JM25-1, a Lidocaine Analog Combining Airway Relaxant and Antiinflammatory Properties

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ABSTRACT

Background: Inhaled lidocaine antagonized bronchospasm in animal models and patients, but adverse effects limited its efficacy. This study evaluated the antibronchospasm potential of the analog JM25-1, exploring in vitro mechanisms and translation to an animal model.

Methods: The effectiveness of JM25-1 was assessed in GH₃ cells, rat tracheal rings, mouse lymphocytes, and human eosinophil systems in vitro, assessing changes in Na⁺ current, contraction, proliferation, and survival, respectively. Lung function and inflammatory changes were studied in ovalbumin-sensitized mice.

Results: The efficacy of JM25-1 was higher than lidocaine in inhibiting carbachol-induced and calcium-induced tracheal contractions (maximum effect inhibition at 1 mM [%]: 67 ± 10 [JM25-1] vs. 41 ± 11 [lidocaine] [P < 0.001] for carbachol; 100 ± 3 [JM25-1] vs. 36 ± 26 [lidocaine] [P < 0.001] for Ca²⁺; mean ± SD; n = 9 each) but lower in Na⁺ current (50% inhibitory concentration = 151.5, n = 8 vs. 0.2 mM; n = 5; P < 0.001). JM25-1 also inhibited eosinophil survival (dead cells [%]: 65 ± 6; n = 4; P < 0.001 at 1 mM) and lymphocyte proliferation (cells in phase S + G₂ [%]: 94 ± 10; n = 6; P < 0.001) at 0.6 mM. Aerosolized JM25-1 (1%) decreased lung eosinophil numbers from 13.2 ± 2.4 to 1.7 ± 0.7 × 10⁴/μm² (n = 7; P < 0.001). Other parameters, including airway hyperreactivity, cytokines, mucus, and extracellular matrix deposition, were also sensitive to aerosolized JM25-1.

Conclusion: These findings highlight the potential of JM25-1, emphasizing its putative value in drug development for clinical conditions where there is bronchospasm. (ANESTHESIOLOGY 2016; 124:109-20)

Bronchospasm is a frequent life-threatening perioperative event during general anesthesia, which can be triggered by pharmacologic and mechanic factors, particularly in those pathological conditions where there is airway inflammation and hyperreactivity.¹ Lidocaine administered intravenously or via aerosol has been used to prevent irritant-induced bronchospasm during anesthesia in patients and animal models.²,³ However, in patients with a reactive airway disease such as asthma, aerosolized lidocaine can itself induce airway irritation and initial bronchoconstriction, indicating that caution in its use is required.¹ When the airway effects of lidocaine and dyclonine (a lidocaine analog displaying longer-lasting and more intense local anesthetic activity) were compared in volunteers with bronchial hyperreactivity, it became clear that the attenuation of bronchospasm is independent of topical airway anesthesia because only lidocaine prevents bronchospasm in response to airway instrumentation. However, aerosolized lidocaine can also provoke bronchospasm by blocking sodium channels and attenuating bronchodilator neurogenic reflexes.

JM25-1, a novel lidocaine analog with limited sodium channel blockade, prevents airway hyperreactivity.

The mechanism of action of JM25-1 and its efficacy in reducing bronchospasm in an experimental model of pulmonary inflammation were evaluated.

What We Already Know about This Topic

- Lidocaine prevents bronchospasm in response to airway instrumentation. However, aerosolized lidocaine can also provoke bronchospasm by blocking sodium channels and attenuating bronchodilator neurogenic reflexes.
- JM25-1, a novel lidocaine analog with limited sodium channel blockade, prevents airway hyperreactivity.
- The mechanism of action of JM25-1 and its efficacy in reducing bronchospasm in an experimental model of pulmonary inflammation were evaluated.

