The adenosine 2A receptor agonist GW328267C improves lung function after acute lung injury in rats

Hans G. Folkesson; Stephanie R. Kuzenko; David A. Lipson; Michael A. Matthay; and Mark A. Simmons

Folkesson HG, Kuzenko SR, Lipson DA, Matthay MA, Simmons MA. The adenosine 2A receptor agonist GW328267C improves lung function after acute lung injury in rats. Am J Physiol Lung Cell Mol Physiol 303: L259–L271, 2012. First published June 1, 2012; doi:10.1152/ajplung.00395.2011.—There is a significant unmet need for treatments of patients with acute lung injury (ALI) and/or acute respiratory distress syndrome (ARDS). The primary mechanism that leads to resolution of alveolar and pulmonary edema is active vectorial Na+ and Cl− transport across the alveolar epithelium. Several studies have suggested a role for adenosine receptors in regulating this fluid transport in the lung. Furthermore, these studies point to the A2A subtype of adenosine receptor (A2AR) as playing a role to enhance fluid transport, suggesting that activation of the A2AR may enhance alveolar fluid clearance (AFC). The current studies test the potential therapeutic value of the A2AR agonist GW328267C to accelerate resolution of alveolar edema and ALI/ARDS in rats. GW328267C, at concentrations of 10−5 M to 10−3 M, instilled into the airspaces, increased AFC in control animals. GW328267C did not increase AFC beyond that produced by maximal β-adrenergic stimulation. The effect of GW328267C was inhibited by amiloride but was not affected by cystic fibrosis transmembrane conductance regulator inhibition. The drug was tested in three models of ALI, HCl instillation 1 h, LPS instillation 16 h, and live Escherichia coli instillation 2 h before GW328267C instillation. After either type of injury, GW328267C (10−4 M) decreased pulmonary edema formation and restored AFC, measured 1 h after GW328267C instillation. These findings show that GW328267C has beneficial effects in experimental models of ALI and may be a useful agent for treating patients with ALI or prophylactically to prevent ALI.

THERE ARE CURRENTLY NO APPROVED drug treatments for patients with acute lung injury (ALI) and/or acute respiratory distress syndrome (ARDS), and there is a significant unmet need for such treatments to be developed. There are ~200,000 patients annually with ALI/ARDS, from various causes, in the United States alone, and the mortality rate is high (17). Aspiration of gastric contents and sepsis are two of the most common clinical events associated with development of ARDS. The mortality rate for ARDS resulting from acid aspiration ranges from 40–50% and from sepsis ranges from 25–35% (15). Supportive care with lung-protective ventilation (1) and a fluid-conservative strategy (20) has succeeded in reducing mortality and morbidity in randomized multicenter clinical trials; however, even in these trials, mortality persists at a high level of ~21–25% (10, 16). Several experimental studies have demonstrated that the primary mechanism that drives alveolar fluid clearance (AFC) (i.e., resolution of alveolar and pulmonary edema) is active vectorial Na+ and Cl− transport across the alveolar epithelium. Two recent review articles summarize much of this work (5, 11).

Several studies have suggested a role for adenosine receptors in regulating fluid transport in the lung (3, 9, 13). Furthermore, these studies point to the A2A subtype of adenosine receptor (A2AR) as playing a role to enhance fluid transport. This suggests that the A2AR may be a valid pharmacological target for drugs that would enhance AFC. The current studies were intended to test the potential therapeutic value of the A2AR agonist GW328267C in accelerating resolution of alveolar edema and ALI/ARDS in a rat model.

GW328267C is a potent and selective agonist at the adenosine A2A receptor and weaker antagonist at the A3 receptor. As a consequence of its A2A agonist properties, GW328267C also modifies the activity of a number of human inflammatory cells, including both the eosinophil and neutrophil, inhibiting cell recruitment and activation. The consequences of A3 antagonism are more difficult to predict, as less is known about the distribution and function of this receptor subtype; however, the compound has about a 100-fold lower affinity for the A3 subtype, so its actions at the A2A receptor predominate. We have tested GW328267C because it has been selectively developed for human use and has been tested in some human studies. GW328267C has a short elimination half-life of ~1 h and is soluble in 0.9% sodium chloride solution (GlaskoSmith-Kline, unpublished observations). It also has high specificity for the A2AR compared with other A2A ligands, so there is less concern for off-target effects of this compound.

The first objective of these studies was to determine whether GW328267C affected epithelial integrity and function, measured as AFC, in normal rat lungs. The second objective was to study the effects of GW328267C on three main causes of ARDS and ALI including, endotoxemia, Escherichia coli (E. coli) pneumonia, and acid aspiration-induced ALI in rats.

MATERIALS AND METHODS

**Animals**

Adult male Sprague-Dawley rats (wt = 300–350 g; Charles River, Wilmington, MA) kept on a 12-h:12-h light/day rhythm with free access to standard rat chow (Purina; Copley Feed, Copley, OH) and tap water were used in the study. All studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Northeast Ohio Medical University, Rootstown, OH.
Surgical Procedures and Ventilation

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt; Nembutal; Abbott, Chicago, IL). A 2.0-mm (I.D.) endotracheal tube (PE-240, Clay Adams; Becton Dickinson, Sparks, MD) was inserted through a tracheotomy, and a 0.58-mm (I.D.) catheter (PE-50, Clay Adams; Becton Dickinson) was inserted in the left carotid artery. Pancuronium bromide (0.3 mg/kg body wt × h; Sicor Pharmaceuticals, Irvine, CA) was administered through the arterial catheter for neuromuscular blockade. Pupil dilation, blood pressure, and heart rate were used as indicators of anesthesia. Additional anesthetic was administered when necessary.

The rats were maintained in the left lateral decubitus position during the experiment, connected to a volume-controlled rodent ventilator (Harvard Apparatus, Nantucket, MA) and ventilated with a FiO₂ of 1.0, a respiratory rate of 45–50 breaths/min, and 2.8 ± 0.2-ml tidal volume. Positive end expiratory pressure was kept at 2–3 cm H₂O. The measured peak airway pressure was 12 ± 4 cm H₂O during the baseline period for all rats. Peak airway pressures, arterial blood pressures, and heart rates were measured with calibrated pressure transducers connected to analog-to-digital converters and amplifiers (AD Instruments, Colorado Springs, CO).

General Experimental Protocol

A blood sample was withdrawn from the carotid artery, 5 min before the fluid instillation, every 30 min during the study, and at the end of the study, for arterial blood gas and pH measurements using an iSTAT blood gas analysis system (Abbott Point of Care, Princeton, NJ). After a 30-min baseline period of stable heart rate and blood pressure, an instillation tube (PE-50) was passed through the tracheal tube into the left lung without interrupting ventilation. The instillation (3–4 ml/kg body wt) was instilled over 20 min by infusing 0.05 ml/min with a 1-ml syringe. The studies lasted 1–4 h, depending on the specific protocol, and from the beginning of fluid instillation.

