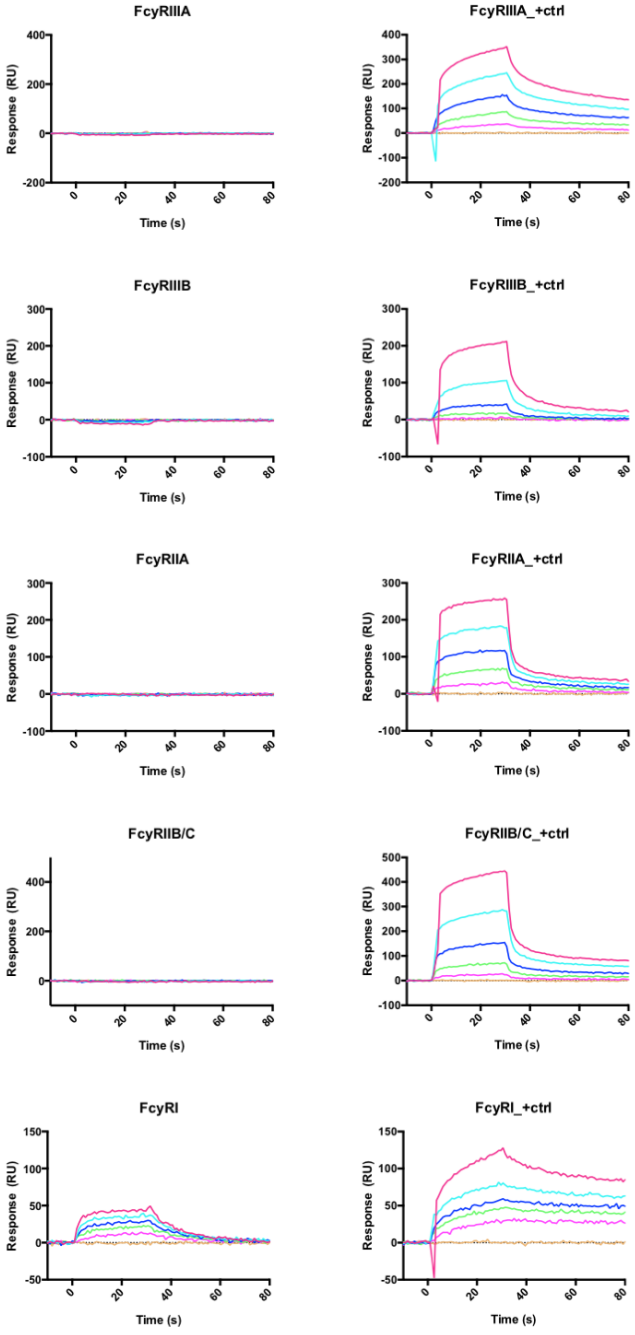

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Fig. 4. Binding specificity of ALD403

