

Dual Inhibition of Cathepsin G and Chymase Is Effective in Animal Models of Pulmonary Inflammation

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Rationale: Mast cells and neutrophils are key contributors to the pathophysiological inflammatory processes that underpin asthma and chronic obstructive pulmonary disease, partly through the release of noxious serine proteases, including cathepsin G (Cat G) and chymase. From this standpoint, a dual inhibitor of neutrophil Cat G and mast cell chymase could protect against these disease-related inflammatory responses.

Objectives: We examined the antiinflammatory pharmacology of RWJ-355871, a dual inhibitor of Cat G and chymase, in animal models of inflammation that evince pathophysiological pathways relevant to asthma and chronic obstructive pulmonary disease to determine the therapeutic potential of this compound.

Methods: In an ovalbumin (OVA)-sensitized rat model, RWJ-355871 was administered to block the mast-cell-mediated increase in paw volume caused by OVA injection. In a sheep asthma model, antigen-induced airway responses were assessed with and without aerosol treatment with RWJ-355871. In a murine tobacco-smoke model of airway inflammation, the effect of RWJ-355871 on smoke-induced neutrophilia was determined.

Measurements and Main Results: Intravenous treatment of OVA-sensitized rats with RWJ-355871 provided dose-dependent reduction in the increase in rat paw volume. In allergic sheep, aerosol pretreatment with RWJ-355871 showed dose-dependent inhibition of the antigen-induced early response, late response, and post-antigen-induced airway hyperresponsiveness. In tobacco-smoke-exposed mice, nebulized RWJ-355871 significantly reduced the smoke-induced neutrophilia from the levels observed in untreated mice.

Conclusions: The preclinical antiinflammatory effects of RWJ-355871 in these animal models of inflammation indicate that this dual inhibitor may have therapeutic utility for treating airway inflammatory diseases involving mechanisms that depend on Cat G and/or chymase.

Keywords: asthma; mast cells; neutrophils; pulmonary disease; serine protease; tobacco smoke; enzyme inhibitor

The prevalence of immune-mediated lung inflammatory diseases has been increasing worldwide. Asthma affects approximately 5% of the global population (1), and chronic obstructive pulmonary disease (COPD) is a major global health problem in terms of morbidity and mortality (2–4). Asthma and COPD have chronic inflammatory components that contribute to their respective pathophysiology. In asthma, mast cells, eosinophils, and Th-2 lymphocytes modulate the allergic inflammatory response, characterized by chronic airway hyperresponsiveness and airway remodeling. In COPD, lung inflammation charac-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Mast cells and neutrophils play an important role in the pathophysiology of asthma and chronic obstructive pulmonary disease, partly by releasing serine proteases, such as cathepsin G and chymase. It is not known if a dual inhibitor of neutrophil cathepsin G and mast cell chymase could ameliorate chronic pulmonary inflammation *in vivo*.

What This Study Adds to the Field

We examined the antiinflammatory pharmacology of RWJ-355871, a potent, dual inhibitor of cathepsin G and chymase, in three animal models of inflammation and determined that it exhibits notable efficacy. The results suggest potential therapeutic utility for treating airway inflammatory diseases that depend on cathepsin G and/or chymase.

terized by activated neutrophils and macrophages causes damage to epithelial and endothelial tissues, with the propagation of lung remodeling (5–7).

Although different cell types are thought to predominate in asthma and COPD, one commonality is that an excess of secreted proteases resulting from the accumulation of leukocytes in the airways and lungs contributes to abnormal airway function and remodeling (8). It is well accepted that tissue damage is accentuated during inflammatory sequelae when the protease activity exceeds the protective capacity of endogenous protease inhibitors (9). Thus, excess levels of neutrophil serine proteases (e.g., cathepsin G [Cat G], elastase, and proteinase 3) and mast cell serine proteases (e.g., chymase and tryptase) can induce bronchoconstriction and/or airway hyperresponsiveness (10) and result in extracellular matrix degradation and tissue remodeling (11–14).

The putative pathological role of excess protease activity in asthma and COPD has spurred substantial interest in identifying novel agents that could be useful for modulating the adverse effects of such excessive protease imbalance. In this context, we identified RWJ-355871 (Figure 1) (15) as a potent, nonpeptide, small-molecule inhibitor of Cat G (EC 3.4.21.20) (11, 12, 16) and chymase (EC 3.4.21.39) (13, 14, 17, 18). Both of these chymotrypsin-like enzymes have adverse effects on matrix proteins: Cat G is associated with activated macrophages and neutrophils, and mast cell chymase triggers the formation of cytokines and chemokines that stimulate the infiltration of inflammatory cells in asthma.

