BRAIN
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Efficacy of MEDI0618, a pH-dependent monoclonal antibody targeting PAR2, in preclinical models of migraine

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Protease activated receptor 2 (PAR2) is a G-protein coupled receptor expressed in meningeal neurons, fibroblasts and mast cells that may be targeted to treat migraine. MEDI0618, a fully humanized PAR2 monoclonal antibody, engineered to enhance FcRn-dependent recycling and currently in clinical development, was evaluated in human and rodent *in vitro* assays, in multiple murine *in vivo* migraine models and in a model of post-traumatic headache.

MEDI0618 bound specifically and with high affinity to cells expressing human PAR2 (hPAR2) and prevented matriptase-induced increase in cytosolic calcium. Similarly, MEDI0618 prevented matriptase-induced calcium in primary fibroblasts and microvascular endothelial cells from human dura mater. MEDI0618 had no effect on hPAR1 receptors. Single-cell calcium imaging of acutely dissociated mouse trigeminal ganglion neurons confirmed expression and functionality of mouse PAR2. Studies *in vivo* used evoked cutaneous allodynia as a surrogate of headache-like pain and, in some experiments, rearing as a measure of non-evoked headache pain. MEDI0618 was administered subcutaneously to C57BL6/J female mice prior to induction of migraine-like pain with (i) systemic nitroglycerin or compound 48/80 (mast cell degranulator); or (ii) with supradural compound 48/80 or an inflammatory mediator (IM) cocktail. To assess possible efficacy against CGRP receptor (CGRP-R)-independent pain, MEDI0618 was also evaluated in the IM model in animals pretreated with systemic olcegepant (CGRP-R antagonist). Migraine-like pain was also induced by inhalational umbellulone, a TRPA1 agonist, in animals primed with restraint stress in the presence or absence of MEDI0618 as well as in a model of post-traumatic headache pain induced by a mild traumatic brain injury.

MEDI0618 prevented cutaneous allodynia elicited by systemic nitroglycerin, compound 48/80 and from supradural compound 48/80 and IM. Systemic olcegepant completely blocked periorbital cutaneous allodynia induced by supradural CGRP but failed to reduce IM-induced cutaneous allodynia. In contrast, MEDI0618 fully prevented IM-induced cutaneous allodynia, regardless of pretreatment with olcegepant. Umbellulone elicited cutaneous allodynia only in restraint stress-primed animals, which was prevented by MEDI0618. MEDI0618 prevented the decrease in rearing behaviour elicited by compound 48/80. However, MEDI0618 did not prevent mild traumatic brain injury-related post-traumatic headache measures.

These data indicate that MEDI0618 is a potent and selective inhibitor of PAR2 that is effective in human and rodent *in vitro* cell systems. Further, blockade of PAR2 with MEDI0618 was effective in all preclinical migraine models studied but not in a model of post-traumatic headache. MEDI0618 may represent a novel therapy for migraine prevention with activity against CGRP-dependent and independent attacks.

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Introduction

In spite of the recent introduction of new therapies, migraine remains a major unmet medical need.[1-6] Pharmacological strategies targeting calcitonin gene-related peptide (CGRP) or its receptor (CGRP-R) for migraine prevention with monoclonal antibodies (mAbs) are effective in many patients,[7,8] but many others do not receive benefit from these therapies.[9-11] Current preventative treatments with CGRP-targeting antibodies have been shown to reduce but not eliminate the number of migraine days per month, and breakthrough migraine attacks are common, suggesting that mechanisms other than CGRP may be causal in promoting migraine even in patients who respond to these therapies. As many as 40%–50% of patients receiving anti-CGRP mAb eventually discontinue treatment, possibly due to a reduction in efficacy.[12-15] Additionally, around 40% of patients who receive CGRP-targeting therapy for migraine prevention have been classified as non-responders, indicating that in this patient group, CGRP is unlikely to be the primary cause of their headaches.[10] Such compelling clinical observations have prompted investigations into non-CGRP-related pathophysiological mechanisms that may contribute to migraine.

In many patients, migraine appears to be associated with triggering events, including exposure to environmental allergens.[1,3,16] There is a strong association between allergy-related activation of mast cells (MC) and headache[17,18] that often manifests with reports of a high incidence of attacks during seasons with high levels of airborne allergens.[16] As migraine has been described as a cycling sensory threshold disorder, attacks might result from MC activation[19] and the release of various granule-associated mediators (i.e. degranulation), including cytokines, chemokines and proteases[20-24] that could activate or sensitize meningeal nociceptors.

Tryptase is the most abundant protease in MC and can induce widespread inflammation via protease-activated receptors (PAR), especially PAR2, contributing to neuronal sensitization.[24-26] PAR2 is a 7-transmembrane G-protein coupled receptor (GPCR) widely expressed throughout multiple body systems, as well as in meningeal neurons, fibroblasts, endothelial cells and MC. For reviews, see Bao *et al.*[25] and Mrozkova *et al.*[26] Activation of PAR2 can result from protease-mediated cleavage of its N-terminal domain at specific sites containing the amino acid serine, which exposes a tethered ligand sequence that binds and activates the receptor.[25,26] Our previous studies, and others, have implicated PAR2 in promoting cephalic pain in preclinical models of migraine.[27-32] Thus, blocking PAR2 might be a viable mechanism for migraine prevention.

PAR2 has previously proved to be a difficult target to manipulate pharmacologically with small- and large-molecule approaches, largely because of extensive and destructive internalization. Here, we report the discovery and characterization of MEDI0618 as a novel, highly specific and fully humanized PAR2 mAb, which has been engineered to exhibit reduced affinity in acid environments, allowing non-destructive recycling via FcRN, and as a result, with pharmacological characteristics and a pharmacokinetic profile that has allowed progression to clinical development for migraine prevention (NCT05714254, NCT04198558). We have evaluated the effects of MEDI0618 in multiple migraine models, including a CGRP-independent model of migraine-like pain. Additionally, as post-traumatic headache (PTH) is a common secondary headache that follows traumatic brain injury (TBI) and often shares clinical characteristics with migraine, likely involving MC degranulation,[33,34] we evaluated MEDI0618 in a preclinical model of PTH.

Materials and methods

Animals

All experimental procedures were performed in accordance with the ARRIVE guidelines, the ethical guidelines of the International Association for the Study of Pain regulations on animal welfare and the National Institutes of Health guidelines for the care and use of laboratory animals. A total of 180 C57BL6/J (Jackson Laboratories) female 6–8-week-old mice were used in this study. Animals were housed four per cage under standard animal husbandry conditions of temperature, humidity and light cycle-controlled environment, with free access to food and water in the University of Arizona animal facility. The oestrous cycle of animals was not monitored as our previous studies have not demonstrated differences in sensory thresholds at different stages of oestrous in mice. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Arizona. Experimenters were blinded to treatments. Mice were randomly divided into control and experimental groups.

Mouse trigeminal culture

Trigeminal neurons were harvested from female mice as previously described.[35] Briefly, following euthanasia and decapitation, the overlying skin, skull and brain were removed to reveal the trigeminal ganglion (TG) in the base of the skull. TG were dissected and

chopped before undergoing sequential digestion in Papain (Merck), followed by Collagenase type II (Merck) and Dispase type II. Neurons were separated from myelin and nerve debris via centrifugation through a Percoll gradient [12.5% over 28% Percoll (Cytiva)] in Leibovitz's L15 + 5% fetal bovine serum (FBS) + 2% 1 M HEPES + 1% penicillin streptomycin (L15 dissection media). Cells were diluted to high density by adding 30 ml L15 dissection media per trigeminal pair. Forty-millilitre volumes of cell mix were spotted onto a 12 mm coverglass precoated with poly-D-lysine (PDL) and laminin (Biocoat #1 German Glass, Corning) and placed in 24-well plates.

Wells were flooded after 30 min cell attachment and maintained in F12 (Ham's) Nutrient Mix + 10% FBS + 1% penicillin streptomycin. TG cultures were incubated at 37°C and 5% CO_2 for 2–4 days *in vitro* before single-cell calcium imaging experiments. All trigeminal culture reagents were from Thermo Fisher Scientific unless stated otherwise.

Immunofluorescence

MEDI0618 was directly conjugated to Alexa Fluor 647 via Lightning-Link® (Abcam; Cat. No. ab269823) as per product instructions. 1321N1 parental and 1321N1-hPAR2.cl8 cells were plated onto PDL-precoated 96-well phenoplates (PerkinElmer) at a density of 40 000 per well and left overnight at 37°C and 5% CO_2 to adhere. For live antibody staining, cells were placed on ice and washed twice with ice-cold assay buffer (HBSS+/+ supplemented with 10 mM HEPES + 5% FCS). Cells were incubated with 30 nM MEDI0618-Alexa647 in assay buffer on ice for 30 min before being washed twice with assay buffer. Imaging was performed directly using an ImageXpress microscope (Molecular Devices) equipped with a ×20 objective and standard 647 excitation and filter sets.

