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MP29-02 reduces nasal hyperreactivity and nasal mediators in patients with house dust mite allergic rhinitis

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MP29-02 reduces nasal hyperreactivity in allergic rhinitis

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Key words

Azelastine hydrochloride, allergic rhinitis, fluticasone propionate, nasal hyperreactivity, substance P

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I.K.K., Y.A.A., B.S., L.V.G., K.T., J.L.C., S.F.S. and P.W.H. designed the study. I.K.K., I.C., Y.A.A, B.S., P.S.S. and A.K. performed practical experiments and data analysis. I.K.K, I.C., Y.A.A., K.T., M.M.W., J.L.C., S.F.S., P.W.H. contributed to the interpretation of the data and statistics. I.K.K. wrote the first draft of the article. All authors drafted and revised the manuscript and approved the final version.

Abstract

Background: Nasal hyperreactivity (NHR) is an important clinical feature of allergic rhinitis (AR). The efficacy of MP29-02 (azelastine hydrochloride (AZE) and fluticasone propionate (FP)) nasal spray on local inflammatory mediators and NHR in AR is unknown. We tested if MP29-02 decreases inflammatory mediators and NHR in AR and if this effect is due to restoration of nasal epithelial barrier function.

Methods: A 4-week double-blinded placebo-controlled trial with MP29-02 treatment was conducted in 28 patients with house dust mite (HDM) AR. The presence of NHR was evaluated by measuring reduction of nasal flow upon cold dry air exposure. The effects of AZE+/-FP on barrier integrity and airway inflammation were studied in a murine model of HDM induced NHR and on reduced activation of murine sensory neurons and human mast cells.

Results: MP29-02 but not placebo reduced NHR ($p<0.0001$ vs. $p=0.21$), levels of substance P ($p=0.026$ vs. $p=0.941$) and β-hexosaminidase ($p=0.036$ vs. $p=0.632$) in human nasal secretions. In wild type C57BL6 mice, the reduction of β-hexosaminidase levels ($p<0.0001$) by AZE+FP treatment upon HDM challenge was found in parallel with a decreased transmucosal passage ($p=0.0012$) and completely reversed eosinophilic inflammation ($p=0.0013$). *In vitro*, repeated applications of AZE+FP desensitized sensory neurons expressing the transient receptor potential channels TRPA1 and TRPV1. AZE+FP reduced MC degranulation to the same extent as AZE alone.

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Conclusion: MP29-02 treatment reduces inflammatory mediators and NHR in AR. The effects of AZE+FP on MC degranulation, nasal epithelial barrier integrity and TRP channels provide novel insights into the pathophysiology of allergic rhinitis.

Introduction

Nasal hyperreactivity (NHR) is defined as an increased sensitivity of the nasal mucosa to environmental, non-specific stimuli, such as temperature/humidity changes, irritants or strong odors, leading to induction of nasal symptoms (1,2). Self-reported NHR is found in two-thirds of the patients with allergic rhinitis (AR) (3). The pathophysiology of NHR is not fully elucidated. It is assumed that the release of various neuropeptides, such as substance P (SP), from afferent nerve fibers upon activation of transient receptor potential (TRP) ion channels, is responsible for the induction of nasal symptoms (1). Binding of SP to the neurokinin receptor 1 on mast cells (MCs) results in the release of mediators in an IgE-independent way (4). Moreover, a subgroup of afferent nerve fibers is responsive to histamine as they express the histamine 1 receptor (H1R), and this cross-talk between neurons and MCs may represent an important bi-directional interaction leading to the induction of symptoms (5).

We previously described a pivotal role of TRPA1 activation on airway sensory nerves in a murine model of non-allergic airway hyperreactivity (6), and the involvement of the TRPV1 signaling pathway in a subgroup of patients with non-allergic, non-infectious rhinitis, also called idiopathic rhinitis (IR) (7). Wouters *et al*. found that sensitization of TRPV1 mediates hypersensitivity in patients with irritable bowel syndrome (8). Sensory neurons are required for allergic airway hyperreactivity and their silencing abolished bronchial hyperreactivity in a murine model of allergic airway inflammation (9). Treatment options to reduce NHR are scarce. Capsaicin treatment reduces the TRPV1-SP nociceptive signaling pathway in patients with IR resulting in reduction of nasal symptoms, NHR, and release of

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SP (7). Additionally, blocking of lung nociceptors resulted in decreased airway inflammation (10).

The novel intranasal formulation of MP29-02, a combination product of azelastine hydrochloride (AZE) and fluticasone propionate (FP) nasal spray, was shown to be safe (11,12) and effective (13–17) in reducing nasal symptoms and improving disease control in patients with AR (18–20). However, no clinical trials investigated the potential effect of MP29-02 on NHR in patients with AR or studied why the combination product is superior compared to either of the molecules alone. Additionally, the effect of AZE with or without FP on sensory TRP channel desensitization and the potential synergistic effect of AZE+FP on human MC degranulation are unknown.

Our group previously showed that FP, besides its anti-inflammatory role, also increases the barrier integrity in epithelial cell monolayers (21,22) and reduces the permeability of FITC dextran 4 kDa (FD4) in serum of mice (21). In the present study, we hypothesize that MP29-02 treatment reduces NHR and local neuro-immune mediators in patients with house dust mite (HDM) induced AR, due to restoration of nasal mucosal barrier integrity and reduced activation of MCs and neurons. More specifically, we investigated if MP29-02 treatment reduced local levels of neuro-immune mediators and the presence of NHR. The effect of AZE+/-FP was evaluated on mucosal barrier integrity, MC degranulation and airway inflammation in a murine model with HDM-induced allergic airway inflammation. Finally, the effects of AZE+/-FP on sensory nerve activation and MC degranulation were studied *in vitro*.

Materials and methods

Study patients

Twenty-eight patients with HDM-induced AR and confirmed NHR were recruited through the outpatient clinic at the Department of Otorhinolaryngology, Head and Neck Surgery, University Hospitals Leuven, Belgium from October 2014 to February 2016. The

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study has been carried out in accordance with the CONSORT guidelines, the Declaration of Helsinki and ICH guideline on Good Clinical Practice. The study was approved by the Institutional Review Board of the University Hospitals of Leuven and registered at clinicaltrials.gov (NCT02238353). All enrolled subjects signed written informed consent.

Patients (between 18 and 60 years) with an ARIA (allergic rhinitis and its impact on asthma)-based diagnosis of persistent moderate/severe AR (≥ 2 nasal symptoms suggestive of AR), positive skin prick test to HDM, positive NHR test (decrease in peak nasal inspiratory flow (PNIF) > 20 % upon cold dry air (CDA) provocation), no clinical signs of infection and without anatomic nasal abnormalities or endoscopic signs of chronic rhinosinusitis were included.