In initial studies, dual-inhibitor RWJ-355871 exhibited potent antiinflammatory activity in rat models of glycogen-induced peritonitis and lipopolysaccharide-induced airway inflammation (15). On the basis of this success, we sought to expand our investigation of RWJ-355871 by determining if it might have

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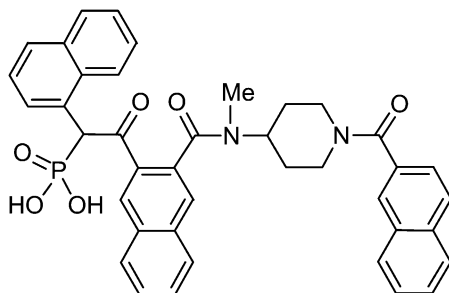


Figure 1. Chemical structure for RWJ-355871.

relevance to the therapeutic treatment of asthma and COPD and thereby reflect the importance of inhibiting Cat G and chymase in these diseases. Thus, RWJ-355871 was examined in three different animal models of inflammation with pathophysiological responses related to those manifested in allergic asthma and/or COPD: (1) mast-cell-mediated inflammation in ovalbumin (OVA)-sensitized rats (19), (2) allergen-induced bronchoconstriction and airway hyperresponsiveness in allergic sheep (20), and (3) tobacco smoke (TS)-induced neutrophilia in mice (21). These experimental models were used because the mechanisms involved in the measured responses were likely to involve activated mast cells and/or neutrophils. The results reported herein, together with earlier pharmacological findings (15), indicate that RWJ-355871 is able to disrupt inflammatory sequelae in animal models associated with inflammatory pathways related to asthma and COPD.

METHODS

Test Compound

Analytically pure RWJ-355871 (also known as JNJ-10311795), [2-[3-[[methyl[1-(2-naphthalenylcarbonyl)-4-piperidinyl]amino]carbonyl]-2-naphthalenyl]-1-(1-naphthalenyl)-2-oxoethyl]phosphonic acid (Figure 1), was synthesized at Johnson and Johnson Pharmaceutical Research and Development, LLC, and used as a tromethamine (Tris) salt (15, 22).

Allergic Inflammation in OVA-Sensitized Rats

Brown-Norway rats were sensitized to OVA by intraperitoneal injection of OVA–aluminum hydroxide on Days 0, 7, and 14 as described elsewhere (19). On Day 21, the rats were challenged with saline (50 μ l) or OVA (50 μ g in 50 μ l of saline) in the intraplantar space of the hind paw, and the paw volume was measured for 4 hours with a plethysmometer (Ugo Basile, Collegeville, PA). RWJ-355871 in 5% aqueous dextrose was administered intravenously to six separate groups of rats ($n = 5$ –9 per group) 30 minutes before OVA challenge in bolus doses of 0.00125, 0.0125, 0.125, 0.25, 0.55, or 0.5 mg/kg followed by infusion of 0.0025, 0.025, 0.25, 0.5, 2.1, or 10 mg/kg/h, respectively, resulting in composite doses of 0.0125, 0.125, 1.25, 2.5, 10, and 45.5 mg/kg, respectively, over the duration of the experiment (4.5 h). As a positive control, dexamethasone (DEX) in saline (VEH2) was dosed at 1 or 10 mg/kg intramuscularly 30 minutes before OVA challenge.

Sheep Asthma Model

Naturally allergic sheep with a history of early and late airway responses and airway hyperresponsiveness after antigen challenge were used (20, 23–25). Mean pulmonary airflow resistance, in units of cm H₂O/L/s, was measured by the esophageal balloon technique (23–25) to assess airway responses to aerosolized *Ascaris suum* antigen (Greer Diagnostics, Lenoir, NC) and carbamylcholine (Carbachol; Sigma Chemical Co., St. Louis, MO). All aerosols were generated with a disposable medical nebulizer (RaindropR; Nelcor-Puritan Bennett, Carlsbad, CA) and were delivered at a V_T of 500 ml and at a rate of 20 breaths per minute as previously described (23–25). Airway responsiveness (reported as PC400) was determined from cumulative con-

centration response curves to aerosolized carbachol dissolved in phosphate-buffered saline (PBS) at concentrations of 0.25, 0.50, 1.0, 2.0, and 4.0% w/v (23–25). For these studies, a baseline PC400 was determined 1 to 3 days before the beginning of treatment. RWJ-355871 was dissolved in PBS (3 ml) and administered by inhalation twice a day for three consecutive days and again at 0.5 hours before *Ascaris* challenge on Day 4. Pulmonary airflow resistance was measured immediately after antigen challenge and then serially for 8 hours after challenge. PC400 was determined 24 hours after challenge to assess the development of airway hyperresponsiveness (AHR). RWJ-355871 was tested at doses of 9, 4.5, and 0.9 mg.