What This Article Tells Us That Is New

- In comparison to lidocaine, JM25-1 was more effective in reducing bronchial smooth muscle constriction, airway hyperreactivity, lung inflammation, and peribronchial fibrosis.
- These data lend support for further investigation of the therapeutic potential of JM25-1 for the treatment of bronchospasm.
inhibited histamine-induced bronchoconstriction, whereas dyclonine showed a more intense irritant response.\(^5\)

Lidocaine acts as local anesthetic by blocking voltage-sensitive Na\(^+\) channels, which are responsible for Na\(^+\) permeability in excitable cells such as neurons and smooth muscle cells.\(^6\) Lidocaine is also known by its ability to inhibit the function of several inflammatory cells, including eosinophils and lymphocytes,\(^7\) paving the way for therapeutic uses to control chronic pulmonary diseases such as asthma. Preclinical\(^8,9\) and clinical\(^10\) studies have pointed out the effectiveness of aerosolized lidocaine for moderate and severe asthma conditions although discrepancies in clinical findings have been reported.\(^15\) In addition, some patients were found non-compliant by displaying adverse side effects, such as clastophobic feelings and initial bronchospasm.\(^10\)

We previously demonstrated that aerosolized JMF2-1, which is a fluorinated lidocaine analog with reduced local anesthetic activity, prevented allergen-induced airway hyperreactivity and inhibited lung eosinophilia, in a mechanism associated with down-regulation of Th2 cytokines and T-cell function.\(^16\)–\(^18\) Because fluorinated anilines have the potential to cause adverse side effects, such as hemolytic anemia and DNA damage,\(^19,20\) we questioned whether JM25-1 (2-diethylamino-N-2,5-dimethylphenyl acetamide), a non-fluorinated lidocaine analog with limited anesthetic activity,\(^16\) could inhibit bronchospasm and airway inflammation, and if so what might be the mechanism implicated. Thus, we hypothesized that nebulized JM25-1 is an alternative to lidocaine without the anesthetic effect.

Accordingly, we demonstrated that JM25-1 is indeed a weak inhibitor of Na\(^+\) channels, but it antagonizes tracheal contraction triggered by distinct stimuli, and increases intracellular cyclic adenosine monophosphate (cAMP) levels in cultured smooth muscle cells. The effectiveness of this innovative compound was also attested in in vitro settings of eosinophil and lymphocyte functions as well as in an animal model in which airway inflammatory changes and respiratory mechanics were assessed.

**Materials and Methods**

All the procedures involving care and use of laboratory animals in this study were examined and approved by the Animal Ethics Committee of the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil (license number: CEUA L-034/09). Human peripheral blood was obtained with informed consent from healthy donors under protocols approved by the Clementino Fraga Filho Hospital Committee on Clinical Investigation, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil (protocol: 190/09).

**GH3 Whole Cell Voltage Clamp Experiments**

Rat clonal pituitary GH\(_3\) cells were cultured in Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) and plated on a glass sheet at 37°C in a 5% CO\(_2\) humidified atmosphere. Ion channel currents in GH\(_3\) cells were recorded by patch clamp.\(^21\) The procedure applied to promote Na\(^+\) channel opening was similar to that described in the Materials and Methods section.\(^17\)

**Isolated Tracheal Preparation and Measurement of Tension**

The responses to carbachol of tracheal ring segments from naïve Wistar rats (180 to 250 g) were performed as described previously.\(^22\) In brief, tissues were allowed to stabilize for 60 min, whereas the bathing solution was exchanged at 10-min intervals. At the end of the equilibration period, the response to carbachol (2.5 × 10\(^{-6}\) M) was recorded. Contractions were measured isometrically with a force-displacement transducer (Ugo Basile, Italy) and recorded by an Isolated Organs Data Acquisition program (Proto5; Letica Scientific Instruments, Spain). After carbachol washout and reestablishment of stable baseline tone, tissues were exposed to either carbachol (10\(^{-9}\) to 10\(^{-3}\) M) or calcium (10\(^{-3}\) to 10\(^{-1}\) mM) in the presence or absence of 1 mM lidocaine or 1 mM JM25-1. To further investigate the mechanism of action of JM25-1, the tracheal rings were pretreated 10 min before its application with 0.1 mM SQ22,536 (adenylate cyclase inhibitor).