At the end of the experiment, a final blood sample was taken and the animal was exsanguinated by transecting the renal artery. The lungs and heart were removed en bloc through a midline sternotomy. A PE-50 catheter was passed into the instilled lung, and residual lung fluid was collected. The protein concentration in fluid aspirated with a catheter wedged into the distal air spaces is a good reflection of the lung fluid protein concentration (6, 12). Protein concentrations in the instillate and in final lung fluid samples were measured with the Lowry method (8). The instillate, aspirate, lungs, and plasma were snap frozen in liquid nitrogen and stored at −80°C until further analysis.

AFV was calculated from the increase in lung fluid albumin concentration over the 1-h study (5, 11) as: 

\[ \text{AFV} = \frac{(V_1 - V_F)V_1}{V_1} \times 100 \]

where \( V_1 \) is the instilled fluid volume and \( V_F \) is the final lung fluid volume calculated from the increase in protein concentration over the 1-h experiment; 

\[ V_F = \frac{(V_1 \times C_1)}{C_F} \]

where \( V_1 \) and \( V_F \) are as above and \( C_1 \) and \( C_F \) are the protein concentrations of the final lung fluid and the instilled fluid, respectively.

Control and Concentration-Response Determination

For control and concentration-response studies, a 5% albumin instillate was prepared by dissolving 50 mg/ml bovine serum albumin (SeraCare Life Sciences, Oceanside, CA) in 0.9% NaCl. In the concentration-response studies, the test compound GW328267C (GlaxoSmithKline, King of Prussia, PA) was added at the following concentrations: 10⁻³ M, 10⁻⁴ M, 10⁻⁵ M, and 0 M (control) to the 5% albumin instillate. The concentrations in the rat studies were chosen to demonstrate a concentration-dependent effect in the various experiments. Studies were done over 1 h to determine the specific effects of the drug on lung fluid balance.

Inhibitor Studies and Comparison Studies to β₂ARs

In these studies, the test compound GW328267C was added to the 5% albumin instillate with and without stimulatory terbutaline, with and without inhibitory amiloride, and with and without inhibitory cystic fibrosis transmembrane conductance regulator (CFTR)inh-172. In these studies, we also injected FITC-labeled albumin intravenously to measure protein movement across the lung endothelium and the epithelial barriers of the lung. We used terbutaline (Sigma, St. Louis, MO) at a concentration of 10⁻⁴ M, we used amiloride (MP Biomedicals, Aurora, OH) at 10⁻⁴ M, and we used the CFTRinh-172 (Sigma) at 10⁻³ M.

Pathological Studies

In these studies, we used a test solution of 5% albumin with and without 10⁻⁴ M GW328267C dissolved in 0.9% NaCl and instilled it into the airspaces of rats to measure AFC in the presence and absence of lung injury produced by HCl, LPS, or *E. coli* (see below for details). Before test solution instillation, we injected FITC-labeled albumin intravenously to measure bidirectional protein movement across the lung endothelial and epithelial barriers. Studies were carried out over 1–2 h to determine the effects of GW328267C on lung fluid balance during these three pathological conditions. For all pathological studies, the blood was sampled for fluorescence and arterial blood gas measurements every 30 min throughout the experiment. All rats were then euthanized and samples (blood, alveolar edema fluid, lung tissue) were obtained. AFC, extravascular lung water, and endothelial-epithelial barrier leak were determined.

HCl studies. To study the effect of HCl, a model of aspiration-induced ALI, we used HCl in the following setting (Fig. 1A). A solution of 100 mosmol/kg of NaCl (1/3 normal saline) was prepared with isotonic 0.9% saline and distilled water. The 100 mosmol osmolality was chosen to match the osmolality of gastric aspirates. HCl was then added to the solution and titrated to a pH of 1.5. In negative control studies, 1/3 normal saline was used as the instillate. These rats were surgically prepared as above. The rats were ventilated for a 30-min baseline of stable blood gases, the vascular tracer (FITC-labeled albumin) was given after 15-min stabilization, and HCl, pH 1.5, was then instilled into the distal airspaces of the lung after the 30-min baseline. The injury was allowed to develop for 1 h. After 1 h, the test solution (5% albumin with and without GW328267C) was instilled to cover the same area of the lung as the acid, and the rats were followed for an additional 1 h.

LPS studies. To study the effect of endotoxin (lipopolysaccharide; LPS), a model of sepsis-induced acute lung injury, a solution of 5 mg/kg body wt LPS (from *E. coli* serotype O111:B5; Sigma) was prepared in isotonic 0.9% NaCl and given intratracheally in the following setting (Fig. 1B). This solution was prepared fresh before all experiments. The rats were instilled intratracheally with 5 mg/kg body wt LPS (from *E. coli* serotype O111:B5) in 1 ml/kg body wt 0.9% NaCl under a brief isoflurane anesthesia. Sixteen hours later, the rats were surgically prepared as described above. The rats were ventilated for a 30-min baseline of stable blood gases, the vascular tracer (FITC-labeled albumin) was given after 15-min stabilization, and the 5% albumin solution with and without GW328267C was instilled into the distal airspaces of the lung.

*E. coli* studies. To study the effect of live *E. coli*, a model of pneumonia-induced ALI, we used freshly prepared *E. coli* (serotype DH5α) at the concentration of 10⁶ cfu/ml added to the 5% albumin instillate and given intratracheally in the following setting (Fig. 1C). The rats were surgically prepared as above. After a 30-min baseline of stable blood gases, an instillation catheter was passed to rest just above the bronchial carina, and 1 ml/kg body wt of the solution containing 10⁶ cfu/ml *E. coli* DH5α was rapidly instilled into the lungs. The vascular tracer (FITC-labeled albumin) was given after 15-min stabilization time, and the 5% albumin solution with and without GW328267C was instilled 2 h later into the distal airspaces of the lungs.

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After a total of 3 h after the live \textit{E. coli} instillation, the rats were euthanized, and samples (blood, alveolar edema fluid, lung tissue) were obtained. BAL studies. Bronchoalveolar lavage (BAL) studies were carried out on separate sets of control, HCl-instilled, LPS-instilled, and live \textit{E. coli}-instilled rats with and without GW328267C to determine whether the influx of inflammatory cells into the lung was affected by GW328267C. These rats were treated as above with the difference that, instead of endothelial-epithelial barrier permeability and AFC measurements, we lavaged the lungs of these rats. Five milliliters of a solution of 0.9% NaCl containing 0.5% lidocaine was introduced into the lungs and mixed with the preexisting pulmonary edema fluid four to five times before finally aspirating the fluid as a sample. This procedure was then repeated four times. Typically a 90–95% recovery of instilled fluid was aspirated as lavage fluid from each rat. Cell numbers were then corrected for the recovery of the BAL fluid.