TS Model of COPD in Mice

Female A/J mice were assigned to three separate groups ($n = 9$ or 10 per group): a sham group (air/vehicle), a TS- and vehicle-treated group (TS/vehicle), and a TS-treated group that received daily aerosol doses of RWJ-355871. Mice were exposed to smoke from 1R1 cigarettes (Kentucky Tobacco Research & Development Center, University of Kentucky, Lexington, KY) or to air for 11 consecutive days by the method described by Medicherla and colleagues (21). Saline (vehicle) or RWJ-355871 (2.2 mg/kg) was administered by inhalation with an ultrasonic nebulizer (Servo Ultra Nebulizer 145 [SUN145]; Siemens-Elema AB, Solna, Sweden) over a 5-minute period 30 minutes before TS exposure. Mice were killed 24 hours after the final TS exposure. Their bronchoalveolar lavage fluid (BALF) was collected by instilling and withdrawing 0.4 ml $\times$ 3 of heparinized (10 U/ml) PBS into the airways and lungs. The BALF cell pellet was resuspended, and a differential cell count was obtained from a cytospin slide stained with Shandon Kwik-Diff (Thermo Fisher Scientific, Waltham, MA). The amount of keratinocyte-derived chemokine (KC; murine homolog of IL-8) in the BALF supernatant was measured by ELISA according to the manufacturer's instructions (Quantikine Murine KC Kit; R&D Systems, Minneapolis, MN).

Animal Regulations

All animal studies were performed according to the guidelines for animal experiments at Johnson and Johnson Pharmaceutical Research and Development, LLC, Mount Sinai Medical Center, and Etiologics, Ltd (Slough, UK). Studies involving animals were performed with the approval and oversight of the Institutional Animal Care and Use Committees at each institution in accordance with federal, state, and local animal use regulations.

Statistical Methods

Sheep data for the 9- and 0.9-mg studies were analyzed by a one-way repeated measures analysis of variance followed by Student–Newman–Keuls *post hoc* test to determine significant differences between animals in the control group and the animals treated with RWJ-355871 (at 9 and 0.9 mg total dose). Data for the 4.5-mg studies (conducted in different sheep) were analyzed using a paired *t* test. Data for the TS-exposure study were evaluated using a one-way analysis of variance followed by a Bonferroni correction for multiple comparisons to test for significance between control and treatment groups. A *P* value of less than 0.05 was considered to be statistically significant. Data for the paw-volume study were analyzed by a Student's *t* test for comparison of the drug-treated groups to the corresponding vehicle groups.

RESULTS

Inhibition of OVA-induced Allergic Inflammation in Rats

Injection of OVA into the hind paw of sensitized Brown-Norway rats caused a reproducible 45 to 60% increase in paw volume. This inflammatory response, which is primarily mediated via the stimulation of mast cells (19), serves as an index of edema and inflammation (Figure 2). In this OVA-challenged rat model, treatment with a bolus intravenous dose of RWJ-355871, followed by an infusion for 4.5 hours, resulted in a dose-dependent inhibition of paw volume, with 90% reduction at a composite dose of 45.5 mg/kg. By comparison, administration of DEX (10 mg/kg, intramuscularly) gave an 80% reduction in paw volume.