Patients were excluded from the study if they had evidence of clinically relevant acute or chronic systemic disorders, infections or malignancies at screening, a known allergic reaction to MP29-02 or any of the components, a change in vision or with a history of increased ocular pressure, glaucoma and/or cataracts, a prolonged use of decongestive nose sprays or oral medication affecting nasal function, a psychiatric or addictive disorder, or if they were on immunotherapy for HDM, had been enrolled in other clinical trials within the last 3 months, were pregnant at screening, if they were current smoker, or used anticoagulation medication (Supplementary Figure 1).

Study medication

Patients were randomized and blinded to receive MP29-02 or placebo nasal spray (Meda Pharmaceuticals Inc. Somerset, NJ) in a 2:1 ratio. The nasal sprays were administered as 1 spray/nostril twice daily for 28 days. Each spray applies 0.137 mL solution containing 137 µg of AZE and 50 µg of FP for the active treatment. The placebo nasal spray was administered in the same vehicle and device.

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Study design

At the screening visit, the skin prick test was performed, and a nasal endoscopy to exclude nasal abnormalities or polyps. The patients were asked to score their nasal symptoms. At baseline visit (V0), the NHR was measured, nasal secretions were collected and nasal symptoms were scored. After enrollment, the first application of the nasal spray was given. At the follow-up visits (V1 and V2) after 7 and 28 days of treatment, respectively, the same procedure was followed as at baseline. At V2, the use of the nasal spray was finished.

Evaluation of nasal hyperreactivity

To evaluate the presence and severity of NHR (23), the validated CDA provocation methodology (23) was used to expose the patients to cold (-10 ˚C) dry (< 10% humidity) air for 15 minutes while breathing through the nose via the mask. Nasal patency was evaluated by measurement of PNIF before and after CDA provocation. PNIF-evaluation (L/min) is a physiologic measure of airflow through both nasal cavities during forced inspiration. NHR was defined as a PNIF-reduction of > 20 % upon nasal exposure to CDA compared to the pre-CDA measurement. The efficacy of MP29-02 was quantified by evaluation of the change in (PNIF-decrease) values compared to baseline and to the placebo treated group.

Evaluation of nasal symptoms and disease control

The nasal symptoms were evaluated by the change from baseline in reflective total 5 symptom score (T5SS; nasal congestion, rhinorrhoea, sneezing and itchy nose and eyes; range 0 - 15) (24) and the visual analogue scale (VAS; range 0 - 10) (25) were evaluated before and after CDA provocation at baseline, V1 and V2. The allergic rhinitis control test

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(ARCT; range 5 – 25, an increased score reflects an improvement in disease control) (26) was evaluated at baseline, at V1 and V2.

Measurement of mediators in nasal secretions

Nasal secretions were collected at baseline, V1 and V2 as previously described (7). The concentrations of SP (3.9 – 500 pg/mL, Cayman Chemical, Ann Arbor, Michigan, USA), interleukin (IL)-5 (10000 – 4.9 pg/mL, BD Pharmingen, San Diego, CA, USA), and eosinophil peroxidase (1.56 – 100 ng/mL, Abbexa, Cambridge,UK) in nasal secretions were measured using ELISA according to the manufacturer's instructions. For correction of the values, the mean value of the blanks was subtracted from the raw data. These values were multiplied by the dilution factor at the time of collection and divided by the total volume of the nasal secretion. Levels of β-hexosaminidase were determined in nasal secretions as previously described (27).

Culture of primary human mast cells

Primary human MCs were cultured from peripheral blood $CD133^+$ precursor cells as described before (28,29) with minor changes. Detailed information on culturing of primary human mast cells can be found as *Supplementary information*.

Mature MCs were sensitized for 24 hours with 10 kU/L myeloma IgE (Calbiochem, San Diego, CA USA). AZE (0-100 µM, n = 3) and FP (0-1000 nM, n = 3) were applied to the MCs 10 minutes prior to activation with anti-IgE antibody (1 µg/mL). MC activity (%$CD63^+$ cells) was monitored by flow cytometry and degranulation was evaluated by measurement of histamine (Reflab, Copenhagen, Denmark) and β-hexosaminidase levels in supernatants (50,000 cells per condition). Maximal activation of the positive control (IgE/anti-IgE) was set

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as highest outcome (100%), and the results of the experimental samples were expressed as a proportion of the positive control.

Murine model of HDM-induced allergic airway inflammation

All animal experiments were approved by the local Committee of Ethics and were performed according to the European Community Council guidelines. Male C57BL/6J mice (8-12 weeks, Envigo, Horst, The Netherlands) were maintained in filter top cages. A murine model of HDM-induced allergic airway inflammation (21) was used to study the effect of AZE +/- FP on epithelial permeability after FD4 application, MC degranulation and lung inflammation (Figure 3A). See the *Supplementary information* for a detailed description.

TRPV1 and TRPA1 expressing cells

Human embryonic kidney cells (HEK293T) were transiently transfected with murine TRPV1. See *Supplementary information* for further details of the functionality experiments. TRPA1-mediated responses were studied in Chinese hamster ovarian (CHO)-K1 cells stably expressing murine TRPA1 (30). To avoid intracellular Ca^{2+} overload via entry through constitutively open TRPA1 channels we did not incubate the cells with the inducer tetracycline (31).

Mouse dorsal root and trigeminal ganglion neurons

Dorsal root ganglion and trigeminal neurons from wild-type C57Bl/6J mice (8–12 weeks, males), were isolated and cultured as previously described (32). The full methodology is provided as *Supplementary information*.

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Functionality measurements by intracellular Ca^{2+} imaging

Cells were incubated with Fura-2 acetoxymethyl ester for 30 min at 37 °C. For recordings, bath solutions prepared in Krebs (containing (in mM): 150 NaCl, 6 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES, 10 glucose and titrated to 7.4 with NaOH) were perfused by gravity via a multi–barreled pipette tip. Intracellular Ca^{2+} concentration was monitored through the ratio of fluorescence measured upon alternating illumination at 340 and 380 nm using an MT-10 illumination system and the Xcellence pro software (Olympus Belgium N.V., Berchem, Belgium).

Statistical analysis

Data were analyzed using Graphpad Prism 5 (La Jolla, CA, USA). Kolmogorov–Smirnov test was used for testing for normality of the distribution. Repeated measures of ANOVA, Friedman, or Kruskal-Wallis test with post-hoc analysis were used to compare multiple groups at different time points. Differences between placebo and active treatment were analyzed using two-tailed unpaired t-test or Mann–Whitney U test. Data are presented as median and interquartile range (IQR) where appropriate. Categorical values were compared using the Fisher exact test. Spearman's *r* was used to determine correlations. Values were considered significantly different when $p < 0.05$.