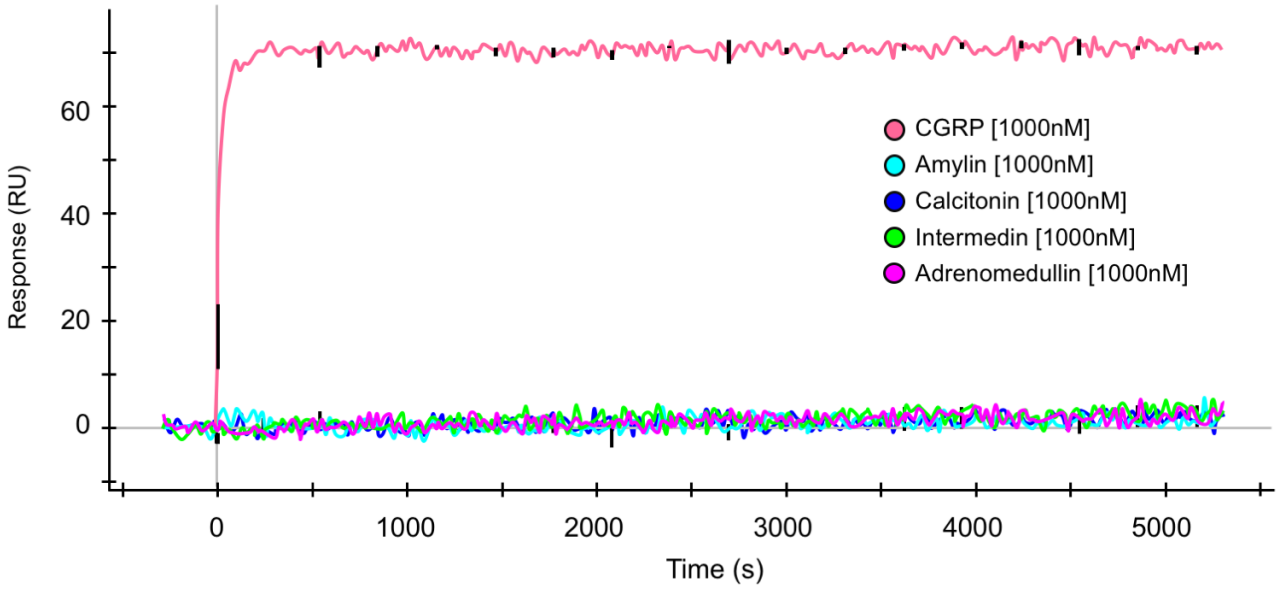

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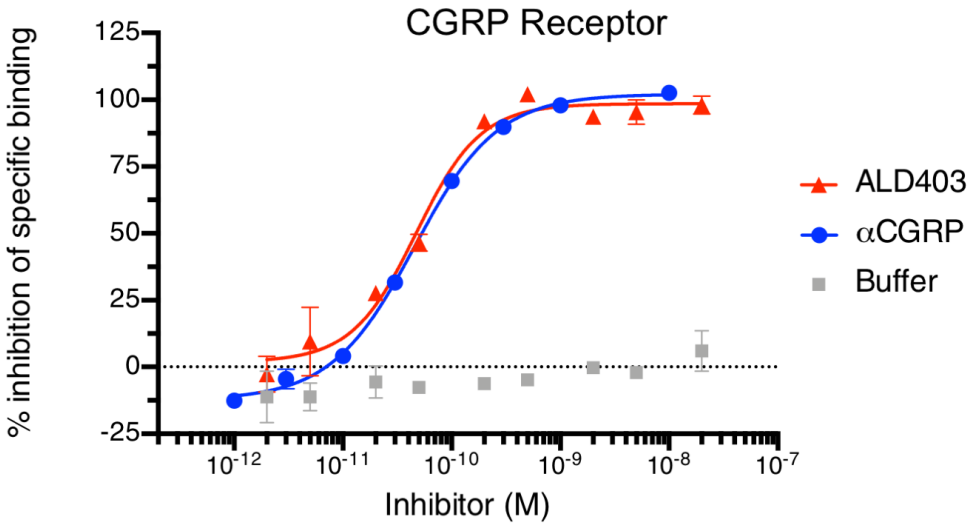
Fig. 5. Inhibition of CGRP binding to A) CGRP and B) AMY1 receptors with ALD403

A.



B.