Extravascular lung water. To measure extravascular lung water in rat lungs, we modified the original method described previously (18). The lungs were rapidly excised, hearts removed, and placed in preweighed sample tubes and reweighed. Water (1 ml) was added, and lungs were weighed again and homogenized using a Tissue Tearor. If extravascular lung water determinations were not done on the day of lung harvest, collected lungs were weighed, water (~1 ml) was added, and lungs were reweighed and stored frozen at −20°C until analysis. Parts of lung homogenates were centrifuged 5 min at 14,000 g. Blood was collected from the rat via the carotid artery catheter to obtain a hemoglobin (Hb) value for rat blood. Hb content was measured on supernatants obtained after centrifugation of the lung homogenates, and blood volumes of the rat lungs were calculated from homogenate supernatant Hb concentration relative to blood Hb concentration. Rat blood wet-dry weights were corrected for blood volume. Drying of lung homogenates, lung homogenate supernatants, and rat blood was carried out using a moisture analyzer (Sartorius, Edgewood, NY) that continuously recorded water loss as samples dried. Each sample was dried at 80–120°C until dry weights reached stability. Typically, this procedure required 15 min/sample. Nonspecific water loss of wet samples and nonspecific rehumidification of dried samples, as may occur when small samples are measured by traditional extravascular lung water techniques, were prevented in this analysis. We verified the technique by comparing it to traditional techniques (6, 12) in adult lungs and found an excellent correlation in extravascular lung water between both techniques.

Lung vascular permeability. For measurement of lung endothelial permeability to protein, the clearance of the vascular tracer protein, FITC-labeled albumin, across the endothelium into the extravascular compartments of the lungs was measured. The total extravascular FITC-labeled albumin accumulation in the lung was calculated by taking the total lung FITC-labeled albumin (in lung homogenate and in the alveolar samples) and subtracting the vascular space FITC-labeled albumin. The FITC-labeled albumin in the vascular space was calculated by multiplying the mean FITC-labeled albumin fluorescence in the plasma by the calculated plasma volume in the lungs, as we have done previously (14). The extravascular accumulation of FITC-labeled albumin in the lung was expressed as plasma equivalents, or the milliliters of plasma that would account for the fluorescence in the lung.

Biochemical and Biomarker Studies

Protein levels in the BAL fluid were measured by the Lowry technique modified for microtiter plates. Von Willebrand factor anti-
gen (vWF) concentrations were measured in plasma and BAL fluid samples by an ELISA assay (IMUBIND vWF ELISA Kit; American Diagnostica, Stamford, CT). Receptor for Advanced Glycation End products (sRAGE) concentrations in airspace fluid and in BAL fluid were also measured by an ELISA assay (Quantikine Human sRAGE ELISA Kit; R&D Systems, Minneapolis, MN).

Transporter Regulation

Total RNA was extracted from lung tissue from each rat using a tissue RNA isolation kit from GE Health Care (Little Chalfont, Buckinghamshire, UK). RNA yield and purity were determined spectrophotometrically at 260/280 nm, and RNA integrity was verified by agarose gel electrophoresis. RT-PCR was carried out using the One-Step RT-PCR kit from EMD Biosciences (San Diego, CA) in a 25-μl system containing 50 ng of total RNA, 1× PCR buffer, 0.2 mM of each dNTP, 2.5 mM MgSO₄, 0.1 μM of each primer, and 1.5 U of rTth DNA polymerase, under the following optimized reaction conditions: 60°C 30 min for reverse transcription, followed by 40 cycles at 94°C for 45 s, 60°C for 2 min, and final extension for 7 min at 60°C. Two pairs of primer sets (+, sense; −, antisense) were derived from the GenBank sequences, and synthesized for the RT-PCR: ENaC (NM_031548); +: 5′-CATGATGTACTGGCAGTTCGC-3′; −: 5′-TGCTGTA-3′; ENaC fragment and

Statistical Analysis

One-way ANOVA with repeated-measures analysis was used to compare samples obtained at several time points from the same animal. One-way ANOVA (factorial) was used when comparing other single groups. Tukey’s test was used as a post hoc statistical test. The gels were scanned by a Typhoon 8610 Scanner and analyzed by TotalLab Quant software. Values are expressed as either means ± SD or means ± SE as indicated in tables and figure legends.

RESULTS

Physiology/Pharmacology Studies

Hemodynamics. GW328267C instilled into the lungs at concentrations of 10⁻³ M, 10⁻⁴ M, or 10⁻⁵ M added to the 5% albumin solution did not significantly alter the hemodynamic parameters measured in this study, compared with the effects observed following instillation of the albumin solution alone (Table 1).

Table 1. Effects of GW328267C on hemodynamic parameters

<table>
<thead>
<tr>
<th>Instillate</th>
<th>HR</th>
<th>Systolic BP</th>
<th>Diastolic BP</th>
<th>MAP</th>
<th>pO₂</th>
<th>pCO₂</th>
<th>pH</th>
</tr>
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<tbody>
<tr>
<td>Baseline (preinstillation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>5% albumin</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 10⁻⁵ M GW328267C</td>
<td>398 ± 45</td>
<td>146 ± 34</td>
<td>129 ± 21</td>
<td>135 ± 25</td>
<td>510 ± 77</td>
<td>38 ± 6</td>
<td>7.4 ± 0.1</td>
</tr>
<tr>
<td>+ 10⁻⁴ M GW328267C</td>
<td>340 ± 62</td>
<td>153 ± 15</td>
<td>130 ± 12</td>
<td>138 ± 12</td>
<td>577 ± 33</td>
<td>37 ± 4</td>
<td>7.4 ± 0.1</td>
</tr>
<tr>
<td>+ 10⁻³ M GW328267C</td>
<td>367 ± 56</td>
<td>139 ± 26</td>
<td>126 ± 22</td>
<td>130 ± 23</td>
<td>543 ± 67</td>
<td>39 ± 4</td>
<td>7.4 ± 0.1</td>
</tr>
</tbody>
</table>

60 min after instillation

| 5% albumin |    |             |             |     |     |      |    |
| + 10⁻⁵ M GW328267C | 405 ± 102 | 136 ± 28 | 129 ± 25 | 131 ± 26 | 486 ± 173 | 41 ± 2 | 7.4 ± 0 |

Values are means ± SD. n = 6–8 per condition. HR, heart rate; BP, blood pressure; MAP, mean arterial pressure.