Results

MP29-02 treatment reduces nasal symptoms and improves disease control

The patient characteristics are shown in Table 1. MP29-02 significantly decreased T5SS at V1 and V2 compared to baseline ($p < 0.0001$) (Figure 1A). In the placebo group, no significant decrease was found at V1 and V2 compared to baseline ($p = 0.11$). At V1 and V2,

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T5SS was significantly lower in MP29-02 compared to placebo treated patients (p = 0.03 (V1) and p = 0.003 (V2), Figure 1A). At V2, the reduction of T5SS post CDA correlated with the reduction in NHR (r = -0.57, p = 0.008), which was not seen in placebo treated patients (r = 0.22, p = 0.66, data not shown). MP29-02 treatment significantly decreased the VAS major symptom at V1 and V2 compared to baseline (p < 0.0001). In the placebo group, no significant decrease was found at V1 and V2 (p = 0.12, Figure 1B). At V2, the reduction in VAS major symptom post CDA correlated with the reduction in NHR in the MP29-02 treated group (r = -0.55, p = 0.017), which was not seen in placebo treated patients (p = 0.24, data not shown). The patients evaluated the effect of MP29-02 on disease control by scoring the ARCT questionnaire. The changes in ARCT from baseline scores at V2 improved compared to V1 (p = 0.014) after MP29-02 treatment. At V2, a better improvement in disease control was found after MP29-02 treatment compared to placebo (p = 0.035, data not shown).

MP29-02 treatment reduces the levels of substance P and β-hexosaminidase in human nasal secretions

The concentrations of SP, β-hexosaminidase, IL-5 and eosinophil peroxidase in nasal secretions after each CDA provocation were measured. SP levels were decreased after MP29-02 treatment at V1 and V2 (p = 0.026). In the placebo group, no decrease was found (p = 0.94, Figure 1C). After MP29-02 treatment, β-hexosaminidase levels were significantly lower at both V1 and V2 compared to baseline (p = 0.004), and significantly different compared to placebo at V2 (p = 0.036). No decrease in β-hexosaminidase was found in the placebo group (p = 0.63, Figure 1D). The concentrations of IL-5 and eosinophil peroxidase were below detection limit.

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MP29-02 treatment reduces nasal hyperreactivity in patients with HDM allergic rhinitis

CDA provocation induced a similar degree of PNIF-decrease in the MP29-02 and placebo group at baseline (p = 0.95). MP29-02 significantly reduced the PNIF-decrease after CDA at V1 and V2 compared to baseline (p < 0.0001). In the placebo group, no change in PNIF values were observed at V1 and V2 compared to baseline (p = 0.21) (Table 2).

AZE+FP inhibits human mast cell degranulation to the same extent as AZE alone

To investigate the effect of AZE and FP on MC degranulation, primary human MCs were incubated with AZE, FP or AZE+FP prior to activation with anti-IgE. AZE was effective at 100 µM and decreased the median relative percentage of $CD63^+$ cells with 42.2% (52.7 - 39.6), β-hexosaminidase release with 42.8% (31.8 - 53.8) and histamine release with 19.8% (18.3 - 21.3). Pre-treatment with FP showed no inhibitory effect on MC degranulation, and no additional effect was found when FP was pre-mixed with AZE compared to AZE alone (Figure 2).

FP prevents allergen passage, AZE reduces mast cell degranulation and AZE+FP completely abrogates airway inflammation in mice

To explore the mechanisms underlying the effectiveness of this combination therapy to reduce NHR on inflammatory mediators, the active compounds AZE, FP or AZE+FP were studied in a murine model of HDM-induced allergic airway inflammation (Figure 3A). The potential effect of the compounds on transmucosal permeability, MC degranulation and lung inflammation, were examined. One hour after FD4 application, AZE+FP and FP treatment, but not AZE, significantly decreased the levels of FD4 in serum (p = 0.012, Figure 3B), confirming that FP reduces transmucosal passage. AZE+FP treatment resulted in a total abrogation of eosinophils and neutrophils in bronchoalveolar lavage (BAL) fluid (p = 0.0013)

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compared to sham, whereas only a partial reduction was observed in FP treated mice (Figure 3C), demonstrating a synergistic effect of AZE+FP on airway inflammation. In addition, all treatments resulted in reduced systemic β-hexosaminidase levels in serum ($p < 0.0001$, Figure 3D) compared to the sham group. Local β-hexosaminidase levels in lung homogenates were significantly decreased in all treatment groups compared to sham treated mice ($p = 0.026$, Figure 3E). These results support the hypothesis that MC degranulation is reduced due to restoration of the barrier integrity by FP.

Repeated applications of AZE+FP abrogates the activation of TRPA1$^+$TRPV1$^+$ neurons in vitro

We previously demonstrated that activation of neuronal TRPV1 and TRPA1 channels is responsible for the development of IR and non-allergic airway hyperreactivity, respectively (6,7). Hence, we sought to determine whether AZE and/or FP could induce desensitization of TRPV1 and TRPA1 expressing neurons.

We evaluated the effect of AZE or FP on sensory neurons isolated from murine trigeminal ganglia. When applied independently, these compounds failed to trigger intracellular Ca^{2+} increase in these cells (Figure 4A, B). In contrast, application of a pre-mixed solution of AZE+FP (30 μM + 1 μM) evoked a rapid intracellular Ca^{2+} increase in ~7% (63 of 915) of the neuronal population. Interestingly, all activated neurons were also sensitive to cinnamaldehyde and capsaicin, indicating the presence of both TRPA1 and TRPV1 channels on these cells (Supplementary Figure 2A, B). To further discern whether these compounds activated TRPA1 and/or TRPV1, we used heterologous expression systems. AZE+FP activated 15% (39 of 263) of CHO cells stably transfected with TRPA1 (Supplementary Figure 2C). Likewise, 49% (91 of 183) of HEK-293T cells transiently overexpressing TRPV1 displayed a robust intracellular Ca^{2+} increase upon application of AZE+FP (Supplementary Figure 2D).

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As it is clinically important to investigate whether repeated applications of AZE+FP can decrease the sensitivity of the TRP channels, murine DRG neurons were exposed three consecutive times to AZE+FP. The $TRPA1^{+}TRPV1^{+}$ double positive neurons only reacted to the first application of AZE+FP and the responses to the two subsequent applications strongly abrogated the Ca^{2+} influx already at the second application (Figure 4C, D). The sensitivity of the TRP channels was not affected by AZE+FP, since low (10 nM) or high doses (1 μM) of capsaicin could still activate TRPV1.

Discussion

This is the first study on reduction of NHR in AR utilizing an objective measure to diagnose NHR and inducing inflammatory mediators. Firstly, in patients with HDM-induced AR, we find a significant decrease in NHR after 7 and 28 days of treatment with MP29-02 and not in the placebo group, which is associated with the reduction in nasal symptoms. In addition, MP29-02 treatment reduces the local levels of SP and β-hexosaminidase after CDA. Secondly, AZE+FP inhibits human MC degranulation to the same extent as AZE alone and that AZE+FP treatment prevents transmucosal passage and abrogates eosinophilic inflammation in a murine model. Thirdly, we show effects of AZE+FP on neuronal desensitization *in vitro*.