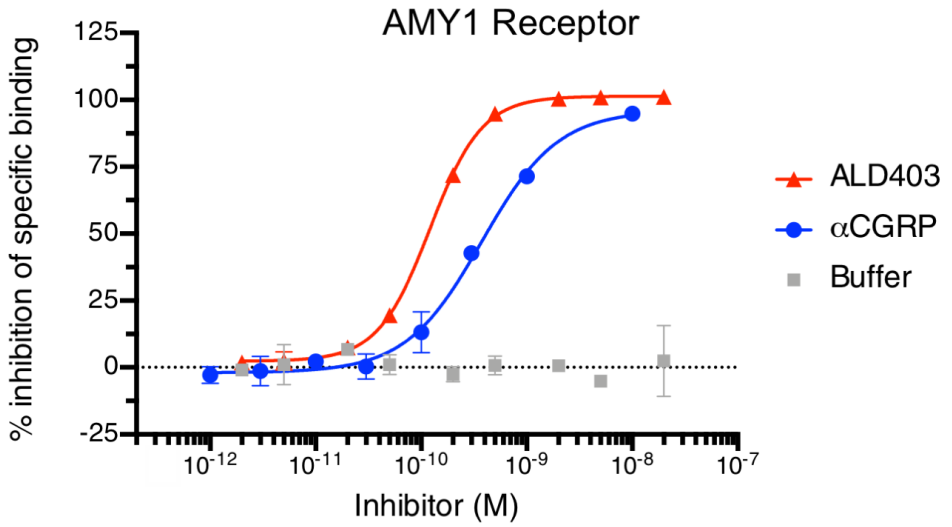


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Fig. 5. Inhibition of CGRP binding to CGRP and AMY1 receptors with ALD403 (cont.)

Assay	**[^{125}I]-α-CGRP Concentration (M)**	**αCGRP IC_{50} (M)**	**ALD403 IC_{50} (M)**
CGRP receptor	3.0E-11	4.5E-11	4.7E-11
AMY 1 receptor	2.0E-10	3.7E-10	1.2E-10

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Fig. 6A. Inhibition of capsaicin-driven increased peripheral blood flow in rodents.

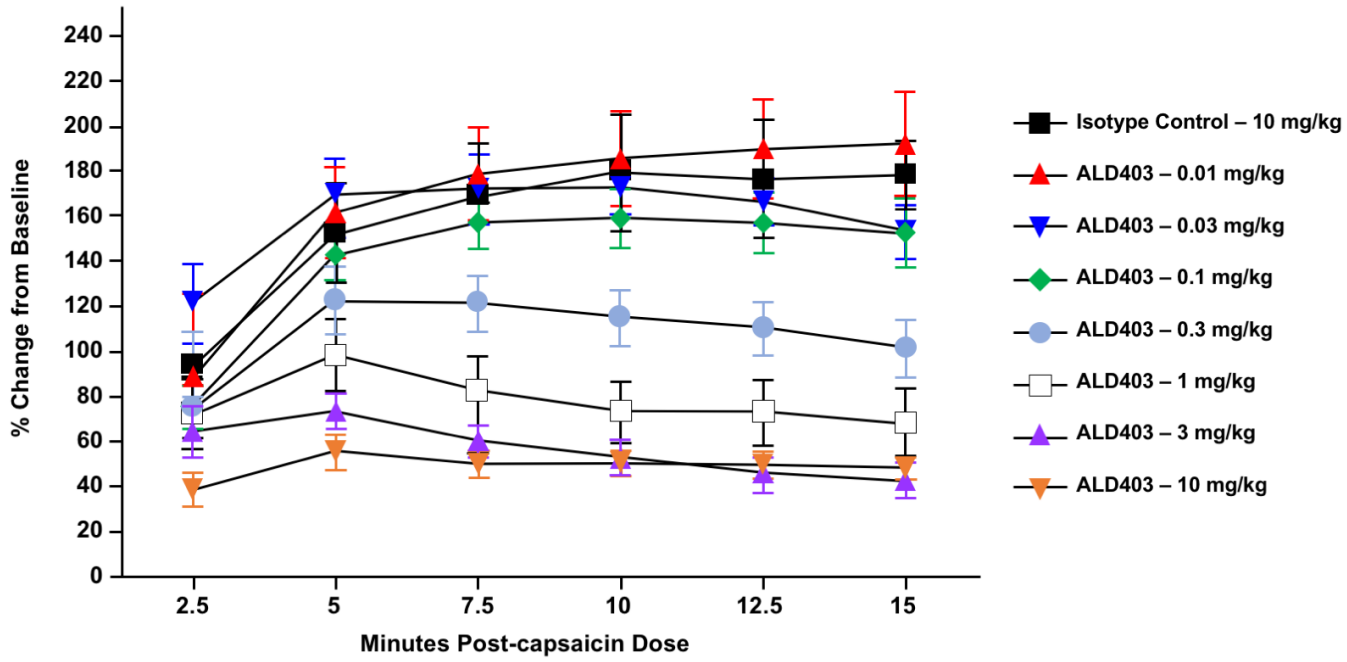

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Fig. 6B. Mean plasma concentration-time profiles of ALD403 in rodents.