Flow cytometry

The 1321N1 parental cell line (lacking PAR2 expression), 1321N1 cells stably expressing human PAR2 (1321N1-hPAR2.cl8) (generated in-house at AstraZeneca) or A549 cells endogenously expressing hPAR2 were pre-stained with LIVE/DEAD™ dead cell stain (ThermoFisher); 7.5×10^6 cells from each line were collected in total and stained in 7.5 ml phosphate buffered saline (PBS) (ice-cold) with 1× violet dead cell stain for 30 min on ice. Cells were then spun down and resuspended in 15 ml fluorescence-activated cell sorting (FACS) buffer (PBS, 3% FCS) at 1×10^6/ml. Cells were then incubated with MEDI0618 on ice for 60 min, followed by washing by centrifugation in ice-cold FACS buffer. Cellular binding of MEDI0618 was detected via direct labelling of antibody with an Alexa Fluor 647 Antibody Labeling Kit (ThermoFisher). Fluorescence was measured on labelled viable cells using a BD LSRFortessa (BD Biosciences) cell analyzer/flow cytometer.

Well-based calcium imaging

PAR2-driven cellular calcium responses were measured using a FLIPR Tetra system (Molecular Devices) in PDL-coated 384-well plates. Cells were plated at 5×10^3/well and loaded with Fluo-8 (Screen Quest™ Fluo-8 No Wash Calcium Assay Kit) for 30 min in a humidified incubator at 37°C, followed by 15 min equilibration at room temperature. The assay buffer was Hanks' Balanced Salt Solution (HBSS) with calcium and magnesium and without phenol red (Gibco) supplemented with 0.1% bovine serum albumin (BSA) and 20 mM HEPES. For determination of antibody (IgG) potency, cells were pretreated with IgGs diluted in assay buffer for up to 1 h at room temperature prior to the addition of the PAR2 agonist matriptase. For CYNOMK1 and LL/2 cells, which express PAR1 receptors, PAR2 responses to trypsin were isolated following a 10 min pretreatment of thrombin (Sigma) at concentrations of 10 nM (CYNOMK1) or 0.5 nM (LL/2) to abolish PAR1 responses.

Single cell trigeminal calcium imaging

Experiments recording cytosolic calcium were undertaken to measure PAR2 activation following treatment with 2-furoyl-LIGRLO-amide (LIGRLO, Peptides International). In a mixed culture, neurons were identified from non-neurons via responsivity to 20 mM KCl. TG cultures were loaded with 5 μM fura-2 ratiometric calcium dye in HBSS+/+ for 30 min at 37°C and 5% CO_2 before transfer to a perfusion chamber (Warner Instruments) containing HBSS. Widefield calcium imaging was performed with an IX81 inverted microscope (Olympus) equipped with a scientific CMOS Orca R2 camera (Hamamatsu). Time series were recorded with a frame rate of 400 ms and using sequential 340 nm (±25 nm) and 387 nm (±11 nm) excitation and 510 nm (±40 nm) bandpass emission capture. Cultures were perfused with HBSS with and without treatments at a flow rate of 1.1 ml/min via a gravity flow perfusion system equipped with a VC-6 valve controller (Warner Instruments) to switch treatment lines. Following baseline collection in HBSS (80 s), cultures were sequentially treated with 10 μM LIGRLO (40 s), HBSS washout (100 s), 20 mM KCl (30 s) and finally HBSS washout (80 s). Regions of interest were drawn around cells of interest, and fluorescence intensity was extracted at each time point to produce traces.

Antibody engineering

Isolation of anti-PAR2 antibodies

Anti-PAR2 antibodies were isolated from either a human single-chain variable fragment (scFv) or fragment antigen-binding region (Fab) phage display library[36] by selection on recombinant human PAR2 N-terminal extracellular peptide (amino acids 1–75). This was followed by screening for specific binding to the PAR2 over PAR1 peptide and human PAR2 expressed on transfected cells. The affinity and potency of the leads (PAR0065 and PAR001) were optimized through randomization of the complementarity determining regions (VHCDR2, VHCDR3 and VLCDR3) to create a phage display library of scFv or Fab mutants. Affinity-based phage display selections[37] were performed on solution phase biotinylated human PAR2 peptide. This approach led to the generation of the lead optimized antibodies P67 and PAR650097.[29]

Compounds and drugs

Compound 48/80 (Sigma) was diluted in saline and administered onto the dura mater at 6.5 nmol/5 μl or intraperitoneally at 2 mg/kg. Umbellulone (UMB; AdipoGen) was prepared as a stock solution of 0.1 M in 100% dimethyl sulfoxide (DMSO) and freshly diluted to 0.01 M with 1× PBS for delivery by inhalation. Nitroglycerin (NTG; American Regent) was freshly diluted in saline at 10 mg/kg from a stock solution of 5.0 mg/ml nitroglycerin in 30% alcohol, 30% propylene glycol and water. CGRP (Bachem) was reconstituted in distilled water and administered onto the dura mater at 1 pg/5 μl. Olcegepant (Tocris) was dissolved using 20% DMSO in saline immediately prior to use and administered intraperitoneally at 1 mg/kg. Synthetic interstitial fluid consisted of 10 mM HEPES, 5 mM KCl, 135 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM glucose (all Sigma). Inflammatory mediators (IM) consisted of 1 mM

bradykinin, 1 mM histamine, 1 mM serotonin (Sigma) and 100 mM PGE2 (Cayman Chemicals) diluted in synthetic interstitial fluid at a pH 4.0. MEDI0618 and the hIgG1 isotype control protein (NIP228) (provided by AstraZeneca) were freshly diluted in 1× PBS and administered subcutaneously (s.c.) at 0.25, 1, 5, 20, 50 or 100 mg/kg. Controls received the respective vehicles at 5 µl or 10 ml/kg. PAR2 agonists Ser-Leu-Ile-Gly-Arg-Leu-NH2 (SLIGRL-NH2), 2-Furoyl-Leu-Ile-Gly-Arg-Leu-Orn-NH2 (LIGRLO) and the reverse peptide control 2-Furoyl-Orn-Leu-Arg-Gly-Ile-Leu-NH2 (OLRGIL) (Tocris) were diluted in PBS + 0.1% BSA to generate a 10 mM stock solution prior to final dilution in assay buffer. Thrombin from human plasma (Sigma) was used to activate PAR1 where detailed and diluted in PBS + 0.1% BSA to generate a 10 µM stock solution prior to final dilution in assay buffer. Recombinant human matriptase (R&D Systems) was diluted in 50 mM Tris-HCl plus 10% glycerol at a concentration of 23 µM prior to final dilution in assay buffer.

Supradural injection

Injectors were modified from commercially available cannulas (Plastic One/Invivo1, part No. C313I/SPC) by inserting a stopper to project a length of 0.65–0.7 mm to maintain dura mater integrity. The injectors were connected to Tygon tubing (Cole Parmer Co.), which was then attached to a 25 µl Hamilton syringe (Hamilton). The injector was inserted through the sagittal and lambdoid suture junction for supradural delivery under brief isoflurane anaesthesia.[38]

Mild traumatic brain injury induction

Mice were lightly anaesthetized and laid on their ventral surface in a prone position on an elevated sheet of tissue paper situated over a plexiglass apparatus with a soft sponge at the bottom. A metal guide tube was directed to the top of the mouse skull between the ears to ensure standardized placement of the weight drop. The 100 g weight was released from a height of 94 cm onto the closed and unfixed skull, resulting in a concussive impact to the head, pushing the mouse through the tissue paper and flipping it down to land on the soft sponge as previously described.[39-41] All mild traumatic brain injury (mTBI) mice experienced both rotational and linear head forces, mimicking to some degree common concussion injuries that involve free head rotation in humans.[42,43] Following the procedure, the righting reflex was recorded, and mice were placed back in their home cages and allowed to recover. All mice awoke within 5 min of the procedure.

Cutaneous allodynia evaluation

Mice were placed in elevated individual Plexiglass chambers with mesh flooring and allowed to acclimate for 3 days for 2 h each day. Periorbital (cephalic) and hindpaw (extracephalic) tactile frequency of response was measured in the same mice following a 2-h acclimation period. The 0.4 g (3.61 N), 0.6 g (3.84 N) and 1 g (4.08 N) von Frey filaments (Stoelting) were applied 10 times, with just enough pressure to cause the filament to display a slight arch to the periorbital (0.4 g) and hindpaw (0.6 g for mTBI and 1 g for migraine experiments) region. Swiping the face, shaking the head and/or turning away from the stimuli were considered positive periorbital responses. Sharp withdrawal, shaking and/or licking the paw were considered positive hindpaw responses. Increased frequency of response represents the development of cutaneous allodynia. Frequency of response was calculated as (number of positive responses/10 × 100%).[29,40,44]

Restraint stress

Restraint stress (RS) priming was performed according to previous studies from our laboratory.[29,44] Mice were placed in plastic restrainers (Plas-labs INC, 551-BSRR) for 2 h each day consecutively for 3 days. Periorbital and hindpaw response frequency was measured at baseline followed by three daily episodes of RS and cutaneous allodynia evaluation on Days 3, 5, 7, 9, 11, 14 and 16 after the first RS.