Vascular permeability. There were no signs of increased pulmonary endothelial permeability (measured as extravascular plasma equivalents) or pulmonary edema formation (measured as extravascular lung water) that could be attributable to GW328267C (Table 2).

AFC. GW328267C, at four different concentrations, was instilled into the airspaces at the same time that the 5% albumin solution was instilled, and the rats were studied for 1 h. The rate of AFC was increased as the instilled concentration of the drug was increased (Fig. 2A).

Lack of additive stimulation by GW328267C of β₂AR-stimulated AFC. We then tested whether GW328267C could stimulate AFC above and beyond that produced by the β₂AR agonist terbutaline. We used GW328267C at 10⁻⁴ M together with 10⁻³ M terbutaline instilled into the distal airspaces at the same time as the 5% albumin solution, and the rats were then studied for 1 h. We have previously shown this concentration of terbutaline to produce maximal stimulation of AFC (6). There were no additive effects produced by GW328267C over terbutaline-stimulated AFC over 1 h (Fig. 2B).

Amiloride-inhibition of GW328267C stimulated AFC. One well-defined and likely mechanism for the increased AFC observed after GW328267C instillation is activation of epithelial sodium channel (ENaC). To test for an interaction of GW328267C with ENaC, we examined its interaction with amiloride, an ENaC blocker. In these studies, we used GW328267C at 10⁻⁴ M with 10⁻⁴ M amiloride instilled into the distal airspaces at the same time as the 5% albumin solution, and the rats were then studied for 1 h. For comparison, we used normal control rats given the same amiloride concentration. Amiloride inhibited GW328267C-stimulated AFC to a greater degree than what was observed in control rats, suggesting that GW328267C stimulated AFC by activation of amiloride-sensitive pathways, e.g., ENaC, over 1 h (Fig. 2C).

Table 2. Effects of GW328267C on lung fluid and protein balance

<table>
<thead>
<tr>
<th>Instillate</th>
<th>Extravascular Plasma Equivalents</th>
<th>Extravascular Lung Water</th>
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<tbody>
<tr>
<td>5% albumin</td>
<td>0.02 ± 0.01</td>
<td>7.79 ± 0.23</td>
</tr>
<tr>
<td>+ 10⁻⁵ M GW328267C</td>
<td>0.01 ± 0.02</td>
<td>8.36 ± 0.15</td>
</tr>
<tr>
<td>+ 10⁻⁴ M GW328267C</td>
<td>0.01 ± 0.02</td>
<td>7.9 ± 0.74</td>
</tr>
<tr>
<td>+ 10⁻³ M GW328267C</td>
<td>0.01 ± 0.02</td>
<td>7.59 ± 0.28</td>
</tr>
</tbody>
</table>

Values are means ± SD. n = 6–8 per condition.
**CFTR<sub>inh-172</sub> inhibition of GW328276C-stimulated AFC.** We then focused our attention on a second described mechanism (CFTR activation) that may be involved in regulating the stimulated AFC after GW328267C instillation. In these studies, we used GW328267C at 10<sup>-5</sup> M with 10<sup>-4</sup> M CFTR<sub>inh-172</sub> instilled into the distal airspaces at the same time as the 5% albumin solution, and the rats were then studied for 1 h. For comparison, we used normal control rats given the same CFTR<sub>inh-172</sub> concentration. CFTR<sub>inh-172</sub> failed to inhibit GW328267C-stimulated AFC, indicating that GW328267C did not stimulate AFC by activation of CFTR<sub>inh-172</sub>-sensitive pathways, e.g., CFTR, over 1 h (Fig. 2D). To verify this observation, we increased the dose of CFTR<sub>inh-172</sub> to 10<sup>-3</sup> M, but the results were the same (AFC = 39 ± 12%, n = 3).

**aENaC expression after GW328267C instillation.** One explanation for the changes in amiloride sensitivity observed is that GW328267C could be related to the change in expression of aENaC mRNA. Thus we carried out an RT-PCR in the animals given the increasing doses of GW328267C and discovered that aENaC mRNA also increased as the GW328267C dose increased (Fig. 3).

**Pathological Studies**

**Hemodynamics.** All three ALIs, HCl instillation, LPS instillation, and *E. coli* instillation, produced a similar decrease in MAP (Table 4). The change was similar in all treatment groups. The changes in MAP that occurred in the treatment groups were not different from each other. Other hemodynamic parameters, i.e., arterial blood gases and pulse, confirmed that we were studying rats with ALI, but administration of GW328267C did not show improvement in these parameters that was better than control.

**Fig. 2. Effects of GW328267C on alveolar fluid clearance.** Values are mean ± SD. *P < 0.05. A: alveolar fluid clearance over 1 h in rats instilled with 0 M (control), 10<sup>-5</sup> M, 10<sup>-4</sup> M, and 10<sup>-3</sup> M GW328267C (n = 6 per group). B: comparison of the stimulatory effect from the β<sub>2</sub>-AR agonist terbutaline (Terb) and GW328267C and determination of additive stimulation from 10<sup>-4</sup> M GW328267C on 10<sup>-5</sup> M terbutaline-stimulated alveolar fluid clearance (n = 6 per group). C: alveolar fluid clearance in control and in 10<sup>-4</sup> M GW328267C-instilled rats with and without intra-alveolar amiloride (Amil) (10<sup>-4</sup> M) (n = 6 per group). D: alveolar fluid clearance in control and in 10<sup>-4</sup> M GW328267C-instilled rats with and without intra-alveolar cystic fibrosis transmembrane conductance regulator (CFTR)<sub>inh-172</sub> (10<sup>-3</sup> M) (n = 4 per group).

**Fig. 3. a-Epithelial sodium channel (ENaC) mRNA expression after instillation of increasing doses of GW328267C in rat lungs.** bp, base pairs; C, control; OD, optical density; GAPDH, housekeeping gene. GW328267C significantly increased a-ENaC mRNA expression in a concentration-dependent manner (P < 0.05).
Pulmonary edema formation. To determine pulmonary edema formation after HCl, LPS, and live E. coli instillation, we examined extravascular lung water (EVLW) and excess lung water (ELW). EVLW and ELW represent excellent measures of pulmonary edema formation. There was a significant increase in EVLW 2 h after HCl instillation that indicated severe pulmonary edema (Fig. 4A). GW328267C by itself did not result in edema formation measured as increased EVLW in any experimental group. However, when GW328267C was administered to the rats that 2 h earlier received HCl, a reduction in EVLW was observed, albeit EVLW remained elevated in those rats (Fig. 4A). In LPS-instilled rats, there was also a significant increase in EVLW 16 h after LPS instillation (Fig. 4B). When GW328267C was administered to the rats that 16 h earlier received LPS, a drastic reduction in EVLW was observed (Fig. 4B). In live E. coli-instilled rats, there was again a significant increase in EVLW 3 h after live E. coli instillation (Fig. 4C), and GW328267C produced a large reduction in EVLW (Fig. 4C).