After treatment with MP29-02, the decrease in NHR may be the result of local reduction in SP and β-hexosaminidase release due to alteration of the local MC and neuronal activation after provocation with CDA. This study provides clinical evidence that MP29-02 is efficient to reduce NHR even with a limited number of subjects. Based on previous *in vivo* studies with AZE, MP29-02 treatment may also affect the release of other mediators than SP and β-hexosaminidase from MCs or immune cells. In a trial including patients with seasonal AR, a single oral dose of AZE reduced leukotrienes in nasal lavages (33). AZE was effective to treat alcohol-induced asthma by inhibiting histamine release (34).

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In patients with asthma and atopy for grass pollen, AZE reduced SP levels in BAL and nasal lavages (35). AZE inhibited the allergen-induced release of histamine and tryptase from the human nasal mucosa (36). A previous *in vitro* study using human MCs demonstrated that AZE inhibits the secretion of IL-6, tumor necrosis factor-α, IL-8, and nuclear factor-κB (37). In the present study, IL-5 and eosinophil peroxidase concentrations were below detection limit and the small volume of nasal secretions, especially after treatment with MP29-02, did not allow us to perform further cytokine analysis.

The inhibitory effect of AZE on *in vitro* degranulation of human cord blood derived MCs and rat skin MCs had been published (38). Also the effect of FP on human MCs is known (39,40). This study provided evidence that FP had no synergy with AZE on the inhibitory effect of human MC degranulation.

We previously demonstrated that FP prevented FD4 passage *in vitro* and in a murine model (21). Presently, this model was used to confirm reduction in FD4 passage and to study the effects of AZE and/or FP on airway inflammation and MC degranulation. The outcomes confirmed that FP prevented transmucosal FD4 passage. Decreased passage of HDM allergens by FP treatment may explain the reduced β-hexosaminidase levels in serum of the mice, as we demonstrated that FP had no effect on MC degranulation. AZE reduced β-hexosaminidase levels, but could not prevent FD4 passage. Taken together, the reduction in β-hexosaminidase levels in nasal secretions and the reduction in NHR may partly be explained by the barrier restoring effect of FP in patients treated with MP29-02.

Previous studies with repeated intranasal applications of capsaicin demonstrated reduction in nasal symptoms, NHR and TRPV1 overexpression in patients with nonallergic rhinitis or IR (7,41). Therefore, it is clinically relevant to know whether repeated applications of AZE+FP would desensitize TRPV1. Recently, Singh and colleagues published the effects of AZE and FP on TRPV1 channels, demonstrating a direct activating effect of AZE on TRPV1 and desensitization of TRPV1 after repeated applications of AZE (42). In the present

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study, we found no effect of AZE or FP on TRPV1, while AZE+FP activated TRPV1 channels. As we used the same concentration of AZE, the contradictory findings may be explained by the difference in cell culture models and settings that were used. In line with the findings of Singh, FP application left TRPV1 channels unaltered. Additionally, repeated applications of AZE+FP abrogated the Ca^{2+} influx in murine DRG neurons at the second and third application. The desensitizing effect of repeated applications of AZE+FP is likely to be induced by excitation of the neurons due to the strong Ca^{2+} influx and inability to transport neurotrophic factors leading to a temporary altered phenotype as described by Anand and Bley (43) and comparable with intranasal capsaicin treatment in patients with IR (7). However, the sensitivity to low concentration capsaicin was not decreased (10 nM). This suggests that both channels are needed to cause a direct activating response in neurons, and that AZE+FP does not covalently bind to TRPV1 so that the receptor remains sensitive to other agonists.

The limitations of this study include the small number of subjects, particularly in the placebo group, due to randomization and many screenings failures because of the strict inclusion criteria of the study.

To conclude, this study provides clinical evidence for MP29-02 reducing NHR together with a reduction in MC and neuro-mediators in nasal secretions of patients with HDM-induced AR. These results can partly be explained by the barrier restoring effect of FP, preventing passage of allergens to the submucosa and the synergistic effect of MP29-02 on the reduction on airway inflammation. The effect of MP29-02 on barrier function and neuro-sensitivity is only at the beginning of being explored.

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Figure legends

Table 1: Patient characteristics

* Data are given as median and interquartile range (IQR)

Table 2: MP29-02 treatment reduces nasal hyperreactivity in patients with HDM allergic rhinitis

Data are shown as median and interquartile range (IQR)

***: $p < 0.0001$ compared to baseline (V0)

V0: baseline, V1: after 7 days, V2: after 28 days

Figure 1: MP29-02 treatment reduces nasal symptoms and β-hexosaminidase and substance P levels in nasal secretions after cold dry air provocation

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A; Total 5-Symptom Score (T5SS) at baseline (V0) and after 7 (V1) and 28 (V2) days of treatment with MP29-02 (n = 18) or with placebo nasal spray (n = 7). **B;** VAS major symptoms at baseline (V0), after 7 days (V1) and 28 days (V2) of treatment with MP29-02 (n = 19) or placebo (n = 7) after provocation with cold dry air. **C;** Levels of β-hexosaminidase in nasal secretions in optical density (OD) after correction and **D;** Concentration (pg/mL) of substance P in nasal secretions from patients with house dust mite induced allergic rhinitis at baseline (V0) and after 7 (V1) and 28 (V2) days of treatment with MP29-02 (n = 19) or placebo (n = 7) nasal spray. The data were corrected for the volume of the nasal secretions and the dilution factor.

Figure 2: Fluticasone propionate shows no synergy with azelastine hydrochloride on inhibiting human mast cell degranulation

A-C; Relative $CD63^+$ expression on MC (top), β-hexosaminidase (centre) and histamine release (bottom) from human mast cells sensitized with myeloma IgE (10 kU/L) for 24 hours, incubated with azelastine (0-100 mM) +/- fluticazone propionate (0-1000 μM) for 10 minutes and then activated with anti-IgE antibody (1 μg/mL) for 30 minutes and stained with FITC-conjugated anti-human CD63. Figures show mean and SEM. n = 2 or 3 individual experiments.

Figure 3: Fluticasone propionate treatment prevents allergen passage and mast cell degranulation in a murine model of house dust mite-induced allergic airway inflammation

Mice (n = 6 per group) were intranasally sensitized with 1 μg house dust mite (HDM) extract or saline (negative control group). At days 7-11, the mice were intranasally pre-treated with azelastine hydrochloride (AZE, 10 mg/kg), fluticasone propionate (FP, 0.3 mg/kg), AZE+FP, or sham, and challenged with HDM extract (10 μg) or saline. At day 11, blood was drawn from the tail 30 minutes after the challenge. Then, FITC-dextran-4kDa (50 mg/mL) was

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applied intranasally and 60 minutes later the bronchoalveolar lavage (BAL) fluid, lung tissue, nasal mucosa, and blood were collected for analysis.

Figure 4: The combination AZE-FP activates sensory neurons from trigeminal ganglia

A-C; Representative traces of intracelullar Ca^{2+} levels in sensory neurons isolated from mouse trigeminal ganglia. Thin horizontal lines correspond to application times of the compounds. Azelastine (30 uM), Fluticasone (1 uM). A+F refers to a solution of Azelastine and Fluticasone premixed to final concentrations of 30 uM and 1 uM, respectively. High K^+ solution consists of Krebs solution modified with 45 mM of K^+.