Umbellulone inhalation exposure

Inhalational exposure to UMB was performed as previously described.[29,44] A half-square gauze was placed in each nose cone of the multi-station isoflurane anesthesia board (Parkland Scientific) and 500 µl of UMB at 0.01 M were pipetted onto each gauze. Mice were individually placed in each nose cone and exposed to UMB under light isoflurane anaesthesia (1.5%–2%) for 30 min.

Environmental bright light stress challenge

Mice were kept in their home cages and received one episode of bright light stress on Day 15 after mTBI, as previously described.[41] Bright light stress was induced by placing two LED lights on both sides of the home cages to deliver approximately 1000 lx for 15 min.

Spontaneous migraine-like headache behaviour assay

Counts of rearing/vertical episodes behaviour were used as an outcome measurement for spontaneous migraine-like headache.[45] Reduction in the number of rearing episodes has been interpreted as suggestive of a spontaneous behavioural state indicative of pain associated with 'headache', in which the headache-like condition supersedes the competing drive to explore the vertical dimensions of the unfamiliar environment. Decreased rearing has also been suggested to potentially reflect changes in intracranial pressure that could be consistent with headache.[45-51] Additional details can be found in the Supplementary material, 'Methods' section.

General experimental design overview

See the detailed general experimental design overview in the Supplementary material, 'Methods' section.

Statistical analysis

Sample size was determined using the GPower 3.1 software, with an established significance level of $P < 0.05$. The Shapiro-Wilk normality test was applied to ensure that the data met the criteria for performing parametric tests. All data followed a normal distribution. Therefore, data are expressed as means ± standard errors of the mean. Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software). Mean differences between two groups in the comparisons of time course experiments for sensory thresholds and spontaneous-like pain evaluation were analysed using two-way ANOVA with Sidak's multiple comparisons *post hoc* test. An unpaired Student's *t*-test was used to analyse the area under the tactile frequency of response curves (AUC). The percentage of maximal possible effect of MEDI0618 was analysed by linear regression. Statistically significant differences were considered when $P < 0.05$. The statistical analysis, numbers of animals used (n), P-values and interaction F-ratios are reported in Supplementary Table 3.

Results

Discovery of MEDI0618, a pH-dependent, inhibitory anti-PAR2 antibody with improved pharmacokinetics

MEDI0618 is an affinity-matured, phage display-derived, anti-PAR2 antibody engineered for optimal *in vivo* pharmacokinetics. The parent antibody P67 showed rapid *in vivo* clearance in rat (164 ml/day/kg; Supplementary Fig. 1 and Supplementary Table 1), which we hypothesized was due to broad receptor tissue expression acting as an antigen sink, then internalization and subsequent lysosomal degradation of the antibody.[52-55] We therefore sought to optimize P67 to promote its release in the acidic endosome and recycling back into the circulation via FcRn. Reduced antibody affinity at acidic pH was engineered by inclusion of histidine residues in the VLCDR3, VHCDR2 and VHCDR3 complementarity-determining regions, resulting in up to 50-fold differential binding at pH 7.4 versus pH 6.0 (Supplementary Fig. 2, Supplementary Table 2 and Supplementary material, 'Methods' section). Pharmacokinetic measurement of PAR2 mAb clearance in rat showed a direct correlation between pH-dependent binding and clearance. MEDI0618 (H129) displayed a 7-fold reduction in clearance, leading to a prolonged elimination half-life and sustained systemic concentration, over a non-pH dependent parent antibody (P67) (Supplementary Fig. 2).

Immunofluorescence live cell staining of 1321N1-hPAR2 cells with MEDI0618 directly conjugated with the fluorophore AF647 revealed clear membrane staining, which was absent in 1321N1 parental cells (Fig. 1A). Flow cytometry of 1321N1 cells overexpressing hPAR2 or A549 cells endogenously expressing hPAR2 also showed that MEDI0618 bound specifically to hPAR2 but not to the parental 1321N1 cells (Fig. 1B). Treatment of 1321N1-hPAR2 cells with matriptase led to a rapid and transient rise in cytosolic calcium, which was fully and potently inhibited by pretreatment (60 min) with MEDI0618, half-maximal inhibitory concentration (IC_{50}) = 100 pM (Fig. 1C and D). MEDI0618 was ~15-fold more potent at hPAR2 than a previously identified antibody, PAR650097 hIgG1.[31] Similar to PAR650097, MEDI0618 had no inhibitory activity at the related protease activated receptor hPAR1, with no significant effect on thrombin-mediated calcium transients in A549 cells observed compared to isotype control (Fig. 1E). In contrast, the PAR1 small molecule antagonist Vorapaxar or a cocktail of anti-PAR1 mAbs potently and completely inhibited the thrombin response (IC_{50} = 26 and 0.3 nM, respectively). No agonistic activity of MEDI0618 was observed (Supplementary Fig. 3) and MEDI0618 showed inhibitory activity at mouse, rat and cynomolgus monkey orthologues of PAR2 (Supplementary Fig. 4).

PAR2 is functionally expressed in trigeminovascular cells of human and mouse

Human dura mater obtained from healthy control donors were used to isolate primary human fibroblasts and microvascular endothelial cells. Calcium imaging from these cells (Fig. 2A–F) showed that the PAR2 recombinant peptide agonist LIGRLO, and, where included, SLIGR but not the reverse peptide OLRGIL, activated a large calcium transient, indicating functional expression of PAR2 in both

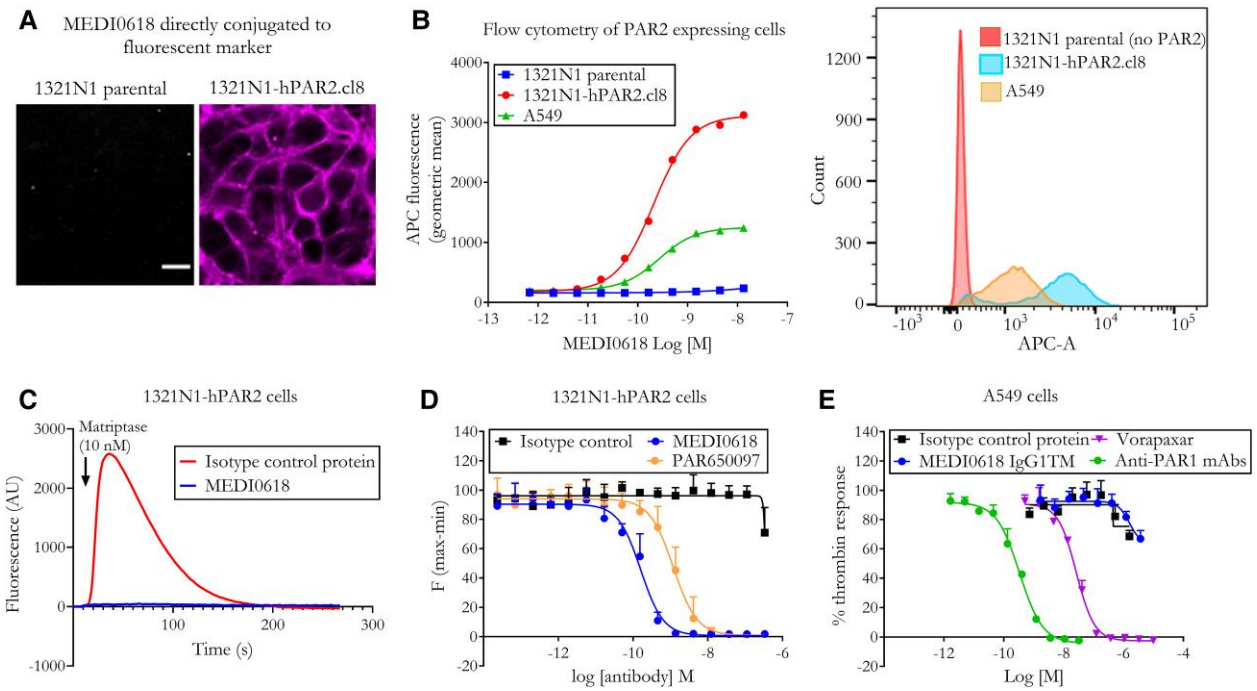

Figure 1 Specificity and potency of anti-PAR2 monoclonal antibody MEDI0618. (**A**) Live staining of PAR2-expressing (1321N1-hPAR2.cl8) or non-expressing (1321N1 parental) cell lines with MEDI0618 directly conjugated to Alexa Fluor 647. Scale bar = 20 µm. (**B**) Flow cytometry of hPAR2 overexpressing cells (1321N1-hPAR2.cl8) or non-expressing cells (1321N1 parental cell line) or A549 cells endogenously expressing hPAR2 live labelled with MEDI0618. (**C** and **D**) Calcium imaging from 1321N1-hPAR2 cells pretreated with MEDI0618 hIgG or an isotype control protein. (**C**) Exemplar raw calcium trace from a single well pretreated with MEDI0618 or isotype control antibody both at 1 nM, followed by PAR2 agonist stimulation with matriptase (10 nM). (**D**) Antibody titration of the anti-PAR2 antibodies MEDI0618 or PAR650097 or an isotype control antibody. Data show the matriptase (10 nM)-mediated calcium signal after preincubation with antibody and normalized to the matriptase response in the absence of antibody treatment. (**E**) Calcium imaging from A549 cells pretreated with MEDI0618, isotype control protein or PAR1 inhibitors (ATAP2 + WEDE15 mAbs) followed by 10 nM thrombin (PAR1 agonist) stimulation. Data are presented as mean ± standard error of the mean, $n = 4$. The concentration of the inhibitor is shown on the x-axis. Data are normalized to the peak thrombin calcium response in the absence of inhibitor pretreatment.

