A Novel 5-Lipoxygenase-Activating Protein Inhibitor, AM679, Reduces Inflammation in the Respiratory Syncytial Virus-Infected Mouse Eye

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Respiratory syncytial virus (RSV) is an important cause of viral respiratory disease in children, and RSV bronchiolitis has been associated with the development of asthma in childhood. RSV spreads from the eye and nose to the human respiratory tract. Correlative studies of humans and direct infection studies of BALB/c mice have established the eye as a significant pathway of entry of RSV to the lung. At the same time, RSV infection of the eye produces symptoms resembling allergic conjunctivitis. Cysteinyl leukotrienes (CysLTs) are known promoters of allergy and inflammation, and the first step in their biogenesis from arachidonic acid is catalyzed by 5-lipoxygenase (5-LO) in concert with the 5-LO-activating protein (FLAP). We have recently developed a novel compound, AM679, which is a topically applied and potent inhibitor of FLAP. Here we show with the BALB/c mouse eye RSV infection model that AM679 markedly reduced the RSV-driven ocular pathology as well as the synthesis of CysLTs in the eye. In addition, AM679 decreased the production of the Th2 cell cytokine interleukin-4 but did not increase the viral load in the eye or the lung. These results suggest that FLAP inhibitors may be therapeutic for RSV-driven eye disease and possibly other inflammatory eye indications.
CysLTs have been measured with human tear fluid after specific allergen challenge and with tears from patients suffering from contact lens-associated giant papillary conjunctivitis (2, 22). Application of CysLTs to guinea pig eye results in increased microvascular permeability, with LTC4 being more potent than LTD4, which in turn is more potent than LTE4 (17). However, there have been no studies of activation or inhibition of the LT pathway in murine RSV-infected eye models. We show here that a topically applied, potent, selective FLAP inhibitor, AM679, decreases RSV-induced CysLTs, the Th2 cytokine IL-4, and eye pathology while not increasing the viral load in the eye or lung. Topical therapy with FLAP inhibitors may be useful to reduce the eye pathology of RSV infection and perhaps other types of inflammatory and allergic ocular diseases.

MATERIALS AND METHODS

Instillation of virus and inhibitor in the eye. Female BALB/c mice, 6 to 8 weeks old, were purchased from Charles River Laboratories. RSV (Long strain, serotype A) was grown on HEp-2 cells and purified on sucrose layers to a concentration of 10^{11} PFU as described previously (4). Dilutions were done in phosphate-buffered saline (PBS) immediately before use to a final concentration of 10^{6} PFU/2 μL. A similarly diluted sucrose solution was used in sham-infected control mice. Mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight), and virus in 2 μL PBS was dropped onto the corneal surface and massaged in with closed eyelids. In each mouse only one eye was treated. The day of the inoculation was considered day 0. The FLAP inhibitor AM679, 3-[[5-[(5S)-1-acetyl-2,3-dihydro-1H-indol-2-ylmethoxy]-3-tert-butylsulfanyl-1H-indol-2-yl]-2,2-dimethyl-propionic acid (Fig. 2), was supplied by Amira Pharmaceuticals. This compound is a potent, selective inhibitor of FLAP, as demonstrated in an in vitro human FLAP membrane binding assay with a 50% inhibitory concentration (IC_{50}) of 2 nM and when assayed as an inhibitor of ex vivo ionophore-challenged mouse and human blood LTB_{4} synthesis with IC_{50}s of 55 nM and 154 nM, respectively. In comparison, the FLAP inhibitor MK886 had IC_{50}s for inhibition of mouse and human blood LTB_{4}, of 540 nM and 1,700 nM, respectively. In an in vivo rat lung LT inhibition model, AM679 inhibited ionophore-challenged production of LTD_{4} and CysLTs with IC_{50} values of 14 nM and 37 nM, respectively. AM679 did not inhibit cyclooxygenase-1 or -2 in human blood when tested up to a final concentration of 100 μM. The data for the FLAP binding assay, blood LTB_{4} inhibition assays, cyclooxygenase assays, and rat lung model were obtained by previously published methods (21). AM679 was diluted in PBS to 60 ng/2 μL, and then each day thereafter for 14 days. This concentration of AM679 in the eye was predicted to give complete LT inhibition but, given the 1,000-fold selectivity determined above, was unlikely to cross over to similar protein targets, such as the cyclooxygenases. At days 0, 2, 4, 6, 8, 10, and 14, three mice from the control group and three mice from the FLAP inhibitor-treated group were sacrificed for ocular disease evaluation and eye and lung tissue preparation for assays described below.