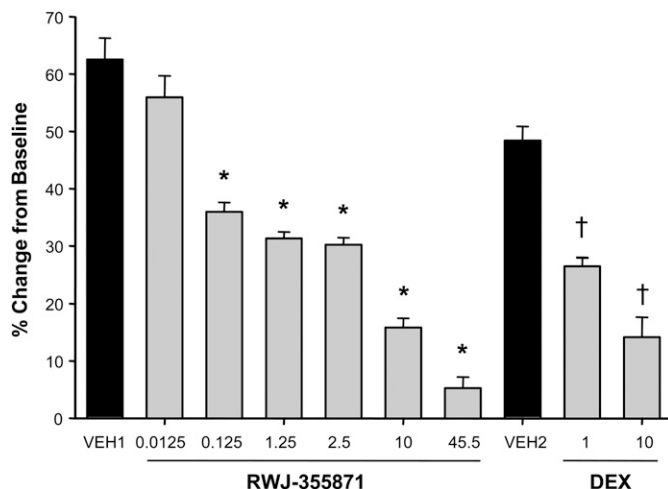


Figure 2. Reduction of paw edema induced by ovalbumin (OVA) injection in sensitized Brown-Norway rats (bars represent total paw volume change over 4 hours). In vehicle-treated rats, OVA challenge caused a 45 to 60% increase in paw volume (black bars). RWJ-355871 in aqueous dextrose (VEH1) was administered intravenously 30 minutes before OVA challenge followed by a constant infusion for 4.5 hours at composite doses of 0.0125 to 45.5 mg/kg (gray bars). As a positive control, dexamethasone in saline (VEH2) was administered at 1 or 10 mg/kg, intramuscularly, 30 minutes before OVA challenge (DEX). Data are reported as mean $\pm$ SE ($n = 5$ –9/group); * or † indicates statistical significance at $P \leq 0.05$ relative to the corresponding vehicle.

Inhibition of Antigen-induced Airway Responses in Allergic Sheep

RWJ-355871 was tested for efficacy against allergic airway responses in the sheep model of asthma (Figure 3) (24). In untreated sheep, aerosol challenge with the antigen resulted in the characteristic early airway response (EAR; 0–4 hours) and late airway response (LAR; 6–8 hours), and these animals developed AHR to inhaled carbachol 24 hours after antigen challenge. Aerosol administration of RWJ-355871 twice daily (9 mg per dose) for three consecutive days and at 0.5 hour before antigen challenge on Day 4 significantly inhibited the EAR and completely blocked the LAR (Figure 3A; Table 1). Treatment with RWJ-355871 also blocked the antigen-induced AHR in these animals (Figure 3B; Table 1). The beneficial effects of RWJ-355871 treatment were lost by reducing the dose to 0.9 mg (Figure 3; Table 1). In a subsequent series of studies, we showed that an aerosol dose of 4.5 mg of RWJ-355871 was also effective in blocking the LAR and AHR (Table 1).

Antiinflammatory Activity in a Murine TS Model

We used a TS model in mice to acquire information on the potential efficacy of RWJ-355871 against airway inflammation relative to COPD (21). The BALF of the chronically TS-exposed mice showed a marked increase (10.1-fold; $P < 0.001$) in the total number of inflammatory cells compared with air-exposed (sham) mice. There were substantial and significant increases in neutrophils (186-fold), macrophages (8.7-fold), eosinophils (142-fold), lymphocytes (114-fold), and epithelial cells (9.3-fold). Although RWJ-355871 did not inhibit the TS-induced increase in the total number of cells in the BALF, the compound reduced the neutrophil burden by 66% ($1.2 \pm 0.3 \times 10^4$ vs. $0.4 \pm 0.1 \times 10^4$ cells; $P < 0.05$). No other cell type was significantly inhibited. Consistent with this neutrophil inhibition, there was a corresponding reduction in the level of KC (murine homolog of IL-8) in the BALF from mice treated with