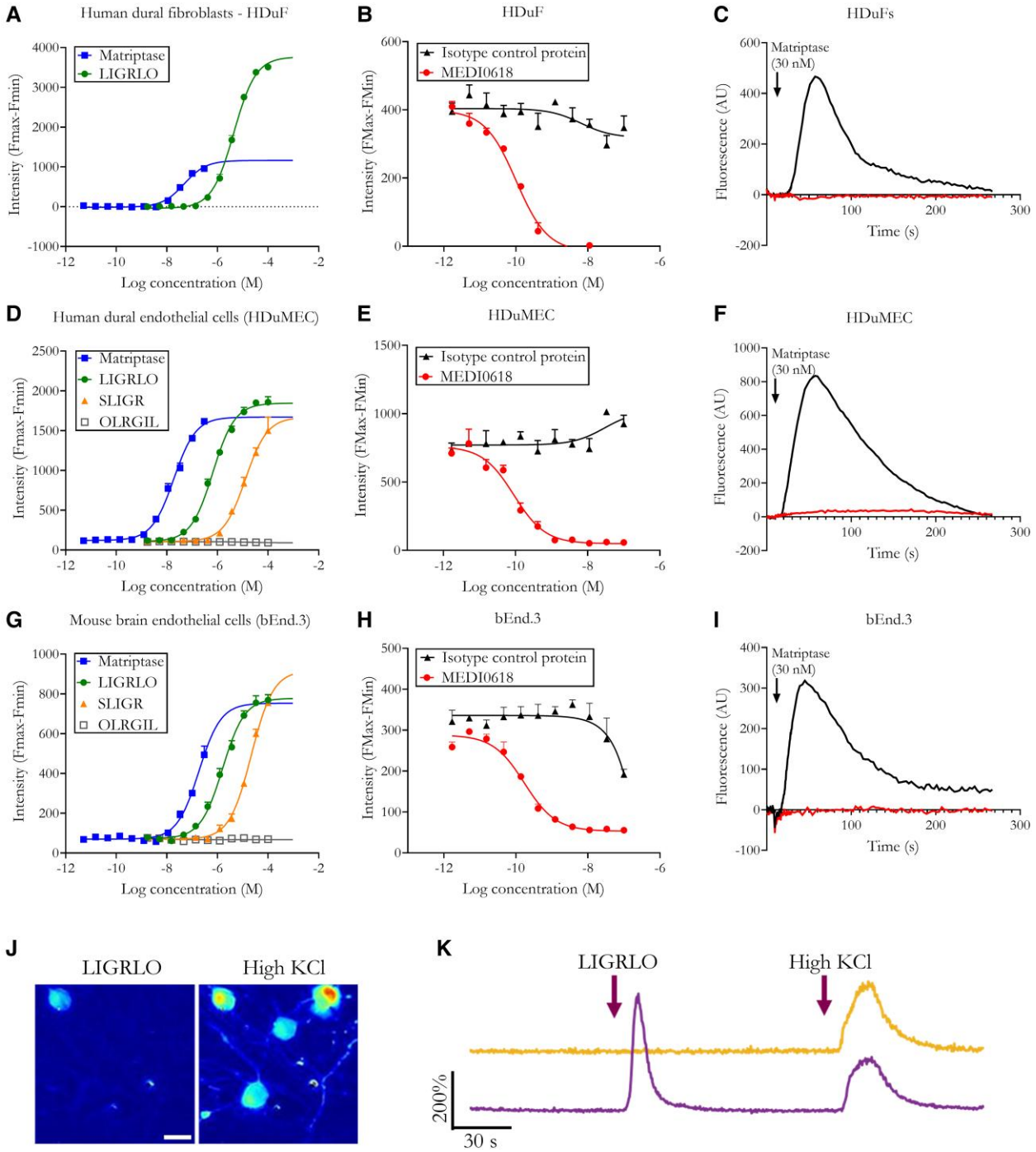

Figure 2 PAR2 functional expression in human and mouse cells relevant to migraine. Whole well calcium imaging from primary human dural fibroblasts (HDuF), human dural microvascular endothelial (HDuMEC) and mouse brain endothelial (bEnd.3) cells. (**A**, **D** and **G**) PAR2 agonists concentration response curve in (**A**) HDuF, (**D**) HDuMEC and (**G**) bEnd.3 cells. (**B**, **E** and **F**) Effect of MEDI0618 and isotype control protein (IgG) on inhibition of matriptase-induced calcium signalling at 30 nM in (**B**) HDuF, (**E**) HDuMEC and (**H**) bEnd.3 cells. (**C**, **F** and **I**) Representative calcium imaging traces of 30 nM matriptase-evoked activity following MEDI0618 or isotype control protein preincubation in (**C**) HDuF, (**F**) HDuMEC and (**I**) bEnd.3 cells. (**J** and **K**) Single-cell calcium imaging from mouse trigeminal neuron cultures. (**J**) Pseudocolour images of fura-2 ratio intensity show a subset of trigeminal neurons activated by treatment with 10 μM LIGRLO in comparison to 20 mM KCl treatment. Scale bar = 20 μm. (**K**) Representation of fura-2 traces recorded from two individual neurons during acute LIGRLO (10 μM) or KCl (20 mM) treatment.

fibroblasts and endothelial cells isolated from human dura mater. Similarly, activation of PAR2 via the endogenous serine protease matriptase generated a calcium transient in both cell types (Fig. 2A and D) which was fully and potently inhibited by MEDI0618 (Fig. 2B, C, E and F; IC_{50} = 108 and 435 pM in human dural fibroblasts and endothelial cells, respectively). Observations in human dural endothelial cells were mirrored in the mouse brain endothelial cell line bEnd.3 (Fig. 2G–I); (MEDI0618 IC_{50} = 168 pM). Finally, we confirmed that PAR2 was functionally expressed in acutely dissociated mouse trigeminal ganglia neurons using single cell calcium imaging (Fig. 2J and K). Neurons were visually identified using phase contrast microscopy and confirmed functionally

by exposure to 20 mM KCl, which activates voltage gated calcium channels in neurons but is inert on glial cells. LIGRLO (10 μM) activated a calcium transient in ~20% of neurons.

PAR2 blockade by MEDI0618 prevents migraine pain-like behaviour induced by mast cell degranulation

Supradural (Fig. 3A and Supplementary Fig. 5A) or systemic (Fig. 3C and Supplementary Fig. 5C) administration of the MC degranulating compound 48/80 induced the development of cutaneous allodynia (i.e. migraine-like pain behaviour), revealed by the increased frequency of response to tactile stimuli at the periorbital (Fig. 3) and hindpaw (Supplementary Fig. 5) regions. Pretreatment with MEDI0618 (50 mg/kg, s.c., 24 h) fully prevented periorbital (Fig. 3) and hindpaw (Supplementary Fig. 5) cutaneous allodynia. The isotype control protein did not block compound 48/80-induced cutaneous allodynia (Fig. 3 and Supplementary Fig. 5). Analysis of the AUC confirmed the preventive analgesic effect of MEDI0618 after compound 48/80 (Fig. 3B and D and Supplementary Fig. 5B and D). Systemic administration of compound 48/80 decreased spontaneous rearing behaviour (vertical episodes) up to 3 h after injection in female mice when compared to the control (Fig. 3E), suggesting the development of spontaneous migraine-like headache behaviour.[45] MEDI0618 fully prevented the decreased rearing provoked by compound 48/80 in comparison to the isotype control protein-treated group (Fig. 3E).

MEDI0618 prevents migraine pain-like behaviour in multiple preclinical models

Umbellulone model

Repeated RS was used as a first-hit priming protocol to sensitize mice to inhalational administration of a TRPA1 agonist as a second-hit stimulus, as previously reported.[44] Following resolution of transient RS-induced periorbital (Supplementary Fig. 6A) and hindpaw cutaneous allodynia (Supplementary Fig. 6D), periorbital (Fig. 4A) and hindpaw (Supplementary Fig. 7A) allodynia was reinstated by inhalational UMB on Day 16. MEDI0618 dose-dependently reduced periorbital (Supplementary Fig. 6B) and hindpaw (Supplementary Fig. 6D and E) cutaneous allodynia. Analysis of the AUC confirmed the analgesic effect of different doses of MEDI0618 after UMB (Fig. 4B and Supplementary Fig. 6C and F). MEDI0618 at 50 mg/kg produced the maximal effect (%) on reducing cutaneous allodynia (Supplementary Fig. 6).