Another way of determining the extent of edema formation in the lung is to determine the amount of excess fluid present in the lung when an edematous (injured) lung wet-dry weight is compared with that in a healthy, normal lung by comparing ELW. There was a significant increase in ELW 2 h after HCl instillation that indicated increased vascular permeability (Fig. 5A). GW328267C by itself did not result in increased vascular leak of proteins measured as increased EPE in any group. However, when GW328267C was administered to the rats that 2 h earlier received HCl, a reduction in EPE was observed, although the EPE remained elevated above the control level (Fig. 5A). There was also a significant increase in EPE 16 h after LPS instillation that indicated increased vascular permeability (Fig. 5B). GW328267C produced a reduction in EPE in the rats that received LPS (Fig. 5B). A similar reduction in EPE was observed when GW328267C was administered 3 h after live E. coli instillation (Fig. 5C).

Pulmonary epithelial permeability. For alveolar edema to form, it is required that the lung epithelia become injured and leaky to solutes (protein) and fluid. To determine whether there was increased alveolar epithelial injury, we measured the accumulation of the vascular protein tracer as the ratio between its concentration in the alveolar space and its plasma concentration. Normally, the pulmonary epithelium is practically impermeable to the tracer, and this was not altered by GW328267C alone (Table 3). There was a significant increase in the alveolar-to-plasma ratio of the vascular protein tracer 2 h after HCl instillation, indicating increased alveolar epithelial barrier injury (Table 3). GW328267C by itself did not result in increased vascular leak of proteins measured as increased EPE in any group. However, when GW328267C was administered to the rats that 2 h earlier received HCl, a reduction in EPE was observed, although the EPE remained elevated above the control level (Fig. 5A). There was also a significant increase in EPE 16 h after LPS instillation that indicated increased vascular permeability (Fig. 5B). GW328267C produced a reduction in EPE in the rats that received LPS (Fig. 5B). A similar reduction in EPE was observed when GW328267C was administered 3 h after live E. coli instillation (Fig. 5C).

Pulmonary vascular permeability. To determine possible causes for pulmonary edema formation after HCl, LPS, and live E. coli instillation, we examined endothelial permeability changes as extravascular plasma equivalents (EPE). EPE is an excellent measure of pulmonary vascular permeability changes during ALI. There was a significant increase in EPE 2 h after HCl instillation that indicated increased vascular permeability (Fig. 5A). GW328267C by itself did not result in increased vascular leak of proteins measured as increased EPE in any group. However, when GW328267C was administered to the rats that 2 h earlier received HCl, a reduction in EPE was observed, although the EPE remained elevated above the control level (Fig. 5A). There was also a significant increase in EPE 16 h after LPS instillation that indicated increased vascular permeability (Fig. 5B). GW328267C produced a reduction in EPE in the rats that received LPS (Fig. 5B). A similar reduction in EPE was observed when GW328267C was administered 3 h after live E. coli instillation (Fig. 5C).

Table 3. Effects of GW328267C on the accumulation of extravascular lung water and the increase in pulmonary epithelial permeability produced by lung injury

<table>
<thead>
<tr>
<th>Injury</th>
<th>Excess Lung Water, ml</th>
<th>Alveolar Plasma Ratio of Tracer</th>
<th>BAL Protein, mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0 ± 0.1</td>
<td>0.01 ± 0.02</td>
<td>3.4 ± 0.9</td>
</tr>
<tr>
<td>GW328267C</td>
<td>1.1 ± 0.1</td>
<td>0.01 ± 0.01</td>
<td>3.1 ± 0.8</td>
</tr>
<tr>
<td>HCl</td>
<td>2.3 ± 0.4*</td>
<td>0.53 ± 0.25*</td>
<td>5.8 ± 0.6*</td>
</tr>
<tr>
<td>HCl + GW328267C</td>
<td>1.9 ± 0.3†</td>
<td>0.25 ± 0.09†</td>
<td>3.9 ± 0.8†</td>
</tr>
<tr>
<td>LPS</td>
<td>2.1 ± 0.6*</td>
<td>0.12 ± 0.03*</td>
<td>7.1 ± 0.8*</td>
</tr>
<tr>
<td>LPS + GW328267C</td>
<td>1.5 ± 0.1†</td>
<td>0.06 ± 0.04†</td>
<td>4.6 ± 0.5†</td>
</tr>
<tr>
<td>E. coli</td>
<td>1.7 ± 0.2*</td>
<td>0.80 ± 0.30*</td>
<td>5.7 ± 0.8*</td>
</tr>
<tr>
<td>E. coli + GW328267C</td>
<td>0.9 ± 0.2†</td>
<td>0.25 ± 0.04†</td>
<td>3.7 ± 0.3†</td>
</tr>
</tbody>
</table>

Values are means ± SD. n = 6–8 per condition. *P < 0.05 compared to control; †P < 0.05 compared to injured. BAL, bronchoalveolar lavage.
permeability. When GW328267C was administered to the rats that 2 h earlier had received HCl, a decrease in the alveolar-to-plasma ratio was observed, although it remained elevated compared with control. There was also a significant increase in the alveolar-to-plasma ratio of the vascular protein tracer 16 h after LPS instillation. When GW328267C was administered to the rats that had received LPS, a decrease in the alveolar-to-plasma ratio was observed. There was also a significant increase in the alveolar-to-plasma ratio of the vascular protein tracer 3 h after live E. coli instillation. Once again, GW328267C decreased the alveolar-to-plasma ratio.

Influx of inflammatory cells into the distal airspaces. To determine whether there was increased influx of inflammatory cells into the lungs, we lavaged the lungs following the induction of the ALI. There was a significant increase in the white blood cell (WBC) counts in the BAL 2 h after HCl instillation, indicating increased cellular influx into the airspaces (Fig. 6A). GW328267C by itself did not affect the BAL WBC counts. However, when GW328267C was administered to the rats that 2 h earlier received HCl, a greater increase in the BAL WBC counts was observed than when HCl alone was administered (Fig. 6A). There was also a significant increase in WBC counts in the BAL 16 h after LPS instillation that indicated increased cellular influx into the airspaces (Fig. 6B). When GW328267C was administered to the rats that 16 h earlier received LPS, a decrease in the BAL WBC counts was observed (Fig. 6B). There was also a significant increase in WBC counts in the BAL 3 h after live E. coli instillation that produced by HCl or LPS (Fig. 6C). However, when GW328267C was administered to the rats that 3 h earlier received live E. coli, no decrease in BAL MØ or PMN counts was observed (Fig. 6C). No changes in or no basophile, eosinophil, or lymphocyte counts in the BAL were observed in any condition.