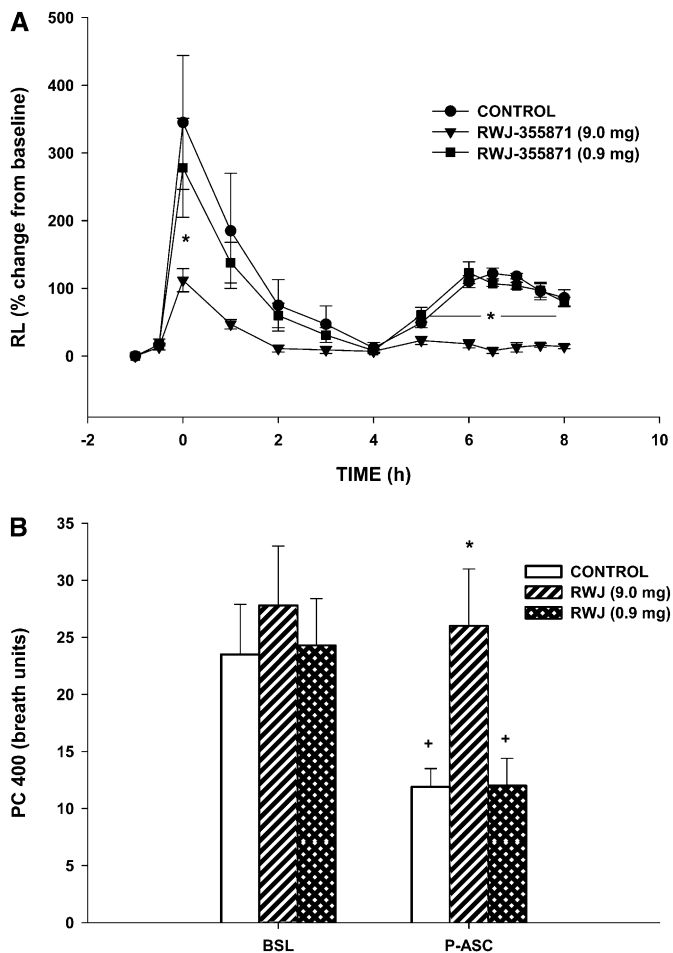


Figure 3. Allergen-sensitized sheep model of asthma. (A) Changes in airway resistance after aerosol administration of 9 mg (closed inverted triangles) or 0.9 mg (closed squares) of RWJ-355871 to sheep twice a day for three consecutive days and then 30 minutes before allergen challenge on Day 4 compared with untreated sheep (control; closed circles). (B) Changes in airway responsiveness to carbachol before (baseline; BSL) and at 24 hours post antigen challenge (P-ASC) in untreated (control; open bars) and compound-treated sheep. The 9-mg dose of RWJ-355871 (hashed bars), but not the 0.9-mg dose (checkered bars), prevented the fall in PC400 seen in untreated sheep after allergen challenge. Values are mean $\pm$ SE for four sheep. * $P < 0.05$ versus control; † $P < 0.05$ versus baseline. Detailed statistical comparisons are given in Table 1.

RWJ-355871. In the TS-exposed mice, KC increased to 55.1 ± 3.8 ng, compared with 12.2 ± 2.5 ng in the sham animals. Treatment with RWJ-355871 reduced the KC levels in TS-exposed mice by a statistically significant 55% to 31.5 ± 5.6 ng.

DISCUSSION

The results of this study show, for the first time, that dual-inhibitor RWJ-355871 protects against pathophysiological responses evoked by allergic and nonallergic inflammatory stimuli in three different model systems. These observations have implications relative to the roles of Cat G and chymase in pulmonary inflammatory disorders involving mast cells and neutrophils, such as asthma and COPD.

RWJ-355871 was previously shown to selectively inhibit mast cell chymase ($K_i = 2.3$ nM) and neutrophil Cat G ($K_i = 38$ nM) (15) without inhibiting mast cell tryptase, neutrophil elastase, or

TABLE 1. RESULTS FROM THE SHEEP ASTHMA MODEL

Treatment	Variables*			
	EAR	Peak LAR	Average LAR	AHR
9 mg RWJ-355871 (n = 4)	112 ± 19 [†]	26 ± 3 [†]	16 ± 3 [†]	0.95 ± 0.15 [†]
0.9 mg RWJ-355871 (n = 4)	277 ± 82	131 ± 14	95 ± 6	0.49 ± 0.07
Control (n = 4)	345 ± 99	130 ± 7	97 ± 6	0.54 ± 0.06
4.5 mg RWJ-355871 (4)	145 ± 36 [§]	28 ± 5 [†]	16 ± 4 [†]	1.03 ± 0.07 [†]
Control (n = 4)	421 ± 145	156 ± 29	108 ± 17	0.53 ± 0.06

Definition of abbreviations: AHR = airway hyperreactivity; EAR = early airway response; LAR = late airway response.

* Variables are expressed as percent change from baseline, analyzed EAR (maximum response between baseline and 4 h after challenge), peak LAR (maximum response between 5–8 h after challenge), average LAR (average response between 5 and 8 h after challenge), and AHR (calculated as the PC400 ratio = the ratio of the postchallenge/prechallenge PC400). A ratio of 1 indicates no change in airway responsiveness; a ratio below 1 indicates the development of AHR.

[†] $P < 0.05$ vs. control.

[‡] Versus 0.9 mg.

[§] $P < 0.10$ vs. control.

proteinase 3. Although it is well known that serine proteases derived from neutrophils and mast cells degrade extracellular matrix components, induce leukocyte migration, and promote tissue remodeling (9–11, 14–16), the adverse effects of Cat G and chymase can extend beyond this. Of specific interest are reports that Cat G is linked with activated macrophages and that chymase triggers the generation of cytokines and chemokines, which serve to recruit various inflammatory cells. Thus, Cat G and chymase are thought to contribute to self-propagating, chronic inflammation. RWJ-355871 is a unique molecule in that it binds well in the active-site regions of Cat G and chymase, which are very structurally homologous, so that both proteases are inhibited by a single entity.