Nitroglycerin and supradural inflammatory mediator models

Systemic NTG and supradural administration of IM induced periorbital (Fig. 4C and E) and hindpaw cutaneous allodynia (Supplementary Fig. 7C and E). Systemic pretreatment with MEDI0618 fully prevented the migraine-like pain behaviour induced by NTG (Fig. 4C and Supplementary Fig. 7C) and IM (Fig. 4E and Supplementary Fig. 7E) when compared with the control group. Analysis of the AUC confirmed the preventive analgesic effect of MEDI0618 after NTG (Fig. 4D) and IM (Fig. 4F).

MEDI0618 prevents CGRP receptor-dependent and independent migraine-like pain

Supradural administration of CGRP induced periorbital (Fig. 5A) and hindpaw cutaneous allodynia (Supplementary Fig. 8A) lasting 1 h after the injection. Pretreatment with the small molecule CGRP-R antagonist, olcegepant, 30 min prior to supradural CGRP fully blocked CGRP-induced cutaneous allodynia (Fig. 5A and Supplementary Fig. 8A). AUC confirmed the preventive analgesic effect of olcegepant in comparison with control-treated mice after CGRP (Fig. 5B and Supplementary Fig. 8B).

In contrast, pretreatment with olcegepant failed to alter periorbital (Fig. 5C) and hindpaw cutaneous allodynia (Supplementary Fig. 8C) after supradural administration of IM. AUC confirmed the lack of analgesic effect of olcegepant after IM (Fig. 5D and Supplementary Fig. 8D). These data suggest that residual migraine-like pain behaviours are independent of CGRP in mice pretreated with olcegepant and receiving supradural IM.

Administration of MEDI0618, 24 h prior to supradural IM, fully prevented CGRP-independent periorbital (Fig. 5E) and hindpaw cutaneous allodynia (Supplementary Fig. 8E) induced by IM in olcegepant treated mice. The AUC confirmed the preventive analgesic effect of MEDI0618 after supradural IM in comparison to isotype control and olcegepant-treated mice (Fig. 5F and Supplementary Fig. 8F), suggesting efficacy of MEDI0618 in CGRP-receptor independent migraine mechanisms.

PAR2 blockade failed to prevent acute and/or persistent post-traumatic headache induced by mTBI

Consistent with previous data from our group in male mice,[41] mTBI produced a transient periorbital (Fig. 6A) and hindpaw (Supplementary Fig. 9A) cutaneous allodynia in females, demonstrated by increased frequency of response to tactile stimulation as a surrogate readout of acute PTH. Tactile thresholds returned to baseline levels around Day 14 after mTBI (Fig. 6A and Supplementary Fig. 9A). On Days 15 and 17 after mTBI, stress from exposure to bright lights or inhalational exposure to a sub-threshold dose of UMB reinstated the periorbital (Fig. 6B and C) and hindpaw cutaneous allodynia (Supplementary Fig. 9B and C), suggesting the expression of persistent PTH. MEDI0618 given repeatedly at 2 h and again on Days 7, 14 and 16 after mTBI failed to prevent the transient periorbital (Fig. 6A) and hindpaw cutaneous allodynia (Supplementary Fig. 9A) or the reinstatement of the PTH-like behaviour after challenges with bright light stress (Fig. 6B and Supplementary Fig. 9B) or UMB (Fig. 6C and Supplementary Fig. 9C). Administration of MEDI0618 or isotype control protein 21 days after mTBI prevented periorbital and hindpaw cutaneous allodynia induced by systemic injection of NTG 24 h later serving as a positive control (Fig. 6D and Supplementary Fig. 9D).

Discussion

MEDI0618 was designed as a next generation PAR2 mAb with enhanced pharmacodynamics and optimal pharmacokinetic properties suitable for clinical development. Unlike the previous lineage of anti-PAR2 mAb, which were subjected to target-mediated clearance and lysosomal degradation,[29] MEDI0618 was engineered for recycling upon target-mediated cell uptake.[56] Our preclinical study demonstrates the potential of PAR2 blockade by MEDI0618 in preventing pain and sensitization relevant to migraine. The present studies demonstrate (i) the *in vitro* characterization of MEDI0618 in human and rodent tissues as a highly selective and fully humanized IgG1TM PAR2 mAb; (ii) the efficacy of MEDI0618 in prevention of evoked and spontaneous migraine-like pain behaviours induced by systemic provocation with a MC degranulator or NTG, as well as

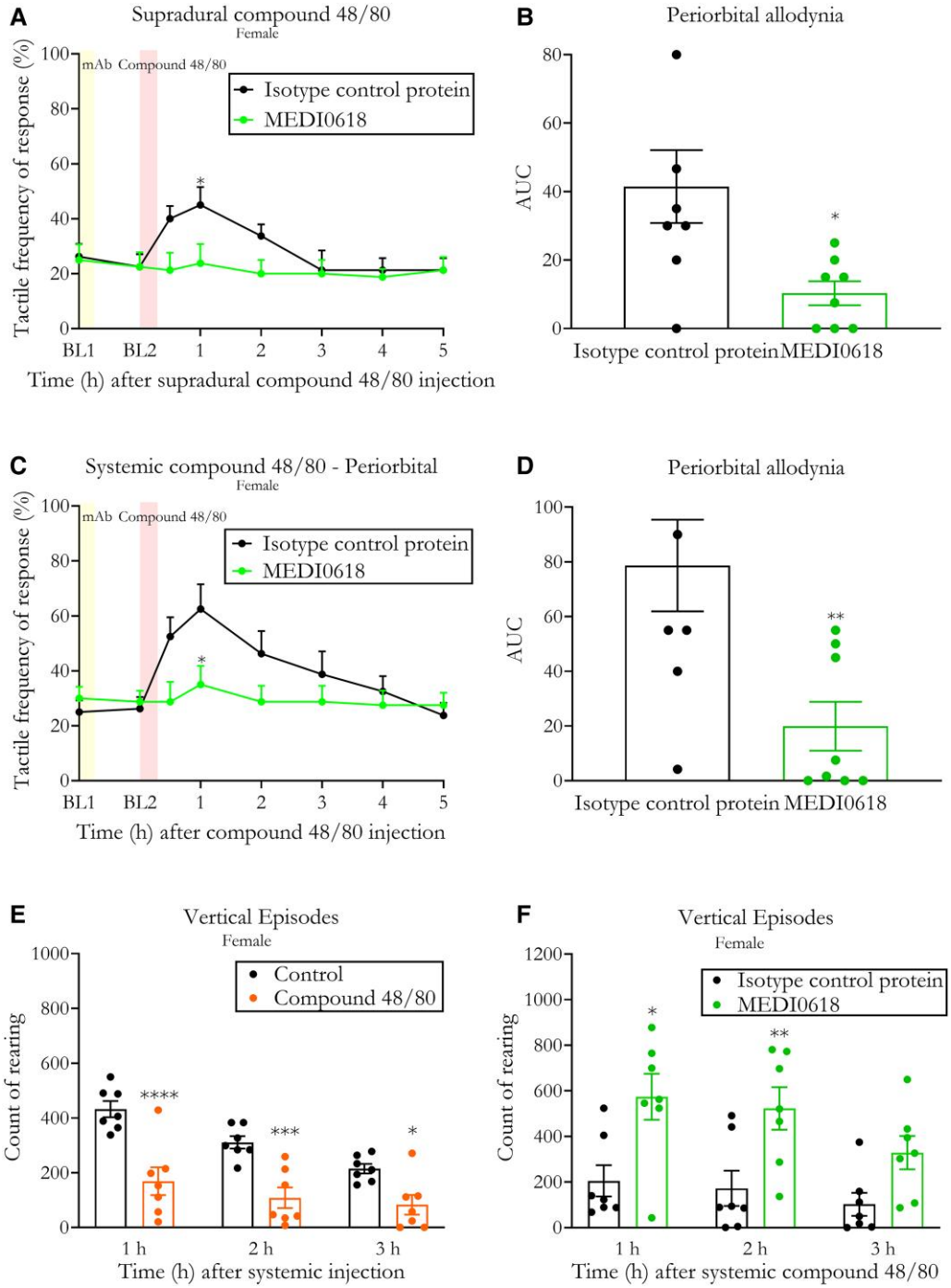

Figure 3 MEDI0618 prevented evoked and spontaneous migraine-like pain behaviour induced by supradural and systemic mast cell degranulation. Data represent tactile responses from different cohorts of female mice receiving isotype control protein or MEDI0618 prior to supradural (**A**) or intraperitoneal (**C**) administration of compound 48/80 and (**E** and **F**) count of rearing/vertical episodes as an outcome measurement of spontaneous migraine-like headache behaviour after systemic administration of compound 48/80. (**A** and **C**) Tactile frequency of response was collected before treatment at baseline (BL1), followed by subcutaneous administration of isotype control protein or MEDI0618 at 50 mg/kg, 24 h prior to testing. Tactile frequency of response for baseline 2 (BL2) was collected right before (**A**) supradural administration of compound 48/80 (6.5 nmol/5 µl) or (**C**) intraperitoneal administration of compound 48/80 (2 mg/kg). Behaviour was evaluated 30 min and 1–5 h after compound 48/80, with cutaneous allodynia revealed by increased frequency of response to tactile stimuli. Area under the curve (AUC) obtained from (**B**) supradural and (**D**) systemic compound 48/80-time courses. Female mice receiving intraperitoneal administration of compound 48/80 at 2 mg/kg or vehicle control were immediately individually placed in a Plexiglas activity box arena for 3 h after the treatment for the quantification of (**E**) vertical episodes. In a separate cohort of animals, MEDI0618 or isotype control protein was administered subcutaneously and 24 h later the mice received intraperitoneal injection of compound 48/80 and were placed in a Plexiglas activity box for the quantification of (**F**) vertical episodes. Data are presented as mean ± standard error of the mean and were analysed using two-way ANOVA followed by Sidak's multiple comparison test (**A**, **C**, **E** and **F**) and unpaired Student's *t*-test (**B** and **D**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ ($n = 7$–8) versus control or isotype control protein. Details of the statistical analyses are provided in Supplementary Table 3.