For measurement of tension development after increasing Ca\(^{2+}\) extracellular concentration during high K\(^+\) depolarization, tissues were exposed to successive cycles of 100 mM KCl stimulations/washouts in Ca\(^{2+}\)-free Krebs solution containing 2 mM ethylene glycol bis(b-aminoethylether)-N,N,N,N-tetraacetic acid until complete desensitization to the 100 mM KCl-evoked contractile response. Next, tracheal rings were immersed in Ca\(^{2+}\)-free Krebs solution containing 100 mM KCl, and the extracellular Ca\(^{2+}\) concentration was step-wise increased by the cumulative addition of Ca\(^{2+}\) (10\(^{-3}\) to 10\(^{-1}\) mM) in the presence or absence of either lidocaine or JM25-1 (1 mM). The preparations were preincubated with tested compounds 20 min before addition of the spasmodic agents. All responses were expressed as percentage of response to 2.5 × 10\(^{-6}\) M carbachol.

**Measurement of Intracellular cAMP Levels in Airway Smooth Muscle Cells**

Smooth muscle cells obtained from guinea pig tracheas were used to measure intracellular cAMP. After the third cell splitting, 10\(^6\) cells per well were grown in 24-well plates. At confluence, monolayer cells were washed with phosphate-buffered saline and incubated with JM25-1 (0.1 to 1 mM) or 0.1 mM forskolin (adenylate cyclase activator) in the presence or absence of 0.1 mM SQ22,536 for 30 min. All incubations were done in the presence of 0.1 mM for 3-isobutyl-1-methylxanthine (Sigma-Aldrich), a phosphodiesterase inhibitor. Lysed cells were collected and the cAMP was determined by using a radioimmunoassay kit (TRK 432–cyclic AMP[\(^{3}H]\) Biotrak assay system; Amersham Pharmacia Biotech, United Kingdom).\(^18\)
Ovalbumin Sensitization, Challenge, and Treatments
Four-week-old (weighing 18 to 20 g) A/J mice were kept in the animal housing facilities at a controlled room temperature (22° to 25°C) and a 12-h (6 AM to 6 PM) light–dark cycle. The sample size (N = 6 to 7 per group) was based on our previous experience with this model. Mice were sensitized on day 0 and boosted 14 days later by a dorsal subcutaneous injection (200 μl) of a mixture containing 50 μg of ovalbumin (grade V; Sigma-Aldrich, USA) adsorbed to 5 mg of Al(OH)3 in sterile 0.9% NaCl (saline). Animals were challenged via intranasal instillation of either ovalbumin (25 μg/25 μl) or vehicle (saline) on days 19 and 20 post sensitization under halothane volatile anesthesia (Cristália, Brazil). A custom-designed dosing chamber was used to deliver JM25-1 (0.25 to 1%) or vehicle (saline) to the animals just after allergen challenge as described in the Materials and Methods section.8

Invasive Assessment of Respiratory Mechanics
Tracheostomized mice were mechanically ventilated, and transpulmonary resistance and compliance were assessed 24 h after the last ovalbumin challenge by using invasive whole-body plethysmography (Buxco Electronics, USA).8 Mice were allowed to stabilize for 5 min and increasing concentrations of methacholine (3, 9, and 27 mg/ml) were aerosolized for 5 min each. Baseline pulmonary mechanics parameters were assessed with aerosolized phosphate-buffered saline.

Measurement of Pulmonary Inflammation, Mucus Production, and Peribronchiolar Fibrosis
Four-micrometer-thick lung sections were stained for eosinophils and neutrophils, mucus secretion, or extracellular matrix deposits as described in the Materials and Methods section.8 The slides were coded and the analyses were carried out in blind manner. The evaluations were made in an image analyzer system (Image-Pro® Plus, 4.1; Media Cybernetics, USA) using digitalized images obtained from a light microscope at a magnification of ×400.

Quantification of Cytokines and Eotaxin
The right lung lobes were removed, immediately frozen in liquid nitrogen, and stored at −80°C. Measurement of cytokine and chemokine proteins in whole-lung homogenates was performed by using commercially available enzyme-linked immunosorbent assay kits (interleukin [IL]-4, IL-5, and IL-13; DuoSet; R&D Systems, USA) and eotaxin-1 (R&D Systems). Results were expressed in picograms of cytokine per milligram of lung tissue.

Analyses of Apoptosis and T-cell Proliferation by Flow Cytometry
Pooled cervical, axial, and inguinal lymph node cells (106 per well) from A/J mice were stimulated with anti-CD3 (10 μg/ml) (BD Biosciences Pharmingen, USA) and treated with JM25-1 (0.1 to 0.6 mM) for 72 h (37°C aerated with 5% CO2). DNA content of retrieved cells was assessed as described in the Materials and Methods section.23 Analyses were performed by using CellQuest software (BD Biosciences PharMingen).

