The TLR7 agonist imiquimod induces bronchodilation via a nonneuronal TLR7-independent mechanism: a possible role for quinoline in airway dilation

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1Division of ENT Diseases, CLINTEC, Karolinska Institutet, Stockholm, Sweden; 2Department of ENT Disease, Karolinska University Hospital, Stockholm, Sweden; Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden; and 4Institute for Environmental Medicine, Karolinska Institutet, Stockholm, Sweden

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Larsson OJ, Manson ML, Starkhammar M, Fuchs B, Adner M, Kumlien Geórén S, Cardell LO. The TLR7 agonist imiquimod induces bronchodilation via a nonneuronal TLR7-independent mechanism: a possible role for quinoline in airway dilation. Am J Physiol Lung Cell Mol Physiol 310:L1121–L1129, 2016. First published April 15, 2016; doi:10.1152/ajplung.00288.2015—Toll-like receptor (TLR) 7 agonists are of particular interest as novel studies have demonstrated that the prototypical agonist imiquimod (R-837) also harbors the ability to induce rapid airway relaxations in vitro, as well as in vivo (11, 22). This further highlights a role for the use of these agonists in the treatment of asthma. To date, relaxation has primarily been attributed to a TLR7-dependent release of neuronally derived nitric oxide (NO), but other additional mechanisms must be considered. The present study was designed to characterize this in depth.

MATERIALS AND METHODS

Animals. Male Dunkin-Hartley guinea pigs (250–750 g) were obtained from Harlan (Horst, The Netherlands). They were housed in groups of five in plastic cages with absorbent bedding in temperature and light-dark cycle (12:12 h) controlled rooms. Food and water were available ad libitum.

Animals were handled in accordance with the Federation for European Laboratory Animal Science Associations guidelines. All animal procedures were approved by the local ethics committee at Karolinska Institutet (Stockholm norra djurförsöksämne; ethical permit nos.: N44-12, N41-14, and N143-14). In total, 60 animals were used for this study.

In vitro pharmacology. Organ bath experiments with guinea pig trachea were performed as described previously (32, 33). Animals were killed by CO2 or an overdose of pentobarbital sodium (Apoteket, Stockholm, Sweden). The trachea was quickly removed and dissected free of surrounding connective tissue in Krebs-Henseleit buffer solution (composition in mM: NaCl 118.5, KCl 4.7, KH2PO4 1.2, MgCl2 1.2, CaCl2 2.5, NaHCO3 25, and d-glucose 11.1). Tracheal segments were cut along the cartilage into eight rings of equal length and mounted in 5-ml organ baths [or myographs for electric field stimulation (EFS) experiments] filled with Krebs-Henseleit buffer solution at 37°C, bubbled with carbon gas (5% CO2 in O2). Changes in smooth muscle force were detected using an isometric force-displacement transducer linked to a Grass polygraph. For denudation experiments, epithelium was removed by mechanical scraping with a scalpel and confirmed via microscopy.

Segments were equilibrated for 60 min, where the force was adjusted to 30 mN. Tracheal reactivity was assessed through the cumulative addition of histamine (0.1 mM to 0.1 mM), and, after a 30-min wash-out period, further pharmacological studies were conducted. Tracheal rings were precontracted with carbachol (100 mM). Once stable preconstrictions were obtained, segments were exposed to cumulative concentrations of imiquimod (0.1–100 μM; Invivogen, San Diego, CA), R-848 (0.1–300 μM; Invivogen), CL-264 (0.1–300 μM; Invivogen).
were cultured in smooth muscle cell growth medium supplemented with 5% fetal calf serum, 0.5 mg/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor, 5 μg/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, NY), and 0.25 μg/ml Fungizone (Gibco); they were maintained in a humidified chamber at 37°C with a constant supply of 5% CO2. Media and supplements were obtained from Promocell, unless otherwise specified. Before experiments, cells were growth arrested with serum-free media for 24 h. Cells were stained with the calcium indicator fluo-4 AM (3 μM, Molecular Probes, Invitrogen, San Diego, CA), and changes in mean fluorescence intensity over time were detected on an BD Accuri C6 Flow Cytometer (BD, San Jose, CA). Analysis was performed with FlowJo software (Tree Star, Ashland, OR).