Ocular pathology detection and tissue collection. Ocular disease was evaluated in the anesthetized mouse using a slit lamp biomicroscope as described previously (19). Pathology was scored on a scale of 0 to 5 as follows: 0, clear eye; 1, slight redness in the corners; 2, moderate redness and injection; 3, conjunctival and corneal injection with ciliary flush; 4, extensive injection, generally associated with some mucus; and 5, most extensive injection, associated with excess mucus. Eyes were examined in a coded fashion, with the reader unaware of the treatment given. When desired, the mouse eyes were removed following the examination. The eyeball, crystallin, and all membranous tissue were carefully and quickly removed. The remaining tissue was weighed and homogenized in a proportional volume (1 to 2 ml) of lys buffer (0.5% Triton X-100, 15 mM Tris Cl, pH 7.4) using a polytron homogenizer. A portion of the homogenate was set aside for viral titer assay without freezing. The remainder was centrifuged at 10,000 × g for 10 min at 4°C, and the supernatant collected and frozen at −80°C for later use in the following assays.

Protein and CysLT assays. The supernatant samples described above were thawed; a sample was assayed for protein (32); and the remainder was precipitated with a final volume of 10% ice-cold methanol, held on ice for 30 min, and then centrifuged at 10,000 × g for 15 min. The denatured protein pellet was discarded, and the lipid-containing supernatant assayed for CysLTs at the appropriate dilutions to be on the linear part of the standard curve using the procedure described in the assay design kit (Ann Arbor, MI) with a sensitivity of ~30 pg CysLT/ml.

Quantification of IL-4. The IL-4 mRNA was quantified by reverse-transcription real-time PCR as described previously (5). In brief, total RNA was isolated from the thawed extracts using an RNeasy mini kit (Qiagen), primers were designed by the Beacon Designer software from Premier Biosoft, and reverse-transcription real-time PCR was performed with the iCycler iQ quantitative PCR system using the iQ SYBR green supermix kit (Bio-Rad). Gene expression measurements were calculated using the manufacturer’s software: GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control. The primers were (forward and reverse [all written 5’ to 3’]) AACTGCTTC CCCCTCCTGTT and TTGGAGGCGCAAAAGATGTC for IL-4 (GenBank accession no. NM_000589.2) and GTGAAGGTCGGAGTCAAC and CAAT CCCCTCTGTTC and TTGGAGGCAGCAAAGATGTC for IL-4 (GenBank accession no. NM_000589.2) and GTGAAGGTCGGAGTCAAC and CAAT CCCCTCTGTTC and TTGGAGGCAGCAAAGATGTC for IL-4 (GenBank accession no. NM_000589.2) and GTGAAGGTCGGAGTCAAC and CAAT CCCCTCTGTTC and TTGGAGGCAGCAAAGATGTC for IL-4 (GenBank accession no. NM_000589.2) and GTGAAGGTCGGAGTCAAC and CAAT CCCCTCTGTTC and TTGGAGGCAGCAAAGATGTC for IL-4 (GenBank accession no. NM_000589.2) and GT.
Assay of RSV. Infective viral titer was determined by serial dilution of the fresh tissue extract and plating on HEp-2 cell monolayer, and the RSV P protein was detected by Western blotting as previously described (3, 4).

Statistical analysis. The pathology scores and ocular CysLT concentrations were subject to a two-way analysis of variance followed by Bonferroni post hoc analysis using GraphPad Prism software (GraphPad Software, San Diego, CA).