D; Average change in intracellular Ca^{2+} amplitude responses during three consecutive applications of a premixed solution of Azelastine and Fluticasone propionate (A+F, 30 μM + 1 μM). Bars represent mean ± s.e.m. **, $p < 0.01$; ***, $p < 0.001$; Dunn's multiple comparison test.

Supplementary information:

Supplementary Figure 1: Flow chart of study patients

Supplementary Figure 2: Azelastine-Fluticasone solution activates trigeminal neurons expressing both TRPA1 and TRPV1

A; Representative traces of intracellular Ca^{2+} levels in trigeminal neurons. A+F, Azelastine (30 μM) + Fluticasone propionate (1 μM); CA, cinnamaldehyde 100 μM; Caps, capsaicin, 1 μM; high K^+, Krebs containing 45 mM K^+.

B; Percentage of trigeminal neurons (total n = 915 neurons) reacting to the different stimuli. The bars are color-coded to match the traces shown in panel A.

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C, D; Representative traces of intracellular Ca^{2+} levels in TRPA1-stably expressing CHO cells (C) and TRPV1-transiently expressing HEK293T cells. A+F, Azelastine (30 μM) + Fluticasone propionate (1 μM); AITC, allylisothiocyanate 100 uM; Caps, capsaicin, 1 μM. In panel C, red traces correspond to CHO cells unsensitive to A+F. In panel D, red traces represent non-transfected HEK293T cells.

Methods section in this article's Online Repository:

Culture of mast cells

$CD133^+$ precursor cells were isolated from buffy coats provided by the local blood bank using positive selection with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were cultured in StemSpan medium (Stem Cell Technologies, Vancouver, Canada) supplemented with human stem cell factor (100 ng/mL Miltenyi biotech), recombinant human IL-6 (50 ng/mL, Gibco, Thermo Fisher Scientific), and pen/strep (200 μg/mL) and cultured in a humidified incubator with 5% CO_2 at 37 ˚C for 8 weeks into mature mast cells. During the first 3 weeks, medium was supplemented with recombinant human IL-3 (2 ng/mL, R&D Systems Inc, Minneapolis, MN USA), and during the last 2 weeks 10% fetal bovine serum (Invitrogen, Thermo Fisher Scientific) was added to the culture medium.

Murine model of house dust mite-induced allergic airway inflammation

Mice (n = 6 per group) were sensitized intranasally (i.n.) with 1 μg HDM extract (Greer Labs Inc, Lenoir, NC USA) or saline at day 0. At days 7-11, 50 μL AZE (10 mg/kg body weight) or FP (300 μg/kg body weight) or AZE+FP or sham (50 μl DMSO 0.6% in saline) was applied i.n., 1 hour prior to a challenge with 10 μg HDM extract. Thirty minutes after the last HDM challenge at day 11, blood was drawn from the tail for β-hexosaminidase

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measurement in serum. To evaluate transmucosal passage, 20 µL of FITC-dextran 4 kDa (FD4, 50 mg/mL) was applied i.n. After 60 minutes, blood samples, bronchoalveolar lavage fluid, lung tissue, and nasal mucosa were collected.

Transfection of TRPV1

For intracellular Ca^{2+} imaging experiments, human embryonic kidney cells (HEK293T) cells were transiently transfected with the mouse TRPV1, in the bicistronic pCAGGS/IRES-GFP vector, using the Mirus TransIT-293 kit (Sopachem n.v./s.a, Eke, Belgium). Transfected cells were seeded on poly-L-lysine-coated (0.1 mg/ml) glass coverslips and grown in Dulbecco's modified Eagle's medium containing 10% (v/v) human serum, 2 mM L-glutamine, 2 U/ml penicillin and 2 mg/ml streptomycin at 37 °C in a humidity-controlled incubator with 10% CO_2. Recordings were performed 18–28 hours after transfection.

Isolation and culture of mouse dorsal root and trigeminal ganglion neurons

Dorsal root ganglion (DRG) and trigeminal (TG) neurons from wild-type C57Bl/6J mice (8–12 weeks, males), as previously described (32). Briefly, the ganglia were dissected, washed in Neurobasal A medium with 10% fetal calf serum, and digested for 45 minutes in medium containing collagenase 1 mg/mL (Gibco, Thermo Fisher Scientific Waltham, Massachusetts, USA) and dispase 2.5 mg/mL (Gibco, Thermo Fisher Scientific). A single cell suspension of DRGs was made using syringes with increasing needle gauges. The neurons were seeded and incubated on poly-l-ornithine/laminin pre-coated chambers (Fluorodish; WPI, Hertfordshire, UK) for 12–18 h at 37 °C in Neurobasal A medium (Invitrogen, Thermo Fisher Scientific) supplemented with glial-derived neurotrophic factor (2 ng/mL, Invitrogen, Thermo Fisher Scientific) and neutrophin-4 (10 ng/mL, PeproTech, Rocky Hill, NJ, USA).

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Table 1: Patient characteristics

	MP29-02 (n=20)	Placebo (n=8)
Age	22.0 (20.3-26.3)	25.5 (20.3-34.8)
Gender (M/F)	8/12	3/5
HDM (mono/poly sensitized)	4/16	6/2
Baseline VAS score	7.0 (5.8-8.7)	6.4 (5.3-7.3)
Baseline PNIF decrease upon CDA exposure	36.4% (26.8-52.5)	37.5% (36.9-44.4)
Major symptoms at baseline:		
- Congestion	6	2
- Sneezing	5	-
- Rhinorrhea	3	4
- Itchy nose	5	1
- Itchy eyes	1	1

Data are shown as median and interquartile range

Table 2: PNIF decrease (%) after CDA provocation before and after treatment

	V0	V1	V2
MP29-02 (n=20)	36.4% (21.4-55.0)	10.9% (0.0-19.0)***	13.6% (6.3-29.0)***
Placebo (n=8)	36.9% (27.7-47.2)	31.3% (11.2-41.0)	24.3% (12.4-33.3)

Data are shown as median and inter quartile range
V0: Baseline, V1: after 7 d, V2: after 28 d
***: $p<0.0001$ compared to V0