AFC. There are mechanisms that work to protect the lung from pulmonary edema formation by actively removing excess alveolar lung fluid. The principal mechanism for this is lung epithelial transport of Na that leads to absorption of alveolar edema fluid. To examine the effect of HCl on AFC and to determine whether GW328267C would be able to enhance AFC, we measured this in HCl-instilled rats with or without the addition of GW328267C. Instillation of HCl 2 h earlier completely abolished AFC and in fact induced secretion of fluid into the airspaces (AFC was negative) (Fig. 7A). GW328267C decreased the rate of alveolar fluid secretion (leak) in these HCl instilled rats despite the lung injury (Fig. 7A). We also measured this in LPS-instilled rats with or without the addition of GW328267C. Instillation of LPS 16 h earlier did not in itself affect AFC (Fig. 7B). GW328267C increased AFC in these LPS-instilled rats (Fig. 7B). Instillation of live E. coli stimulated AFC (Fig. 7C), a phenomenon observed in earlier experimental studies (11). GW328267C further increased the rate of AFC in these live E. coli-instilled rats, despite the lung injury (Fig. 7C).

Biochemical Parameters and Biomarkers

Protein concentration in the BAL. If there was a significant endothelial and epithelial injury following exposure to HCl, LPS, or live E. coli, we might expect to observe elevated protein levels in the BAL. Thus we measured the protein concentrations in the BAL and adjusted for differences in...
recovery. Instillation of HCl 2 h earlier increased BAL protein levels (Table 3). However, GW328267C decreased the BAL protein levels, although not to control/GW328267C levels. Instillation of LPS 16 h earlier also increased BAL fluid protein concentrations. GW328267C administration again reduced the BAL protein levels when administered after LPS injury. In live \textit{E. coli}-instilled rats with or without the addition of GW328267C, instillation of live \textit{E. coli} increased BAL fluid protein concentrations, and GW328267C administration reduced BAL protein levels in live \textit{E. coli}-instilled rats despite the lung injury.

\textit{vWF ag} concentration in plasma and in BAL. \textit{vWF ag} is an excellent marker of endothelial cell injury (11). If there was a significant endothelial injury following exposure to HCl, LPS, or live \textit{E. coli}, we might expect to observe elevated \textit{vWF ag} levels in plasma and in the BAL. Thus we measured \textit{vWF ag} concentrations in plasma and in the BAL and adjusted the values for differences in BAL recovery. We measured BAL \textit{vWF ag} concentration in HCl-injured rats with or without the addition of GW328267C. Instillation of HCl increased plasma and BAL \textit{vWF ag} levels (Fig. 8, A and D). Instillation of LPS earlier also increased plasma and BAL fluid \textit{vWF ag} concentrations (Fig. 8, B and E). GW328267C administration reduced the plasma \textit{vWF ag} levels when administered to LPS-treated rats (Fig. 8B), but did not affect the \textit{vWF ag} levels in BAL when administered together with LPS (Fig. 8E). Instillation of live \textit{E. coli} did not affect plasma (Fig. 8C) but increased BAL fluid \textit{vWF ag} concentrations (Fig. 8F). GW328267C administration did not affect plasma or BAL \textit{vWF ag} levels in live \textit{E. coli}-instilled rats (Fig. 8, C and F).

\textit{sRAGE} concentration in airspace fluid and in BAL. \textit{sRAGE} is a novel marker of alveolar epithelial cell injury (19). If there were a significant alveolar epithelial injury following exposure to HCl, LPS, or live \textit{E. coli}, we might expect to observe elevated \textit{sRAGE} levels in airspace fluid and in the BAL. The BAL fluid \textit{sRAGE} levels were very low and near the detection level of the assay. The levels in the concentrated airspace fluid samples were well in the detection range and thus probably represent the more reliable values. Instillation of HCl 2 h earlier increased airspace fluid and BAL \textit{sRAGE} levels (Fig. 9, A and D). GW328267C decreased the airspace fluid and BAL \textit{sRAGE} levels (Fig. 9, A and D). Instillation of LPS also increased airspace fluid and BAL fluid \textit{sRAGE} concentrations.
GW328267C administration again reduced the airspace fluid and BAL fluid sRAGE levels when administered together with LPS (Fig. 9, B and E). Instillation of live E. coli increased airspace fluid and BAL fluid sRAGE concentrations (Fig. 9, C and F). GW328267C administration decreased airspace fluid and BAL sRAGE levels in live E. coli-instilled rats (Fig. 9, C and F).

**DISCUSSION**

These results provide the first published report of the activity of GW328267C, a compound that is over 100-fold more selective for the human A2A than human A1, A2B, or A3 receptors (GlaskoSmithKline, unpublished observations).
Physiological/Pharmacological Studies

GW328267C had a significant and very reproducible stimulatory effect on AFC when instilled into the lungs at concentrations of 10^{-5} M to 10^{-3} M. The stimulatory effect of GW328267C was at least as great as previously published results with β2AR and dexamethasone stimulation of AFC (6). The finding that GW328267C did not produce additive effects to that observed after maximal β2AR stimulation suggests that, although these two drugs activate different receptors, the receptor-induced signaling converges at some point downstream. This would be consistent with both compounds activating receptors that couple to Gs and lead to an increase in intracellular cAMP.

The increase in the amiloride sensitivity of the AFC in rats treated with GW328267C compared with that of control rats suggests that GW328267C stimulates AFC by activating amiloride-sensitive ENaC channels in the lung epithelium. Although αENaC mRNA expression was increased in a concentration-dependent manner following instillation of GW328267C, these data do not prove that this led to an increased ENaC expression within the time course of these experiments but are consistent with either a transcriptional effect and/or an effect on protein translocation.

We also tested whether CFTR would be activated by the GW328267C compound but found that the CFTR inhibitor, CFTRinh-172, failed to block the GW328267C stimulation of AFC. These data suggest that CFTR is not activated by the GW328267C compound. Factor et al. (3) have demonstrated that adenosine mediates inhibition of AFC in rats via an A1R-mediated pathway that does involve the CFTR. Our data show that the A2AR receptor operates independently of the CFTR.