RESULTS

FLAP inhibitor reduces RSV-induced inflammation in the eye. To determine if a FLAP LT synthesis inhibitor can ameliorate eye inflammation following RSV infection, we treated one eye of drug-treated mice with 60 ng AM679 in 2 μl sterile saline (or one eye of control mice with 2 μl sterile saline only) 40 min after inoculation with 10⁶ PFU RSV and then every day afterward for 13 more days. The RSV-infected eyes from control mice showed ocular inflammation, mucus, and conjunctivitis that peaked 6 to 8 days after infection and largely resolved by 14 days (Fig. 3). The FLAP inhibitor AM679-treated mouse eyes showed significant protection from RSV-induced pathology as early as 2 days continuing to 14 days postinfection. At 6 to 8 days the FLAP inhibitor-treated eyes showed greater than 70% reduction in total pathological scores. Representative eyes from both control and AM679-treated mice through days 2 to 6 clearly demonstrate the reduced inflammation and mucus in the drug-treated animals (Fig. 3).

FLAP inhibitor decreases RSV-induced CysLTs and IL-4 in the eye. RSV eye infection resulted in increased ocular CysLTs beginning about 4 days after infection (Fig. 4A). Treatment with the FLAP inhibitor AM679 resulted in a decrease of more

FIG. 3. FLAP inhibitor AM679 reduces RSV eye pathology. (A) Ocular application of RSV caused an increase in pathology score (as described in Materials and Methods) from 2 to 14 days after challenge. Topical treatment with the FLAP inhibitor AM679 (60 ng/2 μl saline) reduced this effect across all observation days. *** P < 0.001; ** P < 0.01; * P < 0.05, versus RSV control. Bars represent calculations of standard error of the mean. (B) Representative mouse eye photographs of the RSV-alone control versus RSV plus FLAP inhibitor AM679. Day 0, naïve uninfected eye; days 4, 6, and 8, RSV control (no inhibitor) or RSV- and AP670-treated mouse eyes as indicated. At days 0, 2, 4, 6, 8, 10, and 14, three mice from the control group and three mice from the FLAP inhibitor-treated group were sacrificed for ocular disease evaluation and eye and lung tissue preparation.

FIG. 4. Ocular application of RSV caused an increase in CysLTs (A) and IL-4 (B). Assays were done with eye homogenates as described in Materials and Methods. Topical treatment with the FLAP inhibitor AM679 reduced this effect across observation days. *** P < 0.001; ** P < 0.01, versus RSV control. Bars represent calculations of standard error of the mean.
than 90% in the peak 6- to 8-day ocular CysLTs (Fig. 4A). By day 10, CysLT concentrations for both RSV control and RSV drug-treated mouse eyes had almost returned to baseline (Fig. 4A). Previously, a strong correlation between IL-4 mRNA and RSV was reported for human allergic conjunctivitis (14). We therefore measured IL-4 mRNA concentrations for the RSV-infected mouse eyes and found them to be significantly elevated 6 days after RSV infection, and they gradually decreased to near baseline around 14 days (Fig. 4B). Treatment with AM679, in contrast, inhibited more than 80% of the IL-4 increase (Fig. 4B). In this study we did not examine the expression of any other inflammatory cytokines or chemokines.

**FLAP inhibitor does not increase RSV mRNA or protein in the eye or lung.** Infectious RSV progeny in the mouse eye (Fig. 5A) and lung (Fig. 5B) were measured. In the eye, the infectious RSV titer peaked on day 2 and was greatly reduced by day 4. It was further reduced below the detection limit on day 6 through day 14. Treatment with AM679 resulted in a non-significant decrease in infectious virus in the eye at day 2 (Fig. 5A). In the lung, the virus progeny were first measurable on day 4 and showed exponential growth in the 48 h following peak eye titer (Fig. 5B). Treatment with AM679 showed only a minor decrease in viral titer at day 4 and day 6 in the lung (Fig. 5B). In both tissues (eye and lung), the viral titer paralleled an increase in viral protein P, confirming each other. There was a trend for a slight decrease in RSV growth in AM679-treated eye extracts, as shown in representative immunoblots (Fig. 5C and D).

**DISCUSSION**

RSV in the eye drives a strong host inflammatory reaction, with both innate and immune pathologies (3, 5, 27). We show here that an LT synthesis inhibitor that selectively targets FLAP, the membrane protein essential for cellular LT synthesis, effectively reduced the RSV-induced ocular pathology and the RSV-induced increase in CysLTs and IL-4. Importantly the FLAP inhibitor AM679 reduced RSV-induced eye pathology without increasing the viral load in the eye and only modestly decreasing the viral progeny and protein in the lung. There was only weak replication of RSV in the eye in the presence of AM679 and a trend toward a decrease in ocular virus, but this initial reduction may have been magnified during the exponential viral growth phase in the lung. Since AM679 was not designed as an antiviral compound, it was not expected to reduce viral titer or protein, and in fact, other anti-inflammatory strategies, such as antibodies to IL-1 or TNF, actually slightly increased viral loads (3).