Purification and Analyses of Human Eosinophil Survival by Flow Cytometry
Blood eosinophils were isolated from healthy donors as previously described.24 The cells were then cultured in the presence of recombinant human IL-5 (30 ng/ml) (R&D Systems) and lidocaine (0.1 to 1 mM) or JM25-1 (0.1 to 1 mM) for 3 days followed by the addition of fluorescein isothiocyanate–labeled annexin V (1 μg/ml; BD Biosciences Pharmingen) and propidium iodide (1 μg/ml). Cells were immediately analyzed on the flow cytometer (FACScan with Cell Quest Software; BD Bioscience Pharmingen).

Western Blotting Analyses
After isolation, eosinophils were resuspended in 250 μl of Roswell Park Memorial Institute plus ovalbumin 0.1% (0.25 × 106 cells per sample). Thus, the cells were pretreated for 15 min with lidocaine (0.1 to 1 mM) or JM25-1 (0.1 to 1 mM) and stimulated by eotaxin (100 ng/ml) (R&D Systems) for 1 min. Eosinophil pellets were lysed in a 0.5% hexadecyl trimethylammonium bromide (Sigma-Aldrich) Tris buffer (10 mM, pH 7.4) containing 100 mM NaCl, 1 mM EGTA, 1 mM sodium fluoride, 20 mM Na2HPO4, 2 mM Na3VO4, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor (P8340, 1:100; Sigma-Aldrich), and phosphatase inhibitor cocktails (P2850 and P5726, 1:100; Sigma-Aldrich). Lysates were loaded on 10% Bis-Tris gels (Invitrogen, Brazil) under denaturing conditions. Gels were transferred to nitrocellulose membranes (Pierce, USA), blocked overnight with 5% bovine serum albumin, and probed with rabbit anti-human phospho-p38 mitogen activated protein kinase (MAPK) polyclonal antibody that detects phosphorylated Thr-180 and Tyr-182 (1:200 dilution, 9211; Cell Signaling, USA). Membranes were reprobed with rabbit anti-human p38 MAPK polyclonal antibodies; 1:500 dilution (9212; Cell Signaling). Goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase; 1:15,000 dilution (Jackson ImmunoResearch, USA) were used. For Western blotting analyses regarding the in vivo assays, lung-homogenized samples with equal protein concentration (50 μg) were separated on 10% sodium dodecyl sulfate gel electrophoresis and electroblotted onto nitrocellulose membranes. Membranes were blocked overnight with 5% nonfat dry milk and incubated with the primary antibody (anti-GATA-3, 1:1,000 dilution, sc-268 [Santa Cruz Biotechnology, USA]), or phosphor-p38 [1:1,000 dilution; 4515S; Cell Signaling] and β-actin [1:1,000 dilution, sc-477778; Santa Cruz Biotechnology]) for 2 h. Membranes were then incubated with horseradish peroxidase–conjugated goat anti-mouse immunoglobulin G (at 1:3,000; Calbiochem, USA) for 1 h at room temperature. Protein bands were detected by using an electrochemiluminescence plus Western blotting detection system. Western blotting images were analyzed by densitometry using
Gelplot analysis macros in Scion Image Software, version 4.03 (Scion Corporation, USA).

**Statistical Analysis**

Values were shown as mean ± SD or median and interquartile range. Statistical analyses were performed by using GraphPad Prism Software, version 5.0 (USA). Concerning normally distributed data, analyses were done with one-way ANOVA followed by the Student–Newman–Keuls test or two-way ANOVA with post hoc Bonferroni correction. When variables were nonnormally distributed, data were analyzed by using a nonparametric Kruskal–Wallis test with a post hoc Dunn test. Statistical differences were considered significant if P values were less than 0.05 (two-tailed tests). Concentration–response curves were fitted by nonlinear regression using log (inhibitor) versus response–variable slope for patch clamp data and nonlinear regression using log (agonist) versus response–variable slope for tracheal ring contraction data.