In previous studies in rats, RWJ-355871 was found to inhibit neutrophilia in ascites fluid after injection of glycogen and in BALF after tracheal instillation of lipopolysaccharide (15), thereby demonstrating its antiinflammatory potential. The present studies expand these findings to build on the hypothesis that such a dual inhibitor can exert marked antiinflammatory pharmacology in animal models relevant to asthma and COPD. Aerosol administration of RWJ-355871 exhibited antiinflammatory activity in the airways, as evidenced by its effects in TS-exposed mice. Furthermore, the work presented here shows for the first time that RWJ-355871 has antiallergic properties. In an OVA-sensitized rat model, RWJ-355871 prevented an OVA-induced increase in edema as reflected by an increase in paw volume. Previous work in this model indicates that the edema is a mast-cell-mediated event and is sensitive to antihistamines and glucocorticosteroids (19). In the present study, the effects of RWJ-355871 were dose dependent and showed activity at composite doses of 1 and 10 mg/kg, similar to that obtained with 1 and 10 mg/kg of DEX.

RWJ-355871 exhibited dose-dependent effects in the allergic sheep model of asthma, consistent with the findings in OVA-sensitized rats. The test compound exhibited activity against the EAR, which is reflective of acute mast cell degranulation, and on the LAR and AHR. The two latter end points are thought to be the pathophysiological functional events that signify allergen-induced airway inflammation (20, 26). The effect of RWJ-355871 on airway functional changes after antigen challenge is strikingly similar to previous findings obtained with secretory leukocyte protease inhibitor (SLPI) (27) and the tryptase inhibitor APC 366 (28) in this sheep asthma model, with the latter showing similar efficacy in studies involving human antigen challenge (29).

Similar to RWJ-355871, SLPI is a nanomolar inhibitor of Cat G (as well as chymotrypsin), but it also blocks the activity of elastase and trypsin-like serine proteases (27). A 4-day treatment regimen with SLPI (3 mg given four times per day) in the sheep model provided 48% inhibition of the peak EAR, greater than 95% inhibition of the LAR, and 84% inhibition of the post-antigen-induced AHR. Recently, it was reported that chymase can cleave SLPI and that RWJ-355871 (“Inhibitor (B)”) can block this cleavage with an IC_{50} value of 17 nM (30), which suggests that inhibition of chymase may preserve the antiprotease activity of SLPI in the sheep model, thereby contributing to the therapeutic effect observed with RWJ-355871. Although APC 366 inhibited tryptase and trypsin, it showed no activity against elastase or Cat G. Administration of APC 366 for 3 days (18 mg given twice per day with a final dose of 18 mg given on the day of antigen challenge) provided 66% inhibition of the EAR, 74% inhibition of the LAR, and 87% inhibition of AHR (28). These prior results, in conjunction with the fact that the elastase inhibitor α_1 -protease inhibitor (α_1 -PI) had no effect on antigen-induced EAR and LAR (31), suggest that the effect of RWJ-355871 on antigen-induced airway function is related to its ability to block the actions of chymase. This conclusion supplements the reported primary actions of chymase, which are to recruit and sustain eosinophils, cause mucus secretion, and degrade pericellular matrices in contributing to tissue remodeling (14). Although inhibiting cell recruitment is consistent with the effects on the LAR and AHR, our results showing that chymase inhibition significantly reduces the EAR is novel. In the sheep asthma model, it is well documented that the EAR is in part related to mast cell degranulation, as evidenced by the release of histamine and the fact that modulators of the EAR are known to reduce histamine levels in the airways (20, 28, 32). The reason why a chymase inhibitor would show a similar effect is unknown. Although speculative, one explanation would be that the reduction in the EAR in animals treated with RWJ-355871 is linked to a reduction in mediator release from mast cells. However, an alternative explanation could relate to the fact that chymase augments histaminic wheal responses in the skin without causing a response on its own (33) and that chymase inhibitors decrease allergen-induced wheals (14). Because mast cells containing tryptase and chymase infiltrate asthmatic airway smooth muscle (34, 35), it is also possible that treatment with RWJ-355871 affected chymase activation in the airway smooth muscle, resulting in a diminished contractile response without an impact on mediator release. On the basis of the experiments performed herein, it is not possible to determine which of these mechanisms contributes to the reduction in the EAR in the drug-treated animals. In any case, this mechanistic uncertainty does not negate the originality of our findings, and future studies in this direction should help clarify the potential pathways involved.