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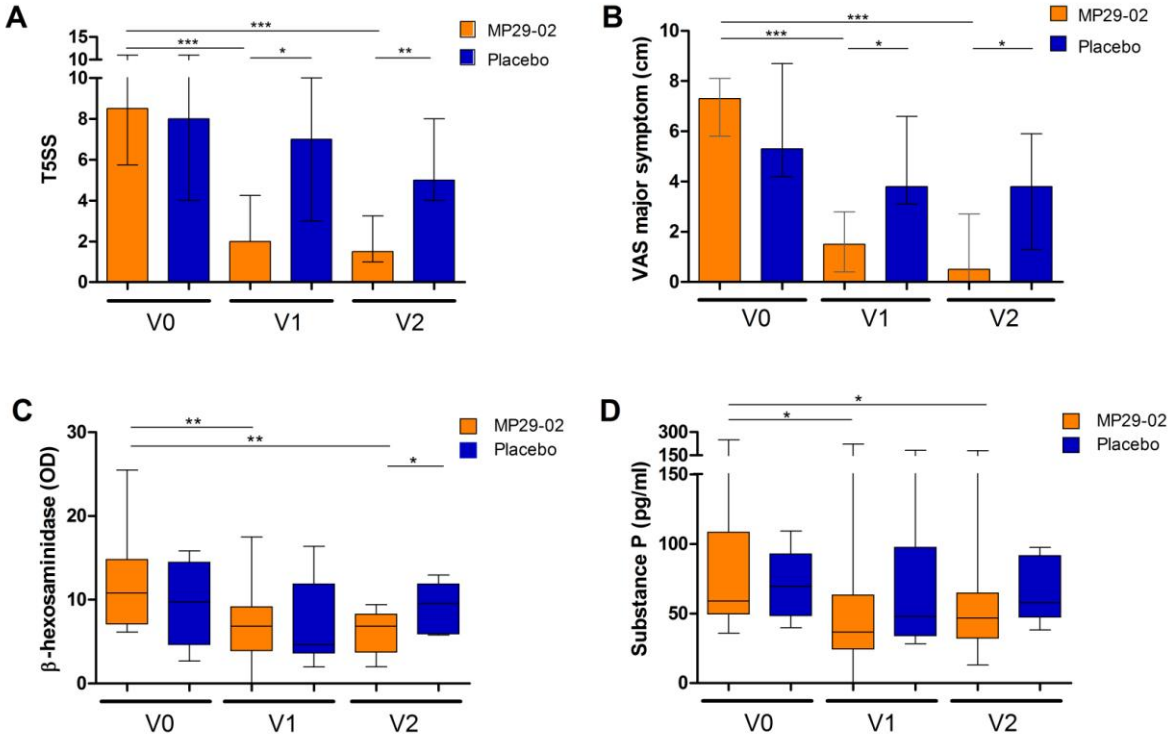

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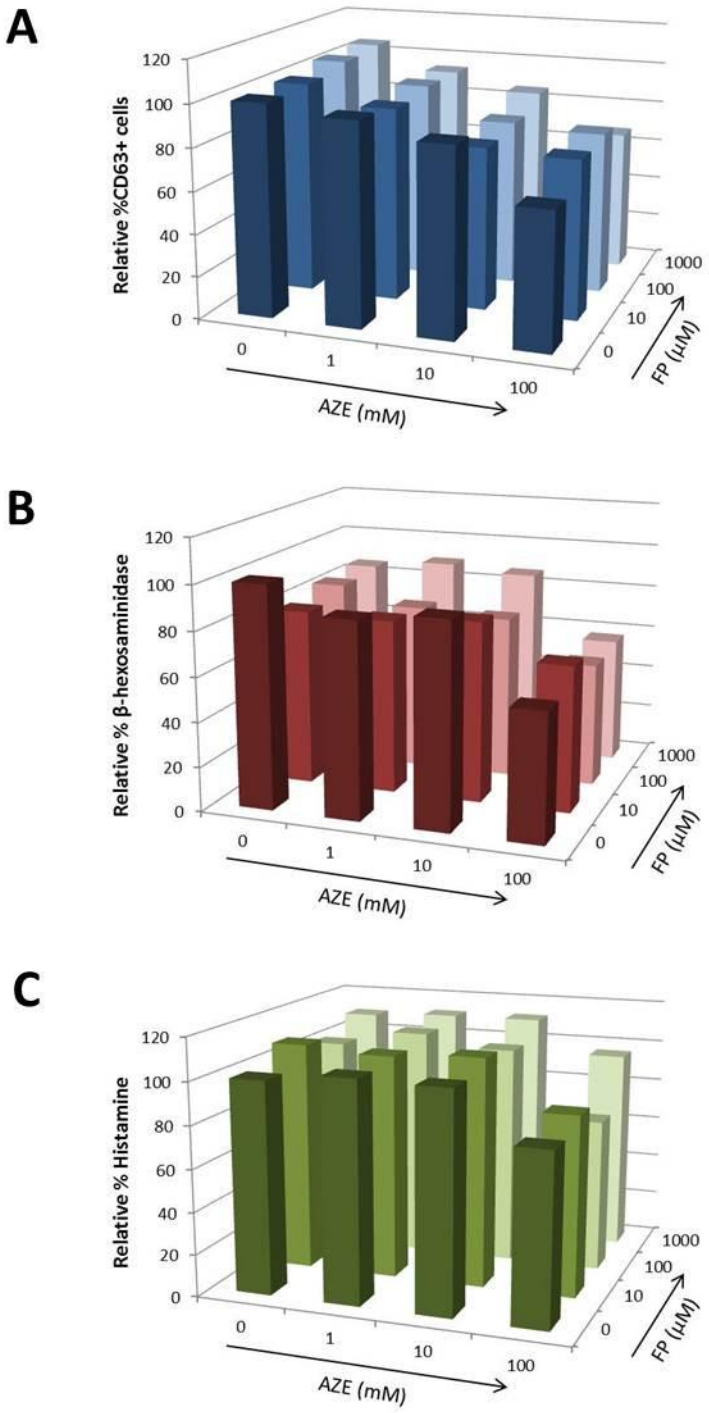

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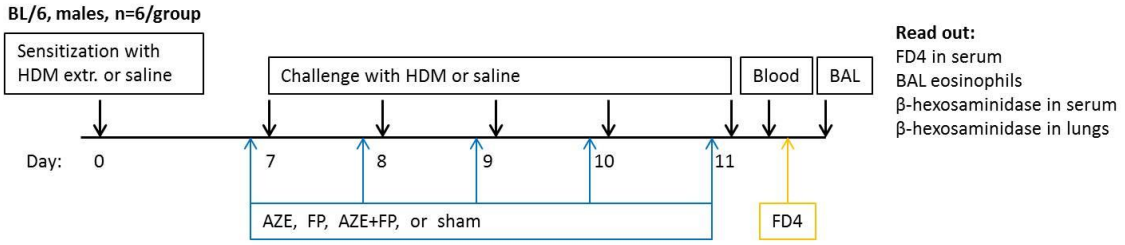

Group	Sensitization	Treatment	Challenge
I	Saline	Sham	Saline
II	HDM	Sham	HDM
III	HDM	AZE	HDM
IV	HDM	FP	HDM
V	HDM	AZE+FP	HDM