**Results**

**JM25-1 Potency in Inhibiting Na+ Channels Is Attenuated When Compared with Lidocaine**

To compare the effectiveness of lidocaine and JM25-1 (chemical structures shown in fig. 1A) in blocking voltage-gated Na+ channels, we performed a patch clamp analysis by using the GH3 cell lineage as target. As expected, a blockade of Na+ currents was observed after exposure to increasing concentrations of lidocaine in the range from 10−5 to 10−3 M, with a 50% inhibitory concentration (IC50) of 0.2 mM. Higher concentrations of JM25-1 were required for blockade of Na+ currents in GH3 cells, resulting in a 758-fold higher IC50 value for the analog (IC50 = 151.5 mM) (fig. 1B).

**Effectiveness of JM25-1 Is Greater Than That of Lidocaine in Inhibiting Rat Tracheal Contractions**

We noted that both lidocaine and JM25-1 inhibited tracheal contraction triggered by cumulative addition of increasing concentrations of carbachol (fig. 2A). As shown in figure 2B, area under the curve (AUC) values for the carbachol concentration–response curves reduced from 374 ± 66 (control) to 190 ± 49 (1 mM lidocaine) (P < 0.001) and 109 ± 34 (1 mM JM25-1) (P < 0.001) (n = 9; mean ± SD). As shown in figure 2C, lidocaine and JM25-1 also inhibited Ca2+-induced tracheal contraction. AUC values for the Ca2+ concentration–response curves reduced from 128 ± 38 (control) to 77 ± 27 (1 mM lidocaine) (P < 0.001) and 8 ± 3 (1 mM JM25-1) (P < 0.001) (n = 9; mean ± SD), as indicated in figure 2D.

**The Adenylyl Cyclase Inhibitor SQ22,536 Attenuates JM25-1 Effects on Tracheal Smooth Muscle Cells**

Preincubation of the adenylyl cyclase inhibitor SQ22,536 (0.1 mM) did not alter carbachol-induced tracheal contraction.
but attenuated the protective effect evidenced by 1 mM JM25-1 (fig. 3A). As shown in figure 3B, AUC values increased from 118 ± 34 (carbachol + JM25-1) to 169 ± 20 (carbachol + JM25-1 + SQ22,536) (\(P < 0.001\)) (n = 7, each group).

We then investigated a putative action of JM25-1 on intracellular levels of cAMP, a signaling pathway known to be involved in smooth muscle relaxation. As shown in figure 3C, JM25-1 concentration-dependently (0.01 to 1 mM) increased intracellular cAMP levels in cultured tracheal smooth muscle cells, achieving at the highest concentration values comparable to that of 0.1 mM forskolin, a known adenylyl cyclase activator. In addition, SQ22,536 inhibited the up-regulation of cAMP levels triggered by JM25-1 (fig. 3C).

**Aerosolized JM25-1 Inhibits Allergen-induced Airway Hyperreactivity**

In attempt to investigate whether the smooth muscle relaxant effects of JM25-1 noted in vitro could be translated into in vivo settings, we performed invasive measurements of airway resistance and lung dynamic compliance responses to inhaled methacholine 24 h after the last ovalbumin provocation. As shown in figure 4A, aerosolized JM25-1 (0.25 to 1%), once a day for 2 days, immediately after ovalbumin challenge, significantly inhibited the state of airway hyperreactivity mainly concerning lung resistance changes. For lung compliance changes, statistically significant values were obtained only for the lowest concentration of methacholine, despite the tendency to return to baseline levels in all doses of methacholine (fig. 4B).
To access the putative effect of aerosolized JM25-1 (0.25 to 1%) on lung inflammatory changes triggered by allergen provocation, we have quantified peribronchiolar eosinophil and neutrophil numbers 24 h after the last provocation. The histologic analysis of the leukocyte infiltrate revealed that the JM25-1 inhibited peribronchiolar eosinophil and neutrophil infiltration in a dose-dependent manner (fig. 5, A–E). It also inhibited allergen-induced mucus exacerbation (fig. 6A) and peribronchiolar collagen deposition (fig. 6B). In another setting of experiments, we demonstrated that aerosolized JM25-1 (0.25 to 1%) also inhibited the increased levels of eotaxin-1 (fig. 7A) and the Th2 cytokines IL-4 (fig. 7B), IL-5 (fig. 7C), and IL-13 (fig. 7D), in lung tissue homogenates of ovalbumin-challenged mice, in parallel with down-regulation of GATA-3 (fig. 7E) and phosphorylated p38 expression (fig. 7F).