The inhibition of the LAR and AHR by RWJ-355871 is consistent with blocking the inflammatory effects of chymase and Cat G. Previous studies in rats indicated that RWJ-355871 blocks increases in the cytokine TNF- α and the chemokine MCP-1 (15), both of which have been linked to AHR. In addition, the ability of RWJ-355871 to block airway neutrophilia correlates with reductions in the LAR and AHR in sheep in that the neutrophil is the predominant cell type recruited to the airway during these events, and a reduction in neutrophil influx is linked to a reduction in these responses (20).

Cathepsin G is traditionally associated with neutrophils, but it is also expressed in the granules of the subset of mast cells that express chymase. In the lung, this mast cell subset tends to reside in bronchial submucosa, although it is also found in

airway smooth muscle (36). Thus, the release of chymase and Cat G could contribute to the AHR by acting on airway smooth muscle, and such a mechanism would be consistent with protection by RWJ-355871. Both chymase and Cat G convert angiotensin I to angiotensin II (37), and an angiotensin II type-1 receptor antagonist can block allergen-induced airway hyperresponsiveness in animal models (38). Because angiotensin antagonists have not been studied in allergic sheep, we are unaware if blockade of chymase- and Cat G-mediated angiotensin II production by RWJ-355871 contributes to protection against the antigen-induced AHR.

Although the above studies in rats and sheep have yielded evidence for the potential antiallergic effects of RWJ-355871, the data from the TS-exposed mice suggest that the compound could be important in modulating inflammation in COPD. TS exposure is a well recognized risk factor for the development of COPD because it produces an abnormal inflammatory response in the lower airways, which leads to tissue damage and destruction (39). Although the murine TS model that we used is subchronic in duration, it represents a state of COPD (21) characterized by an acute inflammatory response in the airways. The TS-induced inflammatory response included increases in all cell types, including a 186-fold increase in neutrophils. RWJ-355871 significantly reduced the TS-induced airway neutrophilia by 66%, and this effect was accompanied by a 55% reduction in levels of the murine homolog of IL-8 (KC). These results are important in terms of tissue remodeling because neutrophil proteases, including elastase and Cat G, are expressed on the neutrophil cell surface. Studies have shown that natural inhibitors, such as α 1-PI, preferentially target elastase, thereby leaving Cat G uncontrolled. This situation could provide a rationale for why selective elastase inhibitors have been ineffective in controlling neutrophil-mediated damage in the airways (40).

Korkmaz and colleagues (41) examined the inhibitory tendencies of purified α 1-PI and of BALF on neutrophil elastase, Cat G, and proteinase 3. Their results indicate that elastase is preferentially inhibited by α 1-PI and that the other two proteases are only inhibited after elastase is completely neutralized. Thus, Cat G and proteinase 3 are not inhibited by α 1-PI as long as there is free elastase available. Experiments with BALF were confirmatory, suggesting that Cat G and proteinase 3 are less rapidly inactivated by inhibitors in BALF. Consequently, these two proteases, if left unchecked, could be responsible for a majority of the damage associated with lung neutrophilia. Our results with RWJ-355871 in preclinical animal models of inflammation suggest that inhibitors of multiple inflammatory proteases may be needed for effective pharmacotherapy in asthma and COPD (42, 43).

The actions of RWJ-355871 against human Cat G and human mast cell chymase were detailed previously, along with molecular structures from X-ray crystallography depicting RWJ-355871 bound within the active-site cleft of each enzyme (15). RWJ-355871 is a very selective inhibitor for Cat G and chymase relative to several other proteases, including tryptase, proteinase 3, leukocyte elastase, thrombin, factor Xa, plasmin, kallikrein, and trypsin, although it is a moderate inhibitor of chymotrypsin ($K_i = 490$ nM) (15). Thus, we surmise that this compound's protective effect in the three experimental models discussed herein derives from the inhibition of Cat G and chymase.