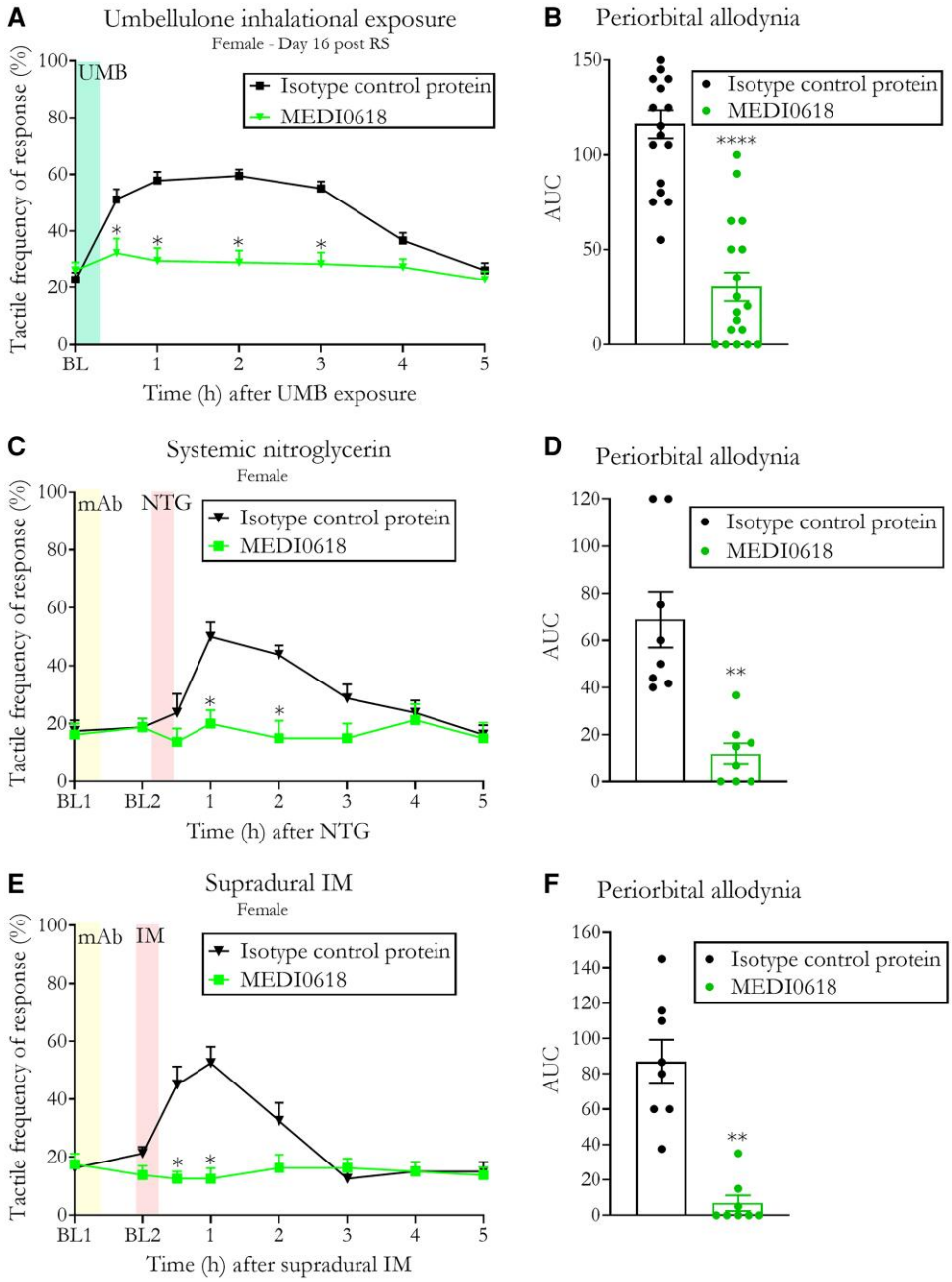

Figure 4 MEDI0618 prevented periorbital cutaneous allodynia induced by migraine-like pain models. Data represent tactile responses from different cohorts of female mice. Inhalational exposure of a subthreshold dose of umbellulone (UMB) on Day 16 after the first restraint stress (RS)-priming, systemic intraperitoneal (i.p.) administration of nitroglycerin (NTG) and supradual injection of inflammatory mediators (IM) were used to induce migraine-like pain behaviour in mice. MEDI0618 or isotype control protein were administered subcutaneously at 50 mg/kg 24 h prior to (**A**) UMB, (**C**) NTG or (**E**) IM time courses. Periorbital tactile frequency of response was collected at baseline (BL, **A**; BL1, **C** and **E**) before and after (BL2) monoclonal antibody (mAb) administration, followed by (**A**) inhalational UMB (0.01 M/500 µl, each gauze; 30 min), (**C**) NTG (10 mg/kg, i.p.) or (**E**) supradural IM (5 µl, each). Area under the curve (AUC) calculation was performed for (**B**) UMB, (**D**) NTG and (**F**) IM time courses. Data are presented as mean ± standard error of the mean and analysed using two-way ANOVA followed by Sidak's multiple comparison test (**A**, **C** and **E**) and unpaired Student's *t*-test (**B**, **D** and **F**). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{****}P < 0.0001$ (UMB, $n = 18$; NTG and IM, $n = 8$) versus isotype control protein. Details of the statistical analyses are provided in Supplementary Table 3.

in preclinical models of direct dural nociceptor activation and stimulation of sensitized nociceptors; (iii) the efficacy of MEDI0618 in prevention of migraine-like pain independent of signalling at the CGRP-R; and (iv) the relevance of PAR2 blockade for migraine, rather than acute or persistent PTH-like behaviours induced by mTBI. Collectively, these observations support the role of PAR2 in migraine pathophysiology and suggest the potential for a preventive therapeutic that may be effective broadly against CGRP-R related and CGRP-R independent migraine mechanisms. Lastly, the data highlight likely differences in pathophysiological mechanisms promoting migraine and PTH despite often overlapping clinical symptoms.

We first confirmed the specificity and potency of MEDI0618 for human PAR2. MEDI0618 specifically bound to human cells with