Measurement of Ca2+ flux in human airway smooth muscle cells. Primary bronchial human airway smooth muscle cells (HASMCs) were obtained from Promocell (Heidelberg, Germany). Cells from four separate patients were used, between passages 4 and 6. Cells were cultured in smooth muscle cell growth medium supplemented with 5% fetal calf serum, 0.5 mg/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor, 5 μg/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, NY), and 0.25 μg/ml Fungizone (Gibco); they were maintained in a humidified chamber at 37°C with a constant supply of 5% CO2. Media and supplements were obtained from Promocell, unless otherwise specified. Before experiments, cells were growth arrested with serum-free media for 24 h. Cells were stained with the calcium indicator fluo-4 AM (3 μM, Molecular Probes, Invitrogen, San Diego, CA), and changes in mean fluorescence intensity over time were detected on an BD Accuri C6 Flow Cytometer (BD, San Jose, CA). Analysis was performed with FlowJo software (Tree Star, Ashland, OR).

In vivo studies. Guinea pigs were ventilated with a flexiVent animal ventilator (Scireq, Montreal, Quebec, Canada). Following anesthesia with fentanyl (500 μg/kg ip), midazolam (30 mg/kg ip), and droperidol (5 mg/kg ip) (all Apoteket), animals were placed on a heating pad (37°C), tracheostomized, and connected to the ventilator via a 16-gauge (2.1 mm) cannula. Guinea pigs were given repeated doses of anesthetic, at 50% of the original dose, every 40 min. Pulse and SO2 levels remained stable throughout the experiment. Airways were precontracted with a continuous infusion of histamine (7.5–12.5 μg/kg min1−1 iv), to produce an approximate fivefold increase in the basal lung resistance. Once a stable baseline had been reached, aerosolized distilled water (dH2O) or imiquimod was given. In concentration-response experiments, increasing concentrations of imiquimod, CL264 (10, 30, or 100 mg/kg iv in vivo) was used to study the involvement of TLR7. In vitro, 30 mg/kg in vivo) and IRS661 were purchased from Sigma Aldrich (St. Louis, MO). Imiquimod, R-848, and CL-264 were purchased from Invivogen. KT-5823 and H89 were purchased from Sigma Aldrich (St. Louis, MO). Moi- quimod, R-848, and CL-264 were purchased from Invivogen. KT-5823, H89, thapsigargin, and TTX were purchased from Tocris Bioscience (Bristol, UK). ONO-8130 was a kind gift from ONO Pharmaceuticals (Japan).

Drugs and materials. NaCl, KCl, KH2PO4, MgCl2, CaCl2, NaHCO3, and d-glucose were purchased from VWR (West Chester, PA). Carbachol, histamine, salbutamol, papavarine, sodium nitroprusside, L-NAME, L-NMMA, Rp-8-Br-PET-cGMPs, indomethacin, and IRS661 were purchased from Sigma Aldrich (St. Louis, MO). Moi- quimod, R-848, and CL-264 were purchased from Invivogen. KT-5823, H89, thapsigargin, and TTX were purchased from Tocris Bioscience (Bristol, UK). ONO-8130 was a kind gift from ONO Pharmaceuticals (Japan).

Statistics. Data was analyzed using GraphPad Prism 6 Software (San Diego, CA). All results are presented as means ± SE. For in vitro pharmacology experiments, the concentration-response curve values for pEC50 and Emax were calculated using nonlinear regression analysis. EC50 values were compared using Student’s t-test. In vivo and Ca2+ mobilization experiments were analyzed using a one-way or a two-way ANOVA (vagotomy), followed by Bonferroni post hoc tests.

RESULTS

Imiquimod relaxes precontracted guinea pig airways in vitro and in vivo. Cumulative addition of imiquimod in vitro resulted in concentration-dependent and rapid complete relaxation of tracheal rings precontracted with carbachol (Fig. 1A, pEC50 = 4.46 ± 0.05, EC50 = 34.8 μM), verifying previously published results (22).