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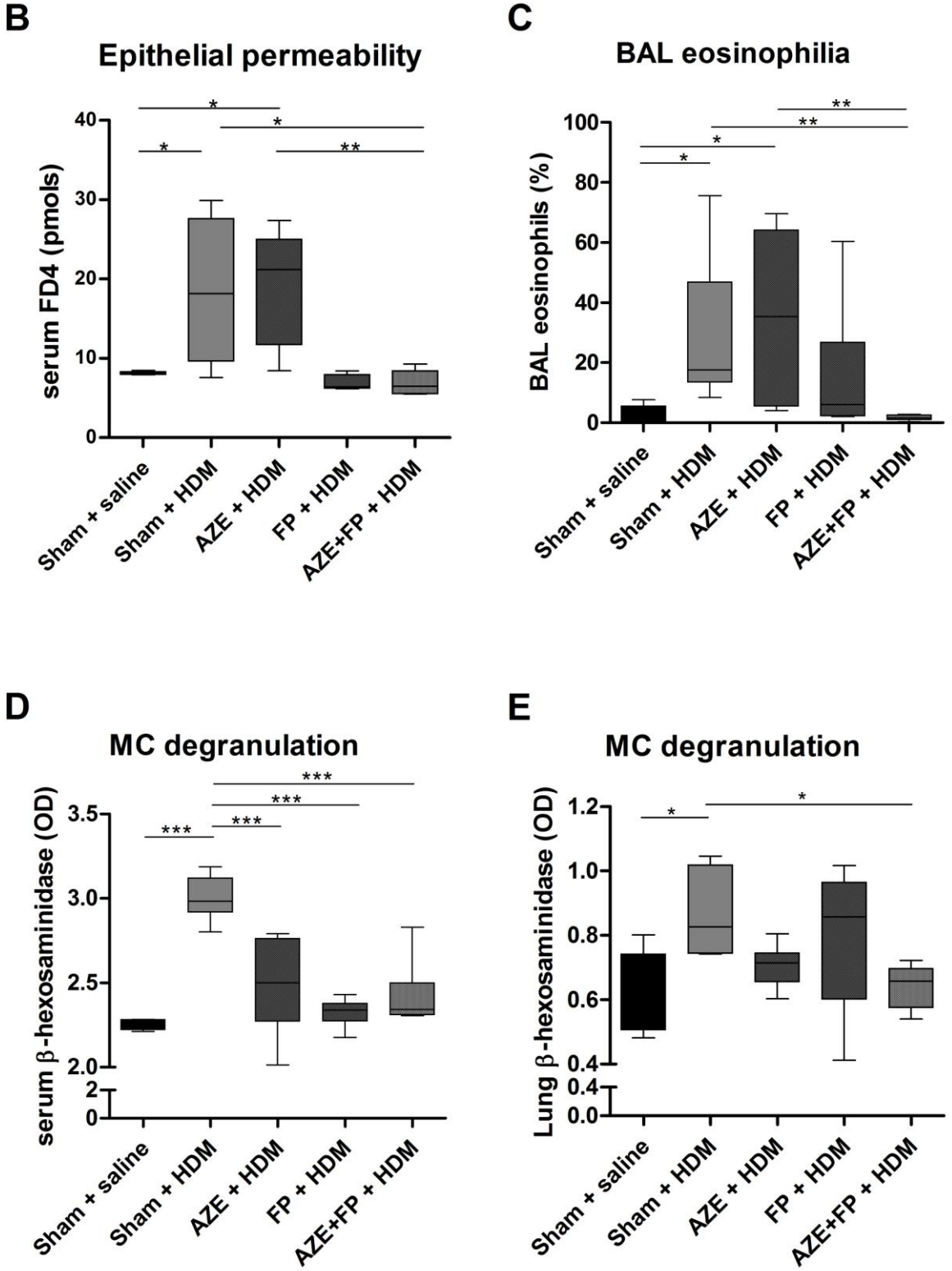

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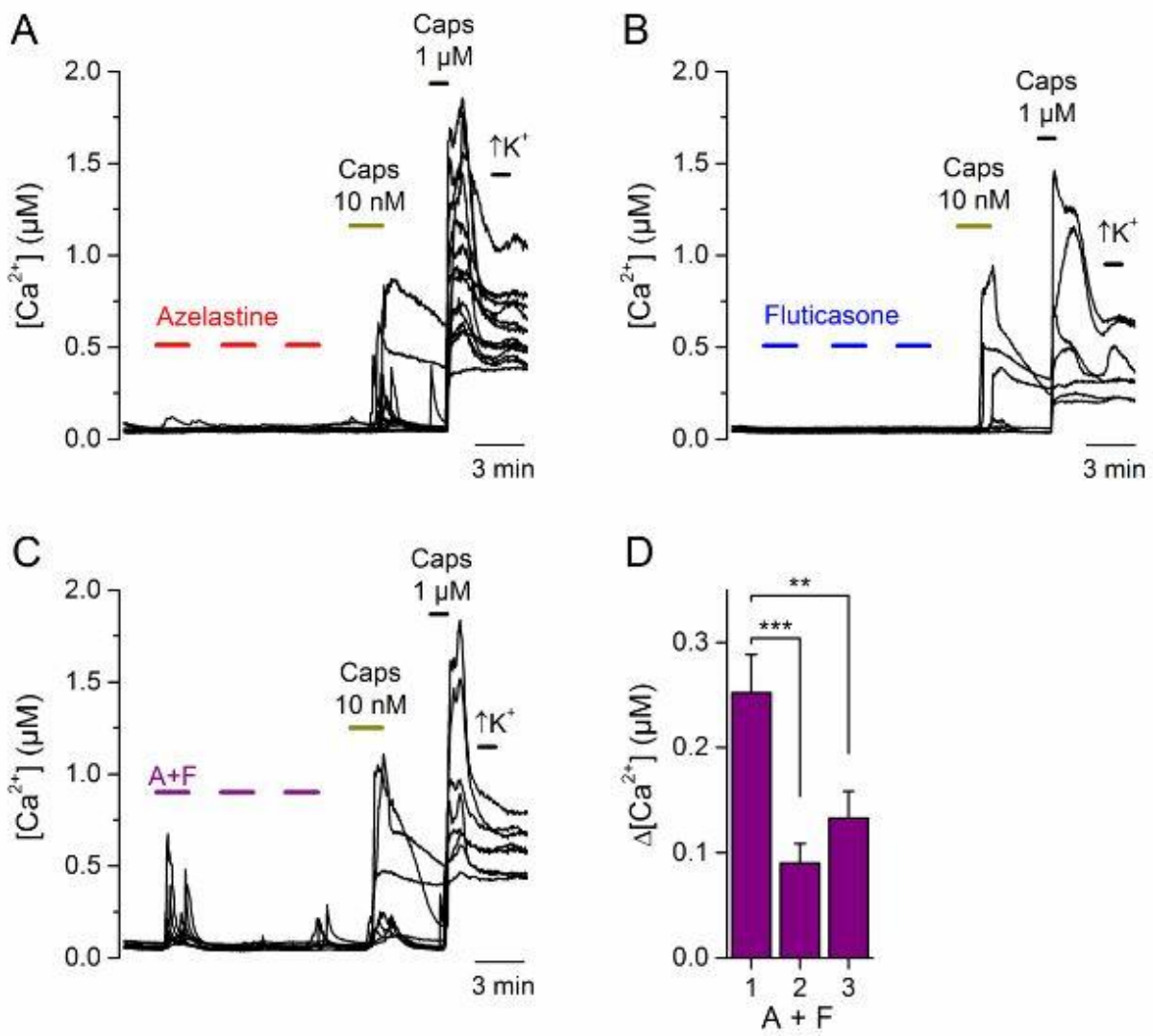