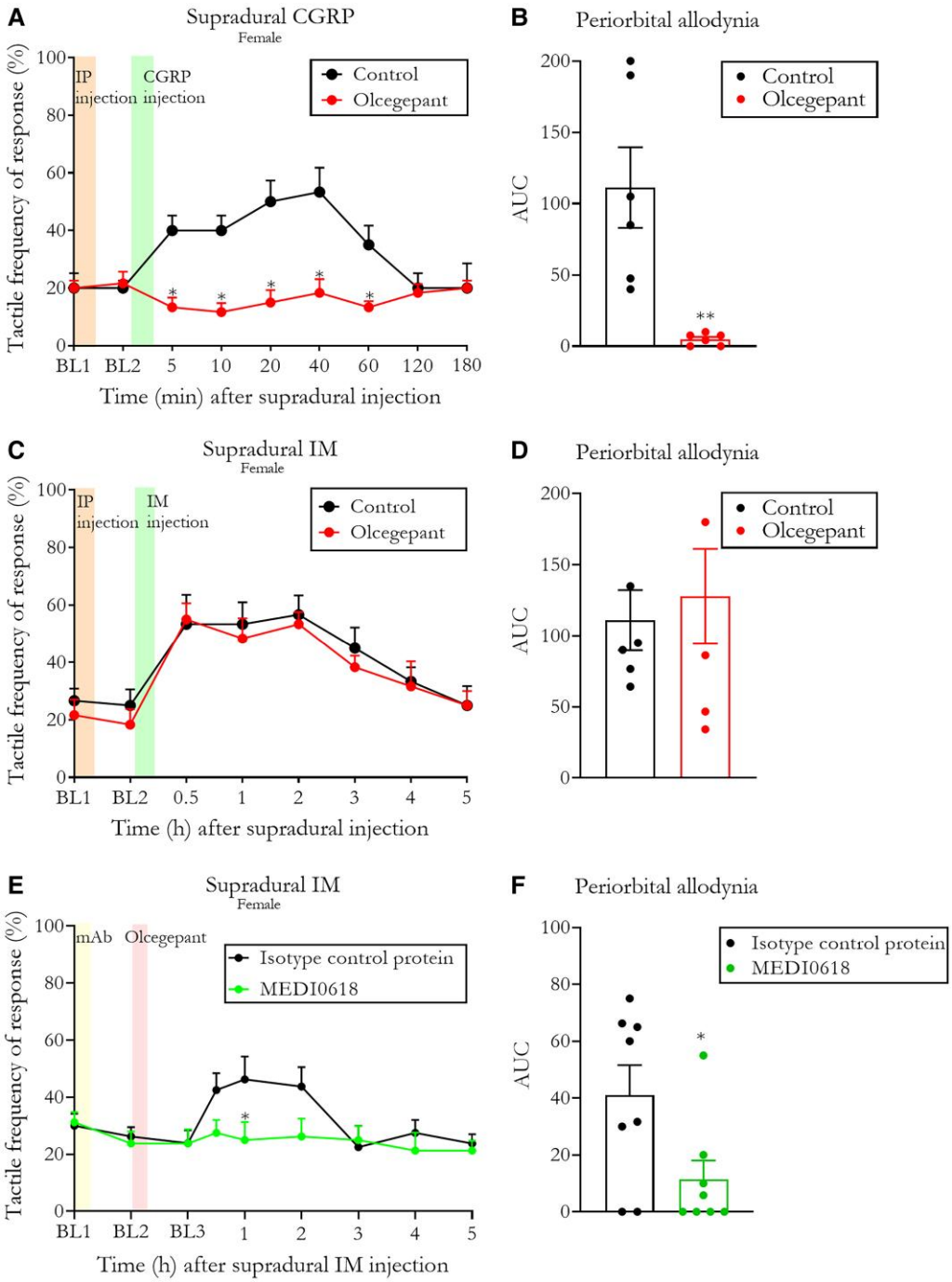

Figure 5 MEDI0618 prevented calcitonin gene-related peptide (CGRP)-dependent and -independent mechanisms involved in migraine-like pain. Data represent tactile responses from different cohorts of female mice. (**A** and **C**) Periorbital tactile frequency of response was collected prior to (BL1) and 30 min after (BL2) intraperitoneal administration of control or the small molecule CGRP-receptor (CGRP-R) antagonist, olcegepant, at 1 mg/kg followed by supradural injection of (**A**) CGRP (1 pg/5 µl, each) or (**C**) inflammatory mediators (IM; 5 µl, each). Cutaneous allodynia was evaluated at indicated times after CGRP or IM. (**E**) Behaviour was recorded before subcutaneous administration of MEDI0618 or isotype control protein, both at 50 mg/kg (BL1). Twenty-four hours later, the tactile frequency of response was assessed prior to (BL2) and 30 min after (BL3) intraperitoneal administration of olcegepant followed by supradural IM and cutaneous allodynia assessment. Area under the curve (AUC) calculation was performed for (**B**) CGRP, (**D**) IM and (**F**) IM/MEDI0618 time courses. Data are presented as mean ± standard error of the mean and analysed using two-way ANOVA followed by Sidak's multiple comparison test (**A**, **C** and **E**) and unpaired Student's *t*-test (**B**, **D** and **F**). $^{*}P < 0.05$, $^{**}P < 0.01$ (CGRP, $n = 6$–8; IM, $n = 6$ and IM/MEDI0618, $n = 8$) versus control or isotype control protein. Details of the statistical analyses are provided in Supplementary Table 3.

PAR2 receptors and prevented matriptase-induced calcium increases in hPAR2-expressing cells without causing agonist actions. This binding profile was consistent across various cell types, including fibroblasts from human donors and both human and mouse endothelial cells. Lastly, MEDI0618 exhibited significant selectivity for hPAR2 over PAR1, with no inhibitory effects observed even at concentrations up to ~40 000 times the hPAR2 IC_{50}. Interestingly, the Fmax response of hDuF cells to the recombinant

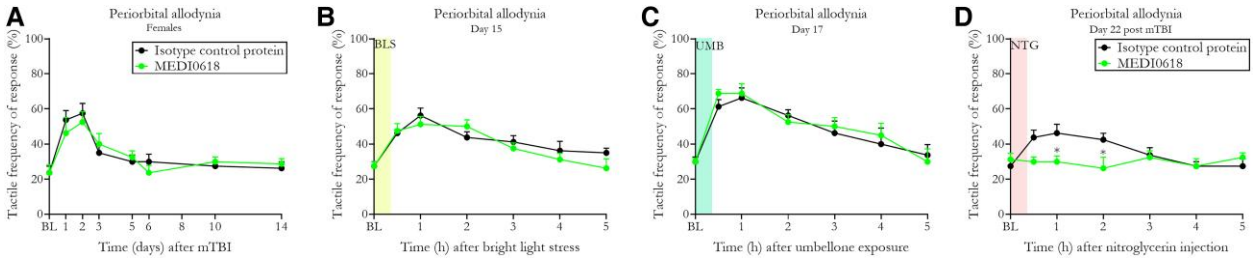

Figure 6 MEDI0618 failed to prevent acute and persistent post-traumatic headache-like behaviours induced by mild traumatic brain injury. (**A**) Periorbital tactile frequency of response was collected before (BL) and on Days 1, 2, 3, 5, 6, 10 and 14 after mild traumatic brain injury (mTBI). MEDI0618 or isotype control protein were administered subcutaneously at 50 mg/kg, 2 h, and on Days 7, 14, 16 and 21 after mTBI induction. On Days 15, 17 and 22 after mTBI (**B**, **C** and **D**, respectively) using the same cohort of animals. The baseline (BL) tactile frequency of response was collected, and mice were immediately (**B**) exposed to a 1000-lx bright light (bright light stress) for 15 min, (**C**) received inhalational umbellulone (UMB, 0.01 M/500 µl) for 30 min or (**D**) intraperitoneal nitroglycerin (NTG) at 10 mg/kg followed by periorbital cutaneous allodynia evaluation. Data are presented as mean ± standard error of the mean and analysed using two-way ANOVA followed by Sidak's multiple comparison test ($n = 8$). Details of the statistical analyses are provided in Supplementary Table 3.

peptide agonist LIGRLO appeared higher than the response to the endogenous agonist matriptase, an observation which was not observed across any of the other cell types tested. LIGRLO and matriptase are reported to be highly selective for PAR2 over other protease activated receptors,[57,58] so it is unlikely that this caused the observed discrepancy. At lower levels of receptor expression, differences in the rate of receptor activation between recombinant peptide ligands and endogenous proteases could conceivably affect Fmax; however, further studies are required to investigate this phenomenon.

Neuronal PAR2 has been shown to play a critical role in numerous pain conditions that are associated with endogenous or exogenous proteases,[59] and activation of PAR2 promotes sensitization of peripheral nociceptors.[27-32,54,60-65] Proteases may also play an important role in activation and/or sensitization of meningeal nociceptors. Relevant to headache-like pain, supradural PAR2 agonists induce migraine-like pain responses that are attenuated in PAR2-deficient mice and also prevented by systemic and local administration of a specific PAR2 antagonist.[28,29] A main source of dural PAR2-activating proteases, including tryptase, are the MC that are located perivascularly and closely associated with neurons and fibroblasts.[24,26,32,66] MC and MC degranulation has been suggested to activate PAR2, resulting in inflammation and headache pain including migraine and PTH.[20,22,33,67-69] Consistent with this, rare human conditions associated with hyperactive MC are associated with migraine,[70-72] and MC products are found in the plasma of patients following a migraine attack.[73-75] Additionally, administration of compound 48/80 triggers hemicranial migraine-like headache in humans.[76] PAR2 antagonists have been shown to inhibit MC degranulation in human and rodent cells.[77,78] The present study, and others, observed that systemic or supradural administration of compound 48/80 promotes migraine-like behaviours in mice.[28,79] Similar to results reported with a recently developed small-molecule PAR2 antagonist,[28] we found that MEDI0618 fully prevented MC degranulation-induced evoked pain behaviour as well as reduction of rearing. Rearing has been interpreted as a measure of headache, possibly due to changes in intracranial pressure that could worsen migraine-like pain.[45]

NTG, a nitric oxide (NO) donor, induces transient headache in healthy individuals but delayed migraine attacks in migraine patients.[80,81] The migraine elicited by NTG and other NO donors is indistinguishable from naturally occurring migraine and includes premonitory symptoms.[80,81] In preclinical studies, NTG produces headache-like pain as well as photophobia.[82] For this reason, rodent studies have commonly employed the NTG model for assessment of migraine mechanisms with high likelihood of translational relevance. Activation of PAR2 mediates cutaneous vasodilatation through NO synthase-dependent mechanisms[83] and promotes acute pain-like behaviour and priming to a subthreshold dose of NTG.[30] Recent work has suggested that NO can react with superoxide to produce peroxynitrite that can promote nociceptor sensitization and pain.[84] Our studies show that pretreatment with MEDI0618 is fully effective in preventing cutaneous allodynia in the NTG model.