**Aerosolized JM25-1 Inhibits Allergen-induced Inflammation, Mucus Production, and Fibrogenesis**

To access the putative effect of aerosolized JM25-1 (0.25 to 1%) on lung inflammatory changes triggered by allergen provocation, we have quantified peribronchiolar eosinophil and neutrophil numbers 24 h after the last provocation. The histologic analysis of the leukocyte infiltrate revealed that the JM25-1 inhibited peribronchiolar eosinophil and neutrophil infiltration in a dose-dependent manner (fig. 5, A–E). It also inhibited allergen-induced mucus exacerbation (fig. 6A) and peribronchiolar collagen deposition (fig. 6B). In another setting of experiments, we demonstrated that aerosolized JM25-1 (0.25 to 1%) also inhibited the increased levels of eotaxin-1 (fig. 7A) and the Th2 cytokines IL-4 (fig. 7B), IL-5 (fig. 7C), and IL-13 (fig. 7D), in lung tissue homogenates of ovalbumin-challenged mice, in parallel with down-regulation of GATA-3 (fig. 7E) and phosphorylated p38 expression (fig. 7F).
with propidium iodide, and analyzed by flow cytometry. Lymph node cells exposed to anti-CD3 provocation in the presence or absence of JM25-1 were permeabilized, stained with JM25-1 (0.1 to 1 mM concentration) and lidocaine (fig. 8B). As shown in the same figures, pretreatment with JM25-1 (0.1 to 0.6 mM) concentration-dependently decreased the proliferative response and increased the percentage of apoptotic cells stimulated by anti-CD3 in vitro.

**Discussion**

This study shows that JM25-1, a novel lidocaine analog with limited impact on Na+ channels, is more effective than lidocaine in inhibiting carbachol-induced and Ca2+-induced rat tracheal contraction in a mechanism, at least partially, mediated by increase in intracellular cAMP levels and the inhibition of Ca2+ influx. Furthermore, allergen-induced airway hyperreactivity, lung eosinophilic inflammation, mucus production, and peribronchial fibrosis can be prevented by aerosolized JM25-1 in actively sensitized mice.

The experiments reported here derive from earlier observations showing that lidocaine prevents bronchospasm caused by distinct stimuli, such as airway instrumentation for general anesthesia or bronchoscopy and many others.2,3 This effect is in part related to the fact that lidocaine can directly relax airway smooth muscle.26–28 However, this alternative application of local anesthetics has to be used with caution because anesthesia of the airways can cause airway irritation and initial bronchoconstriction mediated by blockade of bronchodilator neurogenic reflexes.2,4,29 The purpose of this study was to assess the potential of a novel lidocaine analog, JM25-1, to minimize anesthesia-related adverse effects while improving the antibronchospasm activity achievable with lidocaine.

JM25-1 Induces Apoptosis and Prevents Eotaxin-induced p38 MAPK Phosphorylation in Isolated Human Eosinophils

Because eosinophils are leukocytes notably associated with bronchial reactive diseases, we next evaluated the effect of JM25-1 on isolated human eosinophils. By flow cytometry, we found that JM25-1 (0.1 to 1 mM) concentration-dependently promoted eosinophil apoptosis (fig. 8A) as assessed after annexin V and propidium iodide labeling. The response was apparent at 0.1 mM but achieved significant levels at 0.6 mM and peaked at 1 mM JM25-1. In contrast, the population of apoptotic eosinophils after exposure to 1 mM lidocaine was lower than 20% (fig. 8B).

Then, we performed another set of experiments evaluating the ability of JM25-1 in preventing eotaxin-induced eosinophil activation. By Western blotting, we found that both JM25-1 (fig. 9A) and lidocaine (fig. 9B) inhibited the phosphorylation of p38 MAPK after eotaxin stimulation at 1 mM concentration.