In vivo studies, continuous intravenous infusion of histamine resulted in a stable and reproducible contraction 40–50 min after the start of infusion. Challenge with aerosolized imiquimod (0.3–30 mg/ml) resulted in a concentration-dependent relaxation of contraction, as measured by a reduction in Newtonian resistance (Rn; an approximation of resistance in the conducting airways, Fig. 1B, P < 0.0001) and tissue resistance (G; which reflects energy dissipation in peripheral lung tissue, Fig. 1C, P < 0.0001). A similar trend was evident for tissue elastance (H, a reflection of tissue stiffness in peripheral lung tissue), but did not reach significance (data not shown). Relaxation occurred within 10 s of administration; contraction returned to baseline levels after 10–15 min.

Administration of imiquimod alone had no effect on baseline in vitro or in vivo (data not shown).

Imiquimod relaxes precontracted guinea pig airways independently of the epithelium. The tracheal epithelium is known to release mediators involved in bronchodilation. In this light,
we assessed whether the epithelium was necessary for imiquimod-mediated relaxation. However, denudation of the tracheal epithelium had no significant effect on imiquimod-mediated relaxation (Table 1).

*Imiquimod relaxes precontracted guinea pig airways independently of conventional pathways of bronchodilation.* Using our in vitro and in vivo models, we next examined molecules and associated signaling pathways involved in bronchodilation, including the role of NO and prostanoids, previously implicated in imiquimod-mediated bronchodilation (11, 22), CO, cGMP, PKG, and PKA.

Administration of indomethacin before imiquimod administration in vitro resulted in a small, but significant, decrease in potency (Table 1, \( P = 0.021 \)), compared with administration of vehicle, which is in accordance with previous observations (22). Comparatively, exposure to L-NAME, L-NMMA (NO synthase inhibitors; Fig. 2A, Table 1), ZnPP9 [heme oxygenase 1 (HO-1) inhibitor], and KT-5823 (PKG inhibitor, Fig. 2B, Table 1) did not significantly alter imiquimod-mediated relaxation. Administration of H89 (PKA inhibitor) significantly reduced the potency of salbutamol-mediated relaxation (\( P = 0.014 \)), but had no effect on imiquimod-mediated relaxation of precontracted guinea pig airways in vitro (Table 1).

Intravenous administration of L-NAME in vivo resulted in a small, nonsignificant trend (\( P = 0.08 \)) toward a reversal of imiquimod (3 mg/ml)-induced reduction in \( R_N \), but had no effect on imiquimod-mediated relaxation of precontracted guinea pig airways in vivo. Aerosolized IMQ was added noncumulatively (22), CO, cGMP, PKG, and PKA.

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Imiquimod (IMQ) induces intracellular Ca\(^{2+}\) mobilization and blocks histamine-induced Ca\(^{2+}\) mobilization, in isolated HASMCs. The effect of imiquimod on intracellular Ca\(^{2+}\) mobilization ([Ca\(^{2+}\)]\(_i\)) is a prerequisite of airway smooth muscle (ASM) cell contraction, was subsequently measured, as the data indicated that imiquimod-mediated relaxation was not mediated by conventional bronchodilatory pathways. Surprisingly, addition of imiquimod to HASMC induced a reproducible increase in mean fluorescence intensity, an arbitrary measure of [Ca\(^{2+}\)], concentration (Fig. 5A). The increase in [Ca\(^{2+}\)], was significantly higher than that induced by histamine (Fig. 5, A and B). A similar rise in [Ca\(^{2+}\)], following imiquimod exposure was evident when extracellular calcium was removed (data not shown). Pretreatment with thapsigargin significantly blunted the imiquimod-induced rise in [Ca\(^{2+}\)], in HASMC (Fig. 5C); reciprocally, imiquimod inhibited thapsigargin-induced increases in [Ca\(^{2+}\)], (Fig. 5D), suggesting the endoplasmic reticulum (ER) to be a source of imiquimod-induced [Ca\(^{2+}\)]. To assess if the imiquimod-mediated rise in [Ca\(^{2+}\)], could impact the rise in [Ca\(^{2+}\)], following histamine, HASMC were preincubated with imiquimod for 5 min and subsequently exposed to histamine. Imiquimod preincubation resulted in a concentration-dependent reduction in [Ca\(^{2+}\)], following addition of histamine (P = 0.004) (Fig. 5, E and F), with a potency
similar to imiquimod-mediated bronchorelaxation in vitro (pEC50 = 4.68 ± 0.08).