Caughey's review on mammalian proteases points out that tryptases and chymases can differ across mammalian species (14). However, there is reasonable evidence to suggest that our reported results are related to inhibition of the appropriate proteases. Miller and Pemberton detailed the specificity of

several mammalian mast cell granule serine proteases and noted that mouse, rat, and sheep have serine proteases with chymase-like activity (17). In rats, the chymase analog that shows substrate specificity to human chymase is rat mast cell proteinase-1, which is contained in connective tissue mast cells and would be likely to contribute to the IgE-mediated paw edema used to assess the antiallergic response in the present studies (44, 45). Mast cells in the lungs of mice are concentrated primarily adjacent to the main-stem bronchi (17). These airway mast cells in mice were reported to have a connective tissue-like phenotype; this finding is important because this phenotype contains mouse mast cell proteinase-4, which shows a substrate cleavage specificity similar to that of human chymase (44, 45). Thus, if this chymase analog were to play a role in the responses of TS-exposed mice, then it is reasonable for its actions to have been blocked by RWJ-355871.

In sheep, the largest distribution of mast cells occurs around the peripheral bronchioles and in the parenchyma (17). Collie and colleagues reported that allergen challenge in sensitized sheep is associated with an increase in sheep mast cell proteinase-1 (chymase analog) and tryptase-expressing cells (46), which confirmed and extended earlier studies in sheep in which antigen challenge resulted in an increased number of mast cells in bronchial biopsies (47). Given that the human tryptase inhibitor APC-366 (28), which was effective in human antigen challenge studies (29), blocked antigen-induced responses in sheep, that sheep Cat G and neutrophil elastase have characteristics similar to human Cat G and human neutrophil elastase (48), and that sheep neutrophil elastase activity is blocked by human neutrophil inhibitors (49), sheep proteases appear to be similar to the corresponding human enzymes and thus susceptible to blockade by human protease inhibitors. Because RWJ-355871 does not block tryptase (15), the results of this study support the view that the protective effects of RWJ-355871 on the antigen-induced responses derive from inhibition of chymase and/or Cat G.

Although Cat G is expressed in the granules of the subset of mast cells that express chymase, it is more traditionally associated with neutrophils. In terms of the animal models used in our study, it is apparent that RWJ-355871 inhibited neutrophil influx in TS-exposed mice, which is consistent with the previous report describing the action of RWJ-355871 in two different rat models of neutrophil-mediated inflammation (15). In this connection, studies in double-knockout mice, whose neutrophils lack elastase and Cat G, showed reduced responses to zymosan and immune complex-mediated inflammation in a subcutaneous air pouch (50). The reduced cellular response was accompanied by a significant reduction in the levels of TNF- α and IL-1 β , suggesting that the proteases are important in cytokine processing, consistent with the initial studies of RWJ-355871 in rat models of inflammation (15). Although neutrophils deficient in elastase and Cat G did not undergo CD11b clustering and exhibited defects in reactive oxygen species generation, the addition of exogenous Cat G reversed these abnormal properties (51). Collectively, these findings indicate that Cat G is important for neutrophil recruitment and function. Given that RWJ-355871 has no antielastase activity (15), the reduction in neutrophil recruitment seen in the TS-exposed mice treated with RWJ-355871 is consistent with Cat G inhibition.

In conclusion, we have characterized RWJ-355871, a potent dual inhibitor of neutrophil Cat G and mast cell chymase, in animal models of inflammation that have characteristics relating to allergic asthma and COPD. Our results show that RWJ-355871 is capable of reducing neutrophil influx into sites of inflammation and can counteract asthma pathophysiology. Because the inflammatory proteases Cat G and chymase are

thought to contribute significantly to the conditions of asthma and COPD in humans, especially when endogenous inhibitors are depleted, agents such as RWJ-355871 could hold promise for therapeutic applications (42, 43, 52).

Conflict of Interest Statement: B.E.M. is an employee of Johnson & Johnson, has received more than \$100,001 in compensation, and has numerous patents issued/pending as part of employment, which are assigned to Johnson & Johnson. B.E.M.'s spouse/life partner is an employee of Johnson & Johnson, and has received \$50,001–\$100,000 in compensation, and has numerous patents issued/pending as part of employment, which are assigned to Johnson & Johnson. B.E.M. and B.E.M.'s spouse/life partner hold stock ownership or options in Johnson & Johnson. L.D.G. is an employee of Johnson & Johnson Pharm R&D and holds stock ownership and options in the company. M.N.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.J.H. is an employee of Johnson & Johnson, has received \$50,001 to \$100,000 in compensation, and holds \$50,001–\$100,000 in stock ownership or options from Johnson & Johnson. G.I.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.A.G. is a full-time employee of Johnson & Johnson and holds stock ownership or options obtained as a full-time employee of Johnson & Johnson. W.M.A. received \$10,001–\$50,000 from Johnson & Johnson Pharmaceutical in industry-sponsored grants.

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