**JM25-1 Induces Apoptosis and Inhibits Proliferation of T Cells In Vitro**

Lymph node cells exposed to anti-CD3 provocation in the presence or absence of JM25-1 were permeabilized, stained with propidium iodide, and analyzed by flow cytometry. As expected, stimulation of untreated cells with anti-CD3 monoclonal antibody increased the number of cells in the phase of DNA synthesis or replication (cells in S + G2) (fig. 10A) and decreased the apoptotic cell numbers (cells in sub-G0) (fig. 10B). As shown in the same figures, pretreatment with JM25-1 (0.1 to 0.6 mM) concentration-dependently decreased the proliferative response and increased the percentage of apoptotic cells stimulated by anti-CD3 in vitro.
It is well established that increased cAMP is closely associated with smooth muscle relaxation. Lidocaine failed to augment intracellular levels of cAMP, but it attenuates muscarinic receptor-mediated inhibition of adenyl cyclase in airway smooth muscle. Because JM25-1 effect was attenuated when tracheal rings were pretreated with the adenylate cyclase inhibitor SQ22,536, we next investigated a likely up-regulation by JM25-1 of intracellular cAMP levels in smooth muscle cells. Differently from lidocaine, JM25-1 increased the intracellular basal levels of cAMP in smooth muscle cells derived from guinea pig trachea. In addition, the phenomenon was markedly inhibited by coincubation with SQ22,536. These findings support the interpretation that the anti-spasmodic effect of JM25-1 is probably accounted for by at least two distinct mechanisms: increase in intracellular cAMP levels and Ca\(^{2+}\) influx impairment in respiratory smooth muscle cells.

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**Fig. 5.** Nebulized JM25-1 reduces allergen-induced pulmonary inflammation in A/J mice sensitized and challenged with ovalbumin (OVA). (A–C) Photomicrographs of paraffin-embedded Sirius Red–stained lung sections from ovalbumin-sensitized mice challenged with saline, challenged with ovalbumin, and challenged with ovalbumin and treated with 1% JM25-1, respectively. The number of eosinophils (D) and neutrophils (E) in peribroncholar regions was determined in lung sections by morphometric analyses. Black and white arrows indicate representative eosinophils and neutrophils, respectively. Ovalbumin-challenged mice were treated by exposure to an aerosol of JM25-1 (0.25 to 1%), and all samples for histologic examinations were undertaken 24 h after the last ovalbumin challenge. Data are expressed as mean ± SD (n = 7 for all groups). §P < 0.001 versus saline group, ***P < 0.001 versus ovalbumin-challenged group, one-way ANOVA followed by the Student–Newman–Keuls test. BR = bronchioles.

**Fig. 6.** Nebulized JM25-1 reduces airway remodeling caused by allergen-induced lung inflammation in mice. Quantitative assessment of mucus production (A) and fibrotic changes (B) was carried out in lung sections by morphometric analyses. All histologic examinations were undertaken 24 h after the last ovalbumin (OVA) challenge. Ovalbumin-challenged mice were treated by exposure to an aerosol of JM25-1 (0.25 to 1%), and all samples for histologic examinations were obtained 24 h after the last ovalbumin challenge. Data are expressed as mean ± SD (n = 7 for all groups). §P < 0.001 versus saline group, ***P < 0.001 versus ovalbumin-challenged group, one-way ANOVA followed by the Student–Newman–Keuls test. PAS = periodic acid–Schiff.
As an attempt to access whether the \textit{in vitro} potency and efficacy in down-regulating respiratory smooth muscle contraction could be translated into \textit{in vivo} settings, JM25-1 was tested by nebulization in a well-established animal model of allergic pulmonary inflammation.\textsuperscript{8} We showed that allergen-induced peribronchiolar eosinophil infiltration, mucus exacerbation, and collagen deposition were drastically impaired by nebulization of JM25-1. Airway hyperreactivity, a prominent clinical symptom in asthma, was also inhibited. Notably, the \textit{in vivo} efficacy of JM25-1 was very similar to that of lidocaine in the same animal model,\textsuperscript{8} despite the fact that the analog is much less effective in blocking Na\textsuperscript{+} channels and presented a markedly attenuated anesthetic activity.\textsuperscript{16} It is, therefore, quite unlikely that JM25-1 works by inhibiting neural regulation of allergic inflammation, the most obvious mechanistic hypothesis regarding the pharmacology of lidocaine.\textsuperscript{32,33}