**Imiquimod-induced relaxation is not dependent on TLR7.** The role of TLR7 on imiquimod-induced airway relaxation was subsequently examined, using the TLR7 antagonist IRS661 and other structurally similar and dissimilar TLR7 agonists. Intravenous administration of IRS661 in vivo had no significant effect on imiquimod-mediated reduction in Rn (Fig. 6A) or G (Fig. 6B), when administered before imiquimod. Similarly, no significant difference in imiquimod-induced relaxation in IRS661-treated tracheal rings was found, compared with control (Fig. 6C).

As reported (22), R-848, a TLR7/8 agonist, and imidazoquinoline, with a similar chemical structure to R-837, induced a concentration-dependent relaxation of tracheal rings precontracted with carbachol, but with a lower potency. The structurally dissimilar TLR7 agonist CL264 (19) is a more potent activator of TLR7-specific immune events (10) (data not shown). However, CL264 did not relax precontracted guinea pig trachea (Fig. 6D) and had no effect on histamine-induced increases in [Ca2+]i (Fig. 6, E and F).

**DISCUSSION**

TLR7 ligands have shown preclinical and clinical success in the treatment of allergic disease, due to their ability to modulate and reduce allergic airway inflammation (16, 17, 37, 38). Their recently reported ability to rapidly relax airways (11, 22) has increased their interest in the treatment of pulmonary disease. The present study verified the ability for imiquimod to induce a strong and rapid dilatory effect in isolated guinea pig airways. It also, for the first time, demonstrated that imiquimod could directly relax airways precontracted with histamine in vivo. Epithelial denudation and TTX-mediated blockade of neuronal release ruled out the involvement of epithelium- or neuron-derived mediators. In addition, various pharmacological inhibitors further dismissed the involvement of traditional airway dilatory mechanisms, including NO, CO, and cAMP signaling. Further investigations in isolated ASM cells demonstrated that imiquimod induced a rise in ER-derived [Ca2+]i, which was associated with an inhibition [Ca2+]i, following histamine exposure. Bronchodilatory effects were not affected by TLR7 antagonism, and neither relaxation nor Ca2+ inhibition were evident in response to the structurally dissimilar TLR7 agonist CL264, signifying that this effect was independent of TLR7.

To investigate imiquimod-mediated bronchodilation in vivo, we developed a model based on our laboratory’s previous studies (5, 6) that allowed extensive evaluation of bronchodilators in guinea pigs in vivo. Animals were anesthetized with a novel triple combination anesthetic, which ensured stable surgical anesthesia for up to 4 h in ventilated animals. A continuous intravenous infusion of histamine resulted in a stable airway contraction, enabling the direct measurement of aerosolized bronchodilatory compounds. Using this in vivo setup, changes in Rn, G, and H could be measured. This approach allowed the dissection of changes in resistance in relation to both central (Rn) and peripheral (G and H) airways (31). Decreases in Rn, G, and H were evident within seconds following imiquimod administration, suggesting that imiquimod concentration-dependently diluted histamine-precontracted airways in both central and peripheral compartments. This study is the first to show that imiquimod can reverse established histamine-induced precontractions in vivo.

To study mechanisms of imiquimod-mediated bronchodilation, we evaluated the effect of epithelial denudation and pharmacological inhibition of conventional pathways of bronchodilation. Removal of the epithelium had no effect on relaxation, suggesting that epithelial derived-mediators were...
Fig. 5. Imiquimod (IMQ) induces intracellular calcium mobilization and blocks histamine-induced calcium mobilization. Isolated airway smooth muscle cells were stained with the Ca\(^{2+}\) indicator Fluo-4 and exposed to various compounds, and changes in mean fluorescence intensity (MFI) were determined using a BD Accuri C6 flow cytometer. A and B: cells were exposed to IMQ (100 \(\mu\)M) or histamine (His, 100 \(\mu\)M) (arrow). Values are percent change from baseline (A) or maximum percent change from baseline (B) \(\pm\) SE. ***\(p<0.0001\) using Student’s \(t\)-test \((n=5–15)\). C: cells were pretreated with thapsigargin (Th; 1 \(\mu\)M) or dH2O for 30 min and exposed to IMQ (100 \(\mu\)M, arrow). Values are MFI or maximum MFI \(\pm\) SE (inset). \(*p<0.05\) using a Student’s \(t\)-test \((n=3)\). D: cells were exposed to IMQ (100 \(\mu\)M) or dH2O (arrow) and subsequently exposed to Th (1 \(\mu\)M, arrow). Values are MFI. E and F: cells were preincubated with varying concentrations of IMQ for 5 min and subsequently exposed to His (100 \(\mu\)M, arrow). Values are percentage of maximum vehicle MFI \(\pm\) SE. **\(p<0.01\), ***\(p<0.001\) using one-way ANOVA followed by Bonferroni post-hoc test \((n=4–7)\).