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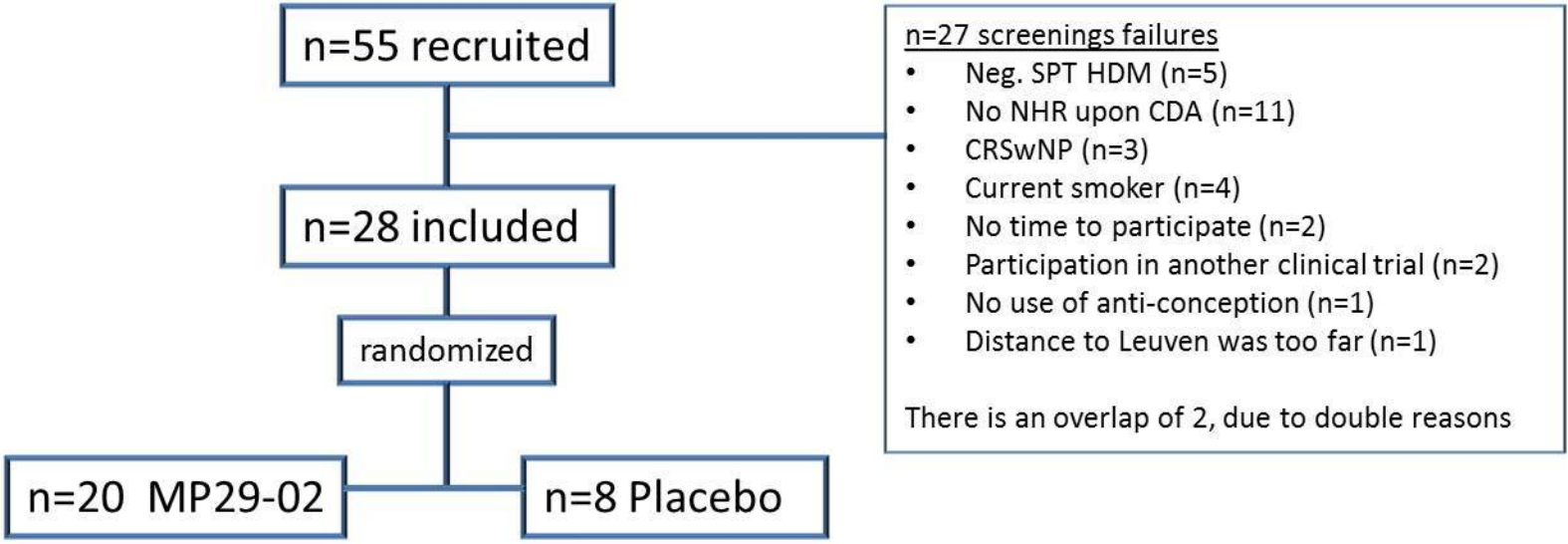
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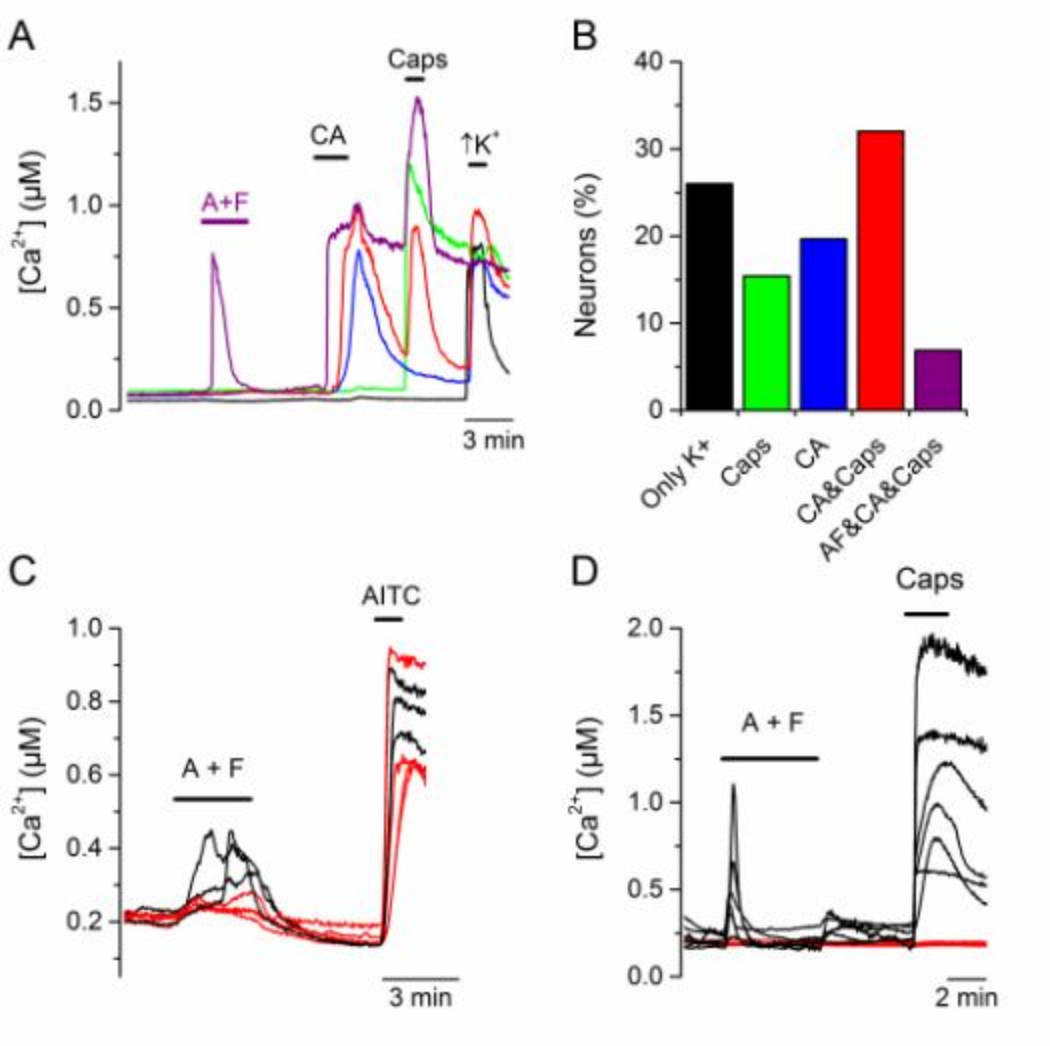
n=8 Placebo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Data S1: Methods section in this article's Online Repository:

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