Pathological Studies

We have carried out studies with GW328267C in three clinically relevant lung injury models, including acid aspiration-induced, endotoxin-induced, and live E. coli-induced ALI. Hemodynamically all three pathological condition injuries reduced MAP. GW328267C did not affect this decline in MAP in any condition. GW328267C did not affect the other hemodynamic properties measured (heart rate, arterial blood gases) or the peak airway pressures in these ALI settings.

All three ALIs resulted in significantly increased edema formation, measured as EVLW and ELW. Acid aspiration resulted in a higher EVLW and ELW than that of either LPS-induced or live E. coli-induced ALI, indicating more severe injury after HCl instillation. The increases in EVLW and ELW following the LPS-induced ALI, and live E. coli-induced ALIs were largely prevented with GW328267C. EVLW and ELW after HCl-
induced ALI was also reduced but not to the same extent as after the other, less severe, injuries.

Pulmonary edema forms as a result of lung endothelial and alveolar epithelial injury, resulting in an increased epithelial permeability to solutes (protein) and fluid. To determine whether the increased EVLW was related to increased vascular endothelial permeability and/or increased alveolar epithelial permeability, we determined EPE, an excellent measure of lung vascular endothelial permeability, and accumulation of the vascular protein tracer in the airspaces of the lungs, a measure of alveolar epithelial permeability, expressed as an alveolar-to-plasma ratio of the vascular protein tracer. With respect to the EPE, there were increased EPE levels of about 20-fold 16 h after LPS, thus indicating that the increase in EVLW after LPS was due to an increased vascular endothelial permeability. With respect to the alveolar-to-protein ratio, this was moderately increased, ~2.5x, suggesting that the alveolar epithelium had been moderately injured. Both the increased EPE and the alveolar-to-plasma ratio were restored to normal or near-normal levels by GW328267C in the LPS-instilled rats. In contrast, HCl-induced ALI resulted in higher increases in both EPE (~65x) and the alveolar-to-plasma ratio (~10x), again supporting the finding that this injury was more severe. GW328267C only partially prevented the increased EPE after HCl instillation and reduced the alveolar-to-plasma ratio by ~50% after HCl instillation. In fact, it is surprising that GW328267C at all was able to prevent any of the epithelial injury that early after injury as the low pH of 1.5 of the HCl would be expected to chemically erode large parts of the alveolar epithelium. After live E. coli instillation, the EPE accumulation was significantly increased (~20%), indicating severe lung injury. GW328267C administration decreased this EPE accumulation by ~50%. With respect to the alveolar-to-plasma ratio, the increase was higher with E. coli than with either HCl or LPS (~15x). Administration of GW328267C to live E. coli-instilled rats decreased this ratio by ~70%, suggesting that GW328267C can affect alveolar epithelial integrity. This indicated that GW328267C protected the lung against pulmonary edema formation in the live E. coli-instilled rats.

Influx of proinflammatory cells into the airspaces of the injured lung is another important characteristic of ALI. LPS-induced ALI resulted in significant increases in WBC counts in the BAL. This increase was reduced by GW328267C administration; however, because these cells migrated into the airspaces over 16 h, it seems likely that GW328267C exposure for 1 h affected the compartmentalization of the WBCs in the lung (i.e., affected WBC binding in the lung, so we could not recover the correct WBC numbers with our technique). HCl induced an apparent small influx of WBCs into the lung airspaces as observed in the BAL analyses. In this setting, GW328267C in fact seemed to increase the acute increase in influx of WBC into the airspaces. This observation was likely due to a decreased adhesion of the WBCs to the endothelial and epithelial cells in the lung after HCl and GW328267C treatment. In fact, we have previously observed a similar scenario in rabbits when CD11/18 inhibitors were instilled together with HCl (4). Instillation of live E. coli also caused an influx of WBCs into the airspaces that was lower than in the other conditions. In the differential cell counts, it was clear that MØs and PMNs were responsible for the increased WBC counts in rats given HCl with GW328267C. In rats given LPS, both MØs and PMNs were increased and GW328267C administration significantly reduced MØ and PMN numbers, although not to control levels. In live E. coli-instilled rats, there was only a trend toward increased PMNs; GW328267C administration did not reduce these numbers.

As a final, and perhaps the most important, test of the effectiveness of GW328267C in ALI, we determined whether GW328267C was able to enhance the rate of pulmonary edema recovery by measuring AFC. In our LPS-induced ALI model, GW328267C increased AFC despite the massive lung injury. As a further test on the effectiveness of GW328267C in the septic ALI, AFC was measured after instillation of live E. coli. When GW328267C was administered to these animals, AFC was restored to the same level as in untreated GW328267C-control rats. Taken together, these findings suggest that, in sepsis-induced ALI, GW328267C may be useful to enhance recovery from pulmonary edema.

In our HCl-induced ALI model, HCl completely abolished AFC and was associated with considerable alveolar edema. GW328267C treatment in these rats did not completely reverse, but did reduce, the rate that fluid accumulated in the airspaces of the lungs. This result suggests that this compound