not involved. Comparatively, the cyclooxygenase inhibitor indomethacin reduced the potency of imiquimod-mediated relaxations without affecting the maximal dilation. This is in accordance with previous observations (22) and suggests that ASM-derived prostanoids are involved, but are not crucial, for imiquimod-mediated effects. Further analysis revealed that the investigated imiquimod-mediated effects were completely independent of other conventional pathways of bronchodilation. CO, which is endogenously produced via the enzyme HO-1 (13), has been shown to dilate guinea pig airways precontracted with histamine in vivo, in a cGMP-dependent manner (5, 6). In vivo or in vitro inhibition of this pathway, through blockade of HO-1, cGMP synthase, or PKG, had no effect on imiquimod-mediated bronchodilation. Similarly, inhibition of PKA, an important effector molecule for cAMP signaling and \(\beta\)2-agonist-mediated bronchorelaxation (4), had no effect.

Vasoactive intestinal peptide and NO, mediators derived from iNANC nerve fibers, are known to potently relax guinea pig airways (18, 36). However, in vivo and in vitro inhibition of NO had no significant effect on imiquimod-induced relaxation, nor did bilateral vagotomy in vivo, which prevents reflex-induced bronchodilation (28, 29). In addition, neuronal blockade with TTX, which prevented EFS-induced relaxation and, as such, inhibited mediator release from iNANC fibers, had no effect on imiquimod-induced relaxation in vitro. These data indicate that neither neuron- or epithelium-derived mediators, nor CO or cAMP, are critical for imiquimod-mediated bronchodilation.

The results in this study demonstrate that imiquimod acts directly on the ASM and interferes with Ca\(^{2+}\) homeostasis. Exposure of ASM cells to imiquimod resulted in a sharp rise in [Ca\(^{2+}\)]\(i\), likely derived from ER stores. Imiquimod subsequently, significantly, and concentration-dependently inhibited histamine-induced mobilization of [Ca\(^{2+}\)]\(i\). The potencies by which imiquimod induced [Ca\(^{2+}\)]\(i\), release and inhibited histamine-induced [Ca\(^{2+}\)]\(i\), were similar to in vitro relaxation, suggesting that disruption of Ca\(^{2+}\) is of importance for bronchorelaxation. However, the mechanism by which a rise in [Ca\(^{2+}\)]\(i\) induces subsequent relaxation is unclear. Previous studies have demonstrated that imiquimod induces rises in [Ca\(^{2+}\)]\(i\) in sensory neurons (24), and PC12 and F11 cell lines (20), with the latter study highlighting a role for the inositol triphosphate (IP\(_3\)) receptor activation in this process. Contractions by histamine and carbachol, both of which were reversed by imiquimod, primarily rely on IP\(_3\) signaling and release of ER-derived Ca\(^{2+}\) to induce contraction (25). Imiquimod may disrupt this pathway either through binding to and blocking the IP\(_3\) receptor (19), or emptying of ER pools, as demonstrated in this study. Alternatively, it has previously been shown that localized increases in [Ca\(^{2+}\)]\(i\) in ASM can activate Ca\(^{2+}\) dependent potassium channels (e.g., BK\(_{Ca}\)), resulting in hyperpolarization and relaxation (9). However, previous studies have
reported minimal involvement of such channels in imiquimod-induced relaxation (22), making this less likely. Surprisingly, despite the sharp rise in \([Ca^{2+}]_i\), imiquimod had no effect on basal airway tone; however, the mechanism underpinning this dissociation is beyond the scope of this study. It remains to be determined how imiquimod disrupts \([Ca^{2+}]_i\) homeostasis and thus induces relaxation, but the results suggest it exerts its effect by acting directly on the ASM.