There was no RSV-induced increase in LTB4 and no consistent inhibition of baseline ocular LTB4 by treatment with AM679 (data not shown). This is in agreement with RSV-induced increases in CysLTs but not in LTB4 in the BALB/c mouse lung (26). It has been shown previously that RSV replicates in eye conjunctival epithelial cells (14), but we do not know which cells are making the initial CysLTs in response to ocular infection by virus. Epithelial cells have been shown to induce expression of dendritic cell HLA-DR antigens (18); thus, it is possible that RSV infection may induce LT biosynthetic genes in conjunctival epithelial cells. In addition, interstitial macrophages may contribute to the first burst of CysLTs, which would then enhance the influx of monocytes, eosinophils, and lymphocytes into the eye. These cells can in turn make CysLTs to amplify ocular inflammatory responses, including incremental chemokine and mucus synthesis.
RSV induces the production of IL-4 and other cytokines by human conjunctival epithelial cells (14), and hence, in the BALB/c RSV-infected eye model a small amount of IL-4 may be produced in the infected conjunctival epithelial cells and then larger amounts by the influx of inflammatory cells. In our study, AM679 dramatically reduced IL-4 concentrations in the eye, presumably largely through inhibition of lymphocyte influx. We did not measure the inhibition of other cytokines by AM679. IL-4 gene polymorphisms in the human 5q31 allergic inflammation cytokine cluster have been shown to link to significant risk for severity of primary RSV disease (13). Close to this cluster is the human 5q35 chromosomal location of the asthma-linked LTC₄ synthase gene that codes for the enzyme that converts LTA₄ to LTC₄ (30). Although systemic steroids can also lower IL-4 in some tissues, there is controversy with respect to the efficacy of systemic steroid use with infants with RSV bronchiolitis (29). This may be due to the fact that although the steroids have a general anti-inflammatory effect on some mediators, they do not inhibit LT production (20).

The CysLTs have been shown to activate human and murine inflammatory cells, including monocytes, eosinophils, and mast cells, through activation of both the CysLT1 and CysLT2 receptors (10). Recently, in addition to the classical CysLT1 and CysLT2 receptors, several G protein-coupled receptors have been shown to be activated with high affinity by CysLTs (25, 28). In the RSV-infected mouse eye, it is unclear which receptor or receptors drive the CysLT pathology. Application of CysLTs to guinea pig eye results in edema and the influx of eosinophils, with LTE₄ being more potent than LTĐ₂, which in turn is more potent than LTC₄ (17, 24). Intriguingly, this CysLT potency profile does not correspond to that for either the CysLT1 or CysLT2 receptor, or GPR17, a third CysLT receptor (7, 9). The ocular effects in the guinea pig eye appear to represent activation of a nonclassical CysLT receptor, such as the LTE₄-activated P2Y12 receptor, which is expressed on platelets (28). It will be of interest in the future to investigate which CysLT receptors are involved in driving the RSV pathology in the BALB/c eye model.

Inhibitors of LT synthesis, 5-LO and FLAP inhibitors, and CysLT1 receptor antagonists have been shown to be effective in reducing asthma symptoms (23, 33). 5-LO and FLAP inhibitors have the potential advantage over CysLT1 receptor antagonists of reducing activation of all LT inflammatory and allergic responses (9, 33). This includes the activation of BLT receptors and also the CysLT activation of not only the CysLT1 receptor but also the CysLT2, GPR17, P2Y12, and perhaps more CysLT receptors. While there are several CysLT1 receptor antagonists on the market for asthma, including Singulair, Accolate, and Onon, the only LT synthesis inhibitor on the market is Zyflo, a relatively weak inhibitor of 5-LO (1, 33). The recent development of novel FLAP inhibitors (10) that are potent inhibitors of all cellular LT synthesis will enable the determination of the clinical benefit of complete inhibition of the LT pathway in lung and ocular inflammatory and allergic diseases.

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