Table 4. Hemodynamics in acute lung injury

<table>
<thead>
<tr>
<th>Instillate</th>
<th>HR</th>
<th>Systolic BP</th>
<th>Diastolic BP</th>
<th>MAP</th>
<th>pO2</th>
<th>pCO2</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline (preinstillation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCl</td>
<td>413 ± 47</td>
<td>159 ± 16</td>
<td>132 ± 17</td>
<td>141 ± 16</td>
<td>566 ± 38</td>
<td>38 ± 5</td>
<td>7.45 ± 0.09</td>
</tr>
<tr>
<td>+ 10^-4 M GW328267C</td>
<td>383 ± 31</td>
<td>161 ± 11</td>
<td>138 ± 11</td>
<td>146 ± 11</td>
<td>546 ± 42</td>
<td>36 ± 3</td>
<td>7.42 ± 0.06</td>
</tr>
<tr>
<td>LPS</td>
<td>403 ± 45</td>
<td>142 ± 21</td>
<td>122 ± 23</td>
<td>129 ± 22</td>
<td>311 ± 138</td>
<td>39 ± 3</td>
<td>7.40 ± 0.06</td>
</tr>
<tr>
<td>+ 10^-4 M GW328267C</td>
<td>411 ± 41</td>
<td>154 ± 21</td>
<td>140 ± 18</td>
<td>145 ± 18</td>
<td>378 ± 109</td>
<td>39 ± 7</td>
<td>7.37 ± 0.05</td>
</tr>
<tr>
<td>E. coli</td>
<td>380 ± 49</td>
<td>155 ± 21</td>
<td>137 ± 13</td>
<td>143 ± 15</td>
<td>575 ± 44</td>
<td>47 ± 8</td>
<td>7.45 ± 0.07</td>
</tr>
<tr>
<td>+ 10^-4 M GW328267C</td>
<td>370 ± 45</td>
<td>173 ± 13</td>
<td>147 ± 17</td>
<td>156 ± 14</td>
<td>556 ± 114</td>
<td>43 ± 5</td>
<td>7.46 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>60 min after instillation of 5% albumin w/wo GW328267C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCl</td>
<td>428 ± 50</td>
<td>98 ± 22</td>
<td>79 ± 22</td>
<td>85 ± 22</td>
<td>112 ± 75</td>
<td>48 ± 11</td>
<td>7.25 ± 0.13</td>
</tr>
<tr>
<td>+ 10^-4 M GW328267C</td>
<td>405 ± 150</td>
<td>90 ± 42</td>
<td>74 ± 44</td>
<td>79 ± 43</td>
<td>81 ± 37</td>
<td>45 ± 9</td>
<td>7.20 ± 0.11</td>
</tr>
<tr>
<td>LPS</td>
<td>471 ± 54</td>
<td>103 ± 25</td>
<td>91 ± 23</td>
<td>95 ± 23</td>
<td>118 ± 66</td>
<td>48 ± 9</td>
<td>7.24 ± 0.15</td>
</tr>
<tr>
<td>+ 10^-4 M GW328267C</td>
<td>540 ± 35</td>
<td>104 ± 20</td>
<td>94 ± 20</td>
<td>97 ± 20</td>
<td>164 ± 121</td>
<td>49 ± 12</td>
<td>7.24 ± 0.05</td>
</tr>
<tr>
<td>E. coli</td>
<td>420 ± 38</td>
<td>99 ± 45</td>
<td>88 ± 47</td>
<td>92 ± 46</td>
<td>252 ± 100</td>
<td>41 ± 15</td>
<td>7.41 ± 0.09</td>
</tr>
<tr>
<td>+ 10^-4 M GW328267C</td>
<td>408 ± 93</td>
<td>110 ± 45</td>
<td>103 ± 37</td>
<td>105 ± 40</td>
<td>149 ± 50</td>
<td>40 ± 13</td>
<td>7.00 ± 0.13</td>
</tr>
</tbody>
</table>

Values are means ± SD. n = 6-8 per condition.

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could be useful in enhancing recovery from acid aspiration-induced ALI.

**Biochemical Parameters and Biomarkers**

To more specifically determine the extent of endothelial injury after ALI, we measured vWF ag as a marker of endothelial cell injury (11). We hypothesized that, if there was significant endothelial injury following exposure to HCl, LPS, or live E. coli, we would observe elevated vWF ag levels in plasma and also in the BAL. Instillation of HCl increased both plasma and BAL vWF ag levels, and GW328267C seemed to decrease the plasma and BAL vWF ag levels. Instillation of LPS also increased plasma and BAL fluid vWF ag concentrations, and GW328267C administration again reduced the plasma vWF ag levels when administered together with LPS. However, GW328267C did not affect the vWF ag levels in BAL. Instillation of live E. coli did not affect plasma but increased BAL fluid vWF ag concentrations. GW328267C administration did not affect plasma and BAL vWF ag levels in live E. coli-instilled rats. In general, as expected because this was an endothelial marker, plasma vWF ag levels were higher than BAL fluid levels. The fact that the various injuries resulted in different vWF ag responses to GW328267C and injury itself may be related to the fact that the lung injury was less severe in some cases, induced various levels of endothelial cell injury, or did not directly injure or involve the endothelial cell. In general, the results still support a role for GW328267C in treating ALI.

sRAGE is a novel marker of alveolar epithelial cell injury (19) and was utilized to detect alveolar epithelial injury, especially to the alveolar epithelial type I cells. We therefore hypothesized that, if there were significant alveolar epithelial injury following exposure to HCl, LPS, or live E. coli, we would observe elevated sRAGE levels in the airspace fluid and in the BAL fluid. Instillation of HCl increased both airspace fluid and BAL fluid sRAGE levels, and the GW328267C decreased both the airspace fluid and the BAL fluid sRAGE levels. Instillation of LPS also increased airspace fluid and BAL fluid sRAGE concentrations, and GW328267C administration again reduced the airspace fluid and the BAL fluid sRAGE levels. Instillation of live E. coli increased the airspace fluid and the BAL fluid sRAGE concentrations, and GW328267C administration again decreased airspace fluid and BAL sRAGE levels in live E. coli-instilled rats. We do not have histological data on the lungs, which is a limitation of these studies. However, these results indicate that there was significant alveolar epithelial injury in all three conditions and that GW328267C reduced the epithelial cell injury in this model, as indicated also by the favorable effects on AFC.

In conclusion, GW328267C was effective in reducing ALI by 1) reducing edema formation (reducing EVLW and ELW), 2) reducing vascular endothelial permeability to protein (reducing EPE, BAL fluid protein levels, vWF ag in plasma and BAL fluid), 3) reducing alveolar epithelial cell injury and epithelial leak (reducing alveolar-to-plasma tracer ratio, sRAGE levels in airspace fluid and in BAL fluid), and 4) altering influx of inflammatory cells into the distal airspaces of the lungs (affecting total WBC counts, differential WBC counts).

Interestingly, there was no significant improvement in the arterial oxygenation with the lower lung water values and higher rates of AFC. Table 4 shows no trend for a beneficial effect except in the LPS group. One explanation may be that GW328267C may have some pulmonary vasodilating effects like a β-agonist, thus increasing pulmonary blood flow to the flooded lung regions, as we have reported with microsphere studies in sheep (2).

Given the relatively short half-life, an intravenous formulation, perhaps by continuous infusion, may be required for human efficacy. With regard to side effects, in safety studies, human tolerability at higher doses has been limited by a well-described adenosine side-effect profile including tachycardia, nausea, and chest discomfort that is directly dose related. Separating these side effects from efficacy has been difficult (GlaskoSmithKline, unpublished observations).

These data suggest that GW328267C may act by multiple mechanisms to protect against lung injury. Increasing functional αENaC would lead to increased fluid clearance from the lung. Activation of A2A receptors on inflammatory cells has been shown to decrease inflammation in a variety of tissues (7) and protects against immune-mediated damage. This could also account for part of the action of GW328267C.

GW328267C was effective in increasing recovery from pulmonary edema associated with ALI by stimulating AFC. It also stimulates AFC in the absence of ALI and may be useful to prevent the development of lung injury in patients at risk of developing ALI.

**DISCLOSURES**

David A. Lipson is employed by GlaxoSmithKline, the pharmaceutical company that developed the drug. GlaxoSmithKline funded the work.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