Imiquimod has previously been shown to upregulate cytokine and chemokine production and alter cell-surface marker expression on ASM, effects that were evident after 24 h (27). However, the speed at which imiquimod exerts its effect would suggest that the effect on ASM does not occur via traditional TLR-dependent pathways. This is corroborated by the finding that, in our models, imiquimod-mediated bronchodilation of guinea pig airways was not dependent on TLR7, as both in vitro and in vivo administration of the TLR7 antagonist IRS661 had no effect on imiquimod-mediated bronchodilation. This was true even when the antagonist was used at in vitro concentrations 100-fold higher than previously shown to be effective (27). Additionally, another TLR7 agonist, CL264, that we and others (7, 19) have shown to be more potent than imiquimod in terms of TLR7 agonism had no effect on precontracted airways or on histamine-induced \([Ca^{2+}]_i\) mobilization in ASM cells. Indeed, previous studies have demonstrated that the rise in \([Ca^{2+}]_i\), following imiquimod administration is independent of TLR7 (20, 24), substantiating that bronchorelaxation by imiquimod occurs predominantly independently of TLR7.

It is likely that the bronchorelaxatory effects of some, but not all, TLR7 agonists are a consequence of their unique chemical structures. Imiquimod and R-848, but not CL264,
quinine and chloroquine have been shown to inhibit calcium release by other agents (14, 30, 35). Indeed, both quinine and chloroquine relax pre-contracted mouse, guinea pig, and human airways, at similar potencies to imiquimod (9, 15, 26, 32) and have been shown to induce [Ca\(^{2+}\)] mobilization (9), as well as inhibit subsequent calcium release by other agents (14, 30, 35). Indeed, both quinine and chloroquine have been shown to inhibit [Ca\(^{2+}\)] mobilization primarily by blocking of IP\(_3\)-IP\(_3\) receptor interactions (30, 35), again raising the possibility that imiquimod may disrupt intracellular IP\(_3\)-mediated signaling pathways. Other studies have demonstrated that TLR7 agonists that lack the quinoline-moiety (loxoribine) do not induce a rise in [Ca\(^{2+}\)], (20, 24), further suggesting that quinoline may be of importance in TLR7-independent mechanisms of imiquimod. It is likely that other bronchodilating TLR7 agonists, such as CL097 and gardiquimod (22), which share similar structural components, dilate airways through similar mechanisms. Quinoline-dependent release and subsequent inhibition of [Ca\(^{2+}\)] responses represent a novel and potential pathway for imiquimod-mediated bronchodilation, independent of classical bronchodilatory mechanisms.

A subset of the data presented in this study can be perceived as lying in contrast to previously published reports (11, 22). In the cited studies, bronchodilation occurs at similar potencies and time frames, as seen in the present study, but instead occurs via a neuronal, NO-mediated and TLR7-dependent mechanism. The present findings do not dismiss these reports; instead, two mechanisms for imiquimod-mediated bronchorelaxation may conceivably exist, and their relative contribution is highly dependent on the experimental protocol employed. For example, the aforementioned studies induced precontraction mainly through neuronal stimulation, whereas our in vivo models induced ASM contraction through nonneuronal mechanisms. Neuronal NO was likely induced by imiquimod in our models, but could have been masked by the additional ability for imiquimod to inhibit histamine-induced Ca\(^{2+}\) mobilization in ASM. The present study supplies an additional and novel TLR7-independent, ASM-dependent mechanism for imiquimod-mediated bronchodilation.

In summary, the present study demonstrates that the prototypical TLR7 agonist imiquimod can directly and rapidly relax precontracted guinea airways in vitro and in vivo. These effects appear to be independent of the epithelium, neuronal activation, and conventional pathways of airway relaxation. Instead, imiquimod acts directly on the ASM and likely causes bronchorelaxation through disruption of Ca\(^{2+}\) homeostasis. This effect is independent of TLR7 and may instead be related to the presence of a quinoline structure. This highlights the potential use of drugs that combine the effects of quinoline with the anti-inflammatory action of TLRs in future treatment of inflammatory and hyperactive airway disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).