Preclinical studies have also directly activated dural nociceptors in order to capture and evaluate the mechanisms underlying the headache phase of migraine. Multiple substances can produce headache-like behaviours in rodents, following application to the dura. These include particularly CGRP and IM cocktails that contain nociceptive substances including bradykinin, serotonin, prostaglandin and histamine and have low pH.[82] CGRP is especially relevant due to the causal link between this peptide and migraine that has been demonstrated by the clinical efficacy of small molecule CGRP-R antagonists as well as monoclonal antibodies targeting either CGRP peptide or the CGRP-R. However, anti-CGRP therapies are not effective in all migraine patients and many patients who are treated with CGRP-based preventive medicines still have breakthrough attacks.[12-15] These observations suggest that mechanisms other than CGRP can promote migraine attacks in many patients.

Our previous report showed that a selective PAR2 antibody effectively and fully prevented pain-like behaviours evoked by supradural CGRP indicating that PAR2 is a key point of convergence for CGRP evoked pain. In contrast, CGRP-R antagonism did not inhibit the headache pain-like effects of supradural PAR2 activators.[29] Likewise, olcegepant, a small molecule CGRP-R antagonist failed to modify the effect of PAR2 agonists in increasing dural artery diameter.[27] These studies suggest that PAR2 signalling might be 'downstream' of CGRP-R. We therefore evaluated the possible effects of blocking PAR2 in CGRP-R independent migraine-like pain, identified as residual pain-like behaviour following antagonism of CGRP-R and activation of dural afferents with an IM cocktail. We first demonstrated that olcegepant could completely block the pain behaviours associated with supradural CGRP suggesting that the dose of olcegepant used in these studies was sufficient to occupy CGRP-R. Following treatment with olcegepant, IM produced cutaneous allodynia suggesting that these pain-like effects might be independent of CGRP-R mechanisms. Importantly, pretreatment

with MEDI0618 prevented periorbital cutaneous allodynia following supradural IM regardless of whether CGRP-R were previously blocked with olcegepant. These effects were notable because the mediators in the IM cocktail not only cause the release of CGRP but additionally activate specific receptors and/or ion channels to produce sensitization and neurogenic inflammation that can promote migraine.[85,86] MEDI0618 blockade of PAR2 may therefore produce effects against multiple mechanisms that can promote migraine.

Environmental irritants are also known to elicit migraine attacks in vulnerable individuals and there is an increase in migraine during seasons with high airborne allergens.[16] The TRPA1 channel is a neuronal sensor for environmental irritants known to promote migraine, including cigarette smoke and some perfumes.[87-89] Importantly, these irritants produce migraine attacks only in people with primary headache disorders, suggesting the likelihood of lowered sensory thresholds for activation of trigeminal nociceptors and pain.[89] Migraine has been termed a cycling sensory threshold disorder, and patients with migraine have lower sensory thresholds even in the interictal pain-free state.[19] PAR2 is co-expressed with TRPA1 in cell bodies of sensory afferents.[62] PAR2 activation leads to sensitization of TRP channels including TRPA1 via phospholipases and phosphokinases[90-92] as well as nociceptive behaviours.[88] We previously reported that repeated restraint stress can sensitize trigeminal ganglion cells to a subthreshold inhalational dose of UMB, a TRPA1 activator, presumably analogous to the pain-producing actions of subthreshold doses of environmental irritants in humans. Pretreatment with MEDI0618 prevented the development of cutaneous allodynia induced by UMB in mice with restraint stress priming. Activation of TRPA1 expressing TG neurons by UMB is likely to result in release of excitatory substances that may engage neuronal PAR2, MC degranulation or both. The anti-migraine effects of MEDI0618 in this model may therefore result from direct or indirect prevention of increased excitability of meningeal neurons,[32,93] decreased activation of trigeminal nociceptors[31] as well as possible inhibition of vasodilation.[27]

The most common outcome of mTBI, also known as concussions, is PTH.[94] PTH may be acute if it resolves within 3 months of the injury, or persistent if the headache lasts for longer than this period. PTH most often presents with a migraine phenotype, yet clinically available migraine medications are effective in a minority (<20%) of PTH patients.[1,94,95] Differences in brain structure, regional volumes, cortical thickness, surface area and curvature as well as fibre tract profiles have been reported in patients with PTH and migraine.[96-98] Given the migraine phenotype of PTH in patients, and as both MC degranulation and increased cortical expression of PAR2 have been reported in rodents after mTBI,[33] we speculated that MEDI0618 might also be effective in this secondary headache. Consistent with our previous reports in this model, we observed a period of transient periorbital and hindpaw allodynia suggestive of acute PTH. Following resolution of the transient allodynia, exposure to a stress or environmental stimulus reinstated periorbital and hindpaw allodynia suggestive of persistent PTH. We previously demonstrated that a monoclonal antibody blocking CGRP signalling prevents acute and persistent PTH if administered soon after mTBI onset but is not effective after resolution of the initial transient allodynia, suggesting that CGRP may have a limited role in sustaining established central sensitization.[41] Unlike the efficacy of MEDI0618 observed in all of the migraine models studied, we did not observe efficacy in this model of mTBI-induced PTH. Our data support the conclusions of studies that reveal differences in the underlying pathophysiological mechanisms that might contribute to PTH and migraine conditions[96-99] and are consistent with the relative lack of evidence of efficacy of anti-migraine drugs in patients with PTH.[95] The current study provides further evidence that migraine and PTH are mechanistically distinct types of headaches.

A limitation of our study includes the uncertainty as to the specific site of action for PAR2 in migraine pain, which could include MC, fibroblasts and meningeal neurons, and will require future investigation, but our findings strongly support the relevance of PAR2 as a pharmacological target for migraine prevention. Additionally, we note that these studies were performed using female but not male mice. Future studies should investigate the efficacy of the PAR2 antibody in male animals. We also note that we did not consider possible influences of the oestrous cycle. Female sex hormones have long been recognized as factors in promoting migraine across all life stages experienced by women. In the present study, disregard for the possible influence of the oestrous cycle may be beneficial in addressing the variability observed in human populations and in increasing the translational relevance of our findings.

In summary, MEDI0618 was effective in preventing periorbital allodynia in all migraine models studied. Such broad activity has not been observed in preclinical migraine models with interventions aimed at CGRP or PACAP38.[100,101] Additionally, hindpaw allodynia was also blocked, suggesting prevention of central sensitization (shown in the Supplementary material). PAR2 blockade may therefore offer a broader therapeutic strategy for migraine prevention, potentially providing therapeutic benefit against both CGRP-dependent and CGRP-independent mechanisms of migraine initiation. MEDI0618, which has been shown to be safe and well-tolerated in healthy volunteer phase I clinical trials, could be an advantageous therapeutic strategy for migraine prevention in both CGRP responders and non-responders. In addition, clinical studies with MEDI0618 in migraineurs will for the first time test whether blockade of PAR2 can effectively prevent migraine.

Data availability

Data are available upon reasonable request.

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Competing interests

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Praxis, AYYA Biosciences, Revance, Pfizer. Honoraria: American Academy of Neurology, Headache Cooperative of the Pacific, Canadian Headache Society, MF Med Ed Research, Biopharm Communications, CEA Group Holding Company (Clinical Education Alliance LLC), Teva (speaking), Amgen (speaking), Eli Lilly (speaking), Lundbeck (speaking), Pfizer (speaking), Vector Psychometric Group, Clinical Care Solutions, CME Outfitters, Curry Rockefeller Group, DeepBench, Global Access Meetings, KLJ Associates, Academy for Continued Healthcare Learning, Majallin LLC, Medlogix Communications, Medica Communications LLC, MJH Lifesciences, Miller Medical Communications, WebMD Health/Medscape, Wolters Kluwer, Oxford University Press, Cambridge University Press. Non-profit board membership: American Brain Foundation, American Migraine Foundation, ONE Neurology, Precon Health Foundation, International Headache Society Global Patient Advocacy Coalition, Atria Health Collaborative, Arizona Brain Injury Alliance, Domestic Violence HOPE Foundation/Panfila. Research support: Department of Defense, National Institutes of Health, Henry Jackson Foundation, Sperling Foundation, American Migraine Foundation, Henry Jackson Foundation, Patient Centered Outcomes Research Institute (PCORI). Stock options/shareholder/patents/board of directors: Aural analytics (options), Axon Therapeutics (shares/board), ExSano (options), Palion (options), Man and Science, Healint (options), Theranica (options), Second Opinion/Mobile Health (options), Epien (options), Nocira (options), Matterhorn (shares), Ontologics (shares), King-Devick Technologies (options/board), EigenLyfe (shares), AYYA Biosciences (options), Cephalgia Group (shares/board), Atria Health (options/employee). Patent 17189376.1-1466:vTitle: Onabotulinum Toxin Dosage Regimen for Chronic Migraine Prophylaxis (Non-royalty bearing). Patent application submitted: Synaquell® (Precon Health). J.E.L., P.T., A.S.W., P.N., S.P., G.M., I.G., C.D., T.C. and I.C. are employees of AstraZeneca. The other authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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