In the literature, there are many pieces of evidence indicating that local anesthetics have indeed actions beyond
local anesthesia and Na⁺ channel blockade.²⁸,³⁴–³⁶ For instance, in vivo and in vitro investigations revealed that lidocaine can inhibit the expression of GATA-3,⁸ a pivotal transcription factor expressed in several immune-competent cells, necessary and sufficient for Th2 cytokine gene expression.³⁷ Similar to lidocaine, JM25-1 also reduced the expression of GATA-3 in lung tissue samples recovered from allergen-challenged mice, in line with decrease in the levels of several proinflammatory cytokines and chemokines after allergen provocation. In parallel, JM25-1 also reduced the expression of phosphorylated p38 in the lung of asthmatic mice. Notably, phosphorylation of p38 MAPK is a relevant event in the signaling cascade of eotaxin-induced eosinophil activation.³⁸,³⁹

We then examined the putative direct impact of JM25-1 on survival and activation of eosinophils and Th2

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**Fig. 8.** JM25-1 induced apoptosis of isolated human peripheral eosinophils. Eosinophils were cultured in the presence of interleukin-5 (30 ng/ml) for 72 h in the absence or presence of lidocaine or JM25-1. Column scatter graphs represent the percentage of cells in apoptosis. Eosinophils were treated with JM25-1 at 0.1 to 1 mM (A) or lidocaine (B). Horizontal lines represent the mean value and the open circles represent the median (n = 4, four different eosinophil donors). Results were analyzed by Kruskal-Wallis test followed by the Dunn multiple comparison test. *P < 0.05, **P < 0.01, and ***P < 0.001 versus untreated group.

**Fig. 9.** JM25-1 prevented eotaxin-induced p38 mitogen activated protein kinase (MAPK) phosphorylation in human eosinophils. Isolated human peripheral eosinophils were pretreated or not with JM25-1 (0.1 to 1 mM) (A) or lidocaine (0.1 to 1 mM) (B) for 15 min and stimulated with eotaxin (100 ng/ml, 1 min). Cell lysates were analyzed by Western blotting for phosphorylated p38 MAPK expression. The membrane was reprobed with anti-p38 as the loading control, respectively (the images represent one of the three independent experiments with similar results). Column scatter graphs represent the densitometry analysis of the bands presented as relative intensity of phosphorylated p38 MAPK (pp38) with respect to total p38 MAPK (p38). Horizontal lines represent the mean values (n = 3, three different eosinophil donors). Results were analyzed by Kruskal-Wallis test followed by the Dunn multiple comparison test. §P < 0.05 versus untreated group, *P < 0.05 versus eotaxin-stimulated group not treated with JM25-1 or lidocaine.
cells. Apoptosis of eosinophils and Th2 lymphocytes is an endpoint that may promote resolution of inflammation in asthma and other bronchial reactive diseases. In prior studies, human eosinophil survival and activation were shown to be susceptible to lidocaine in vitro. In our conditions, the potency and efficacy of JM25-1 to induce apoptosis of human eosinophils were undoubtedly higher than that of lidocaine, whereas its ability to inhibit eosinophil-induced activation of p38 MAPK appeared to be comparable. Moreover, JM25-1 dose-dependently inhibited anti-CD3-induced proliferation of mouse T cells and pushed them to apoptosis, adding support to the interpretation that JM25-1 has indeed potential to benefit patients with lung inflammatory diseases such as asthma.

In conclusion, JM25-1, a lidocaine analog with limited impact on Na+ channels and beneficial effects on airway smooth muscle and lung inflammation, holds promising perspectives as an alternative for treating life-threatening conditions marked by airway hyperreactivity and bronchoconstriction.

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Competing Interests

Drs. Martins and Costa are coinventors in a patent addressing application of JM25-1 for asthma treatment. The other authors declare no competing interests.

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References


Fig. 10. Effect of JM25-1 on proliferation (A) and the subdiploid DNA (sub-G0/G1) content (B) of lymph node cells recovered from A/J mice subjected to in vitro stimulation with anti-CD3. The percentage of cells undergoing DNA fragmentation was determined by propidium iodide staining (flow cytometry) performed within 72 h after exposure to anti-CD3. Data are expressed as mean ± SD (n = 6). *P < 0.05 versus anti-CD3-stimulated but untreated cells, one-way ANOVA followed by the Student–Newman–Keuls test.