Sulforhodamine B interacts with albumin to lower surface tension and protect against ventilation injury of flooded alveoli

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Kharge AB, Wu Y, Perlman CE. Sulforhodamine B interacts with albumin to lower surface tension and protect against ventilation injury of flooded alveoli. J Appl Physiol 118: 355–364, 2015. First published November 20, 2014; doi:10.1152/japplphysiol.00818.2014.—In the acute respiratory distress syndrome, alveolar flooding by proteinaceous edema liquid impairs gas exchange. Mechanical ventilation is used as a supportive therapy. In regions of the edematous lung, alveolar flooding is heterogeneous, and stress is concentrated in aerated alveoli. Ventilation exacerbates stress concentrations and injuriously overexpands aerated alveoli. Injury degree is proportional to surface tension, $T$. Lowering $T$ directly lessens injury. Furthermore, as heterogeneous flooding causes the stress concentrations, promoting equitable liquid distribution between alveoli should, indirectly, lessen injury. We present a new theoretical analysis suggesting that liquid is trapped in discrete alveoli by a pressure barrier that is proportional to $T$. Experimentally, we identify two rhodamine dyes, sulforhodamine B and rhodamine WT, as surface active in albumin solution and investigate whether the dyes lessen ventilation injury. In the isolated rat lung, we micropuncture a surface alveolus, instill albumin solution, and obtain an area with heterogeneous alveolar flooding. We demonstrate that rhodamine dye addition lowers $T$, reduces ventilation-induced injury, and facilitates liquid escape from flooded alveoli. In vitro we show that rhodamine dye is directly surface active in albumin solution. We identify sulforhodamine B as a potential new therapeutic agent for the treatment of the acute respiratory distress syndrome.

acute respiratory distress syndrome; ventilation injury; surface tension; alveolar mechanics; rhodamine

THE ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS) has an incidence of ~200,000 cases per year in the United States and a mortality rate exceeding 35% (21, 24). In ARDS, inflammation increases the permeability of the alveolar-capillary barrier such that alveoli are flooded by proteinaceous edema liquid. At the onset of ARDS, discrete alveoli of the dependent lung are flooded; in severe ARDS, essentially all alveoli of the dependent lung are flooded, and discrete alveoli of the nondependent lung are flooded (30). Thus, regardless of disease severity, there is a region of the lungs with heterogeneous alveolar flooding. In such a region, lung ventilation overexpands aerated alveoli to a degree that is proportional to surface tension, $T$ (19, 38). We have recently demonstrated that plasma proteins in edema liquid are unlikely to elevate $T$ above normal levels in ARDS (11). Even with normal $T$, however, ventilation-induced overexpansion is sufficiently severe as to be mechanically injurious (38). A reduction in $T$ below normal reduces, but does not eliminate, overexpansion injury (11, 38).

We have previously presented theory describing a means through which lowering $T$ directly lessens overexpansion injury (38). In a new theoretical analysis, below, we introduce an additional means through which lowering $T$ may, indirectly, lessen injury. Surface tension can be reduced in the isolated lung, despite the presence of plasma proteins, by alveolar instillation of exogenous surfactant (11). This route is not available in vivo, however, and in six clinical trials exogenous surfactant delivery via the trachea has failed to reduce mortality in ARDS (4). It is possible that surfactant transport from the trachea to the alveolus is problematic and may underlie the clinical inefficacy of surfactant therapy. Working in an isolated rat lung model, we report here that two rhodamine dyes carrying positive iminium charges, sulforhodamine B (SRB) and rhodamine WT (RWT), can lower surface tension and reduce ventilation injury in areas with heterogeneous alveolar flooding. The dyes lower $T$ only in the presence of albumin, and SRB is known to bind to albumin (12, 33). In ARDS, with increased barrier permeability to albumin, SRB or RWT might be delivered via the vasculature to the alveolar liquid phase. SRB is approved as a food dye in Japan (32) and may, in particular, be suitable for clinical use. SRB is a potentially new therapeutic agent for ARDS that might be administered via a new route to lessen ventilation injury.

THEORY

If heterogeneous flooding is responsible for injurious over-expansion of aerated alveoli adjacent to flooded alveoli (38), then redistributing liquid more equitably between alveoli should reduce overexpansion injury. To promote liquid escape from/clearance of discretely flooded alveoli, it is necessary to understand what traps liquid in such alveoli.

In flooded alveoli, the air-liquid interface forms a meniscus (1, 11, 19). According to the Laplace relation, and as depicted in Fig. 1, liquid-phase pressure, $P_{LIQ}$, to one side of the meniscus is less than air pressure, $P_{ALV}$, on the other side by an amount that is proportional to surface tension and inversely proportional to meniscus radius:

$$ P_{LIQ} = P_{ALV} - 2T/r_m. \tag{1} $$

From the center of the flooded alveolus to the border between the flooded alveolus and a neighboring alveolus, interfacial curvature changes. At the border, the free end of the septum terminates at the alveolar duct and has a saddle-shaped geometry specified by two orthogonal radii of curvature (2, 23, 36). As the alveolar liquid phase is continuous (2), the air-liquid interface should likewise have a saddle-shaped geometry at the border with the adjacent alveolus. In the plane of Fig. 1, the interface is convex with a small radius $r_{INT\cdot BORD}$ that is similar in magnitude to half the thickness of the alveolar septum. In the plane of the septum, the interface is concave with a large radius equal to that of the alveolar duct,
Causes a decrease in H₂O and then deflate them to a constant PALV of 5 cm H₂O. Reducing stress concentrations between adjacent alveoli, including flooded alveolus PLIQ, there is a pressure barrier, PLIQ-BORD, which exceeds PALV (see text) and thus bulk liquid-phase pressure in the flooded alveolus (37). Lowering T should therefore reduce the pressure barrier both directly, as ΔPb, that traps liquid in the flooded alveolus, ΔPb is proportional to surface tension T.

\[ \Delta P_b = P_{LIQ\cdotBORD} - P_{LIQ} = T \left( 1/r_{INT\cdotBORD} - 1/r_{DUCT} - 2/r_M \right). \]  

As rDUCT is much greater than rINT-BORD, PLIQ-BORD is greater than PALV.

With constant PALV along the interface, liquid-phase pressure must increase from P_LIQ < P_ALV below the meniscus of the flooded alveolus (Eq. 1) to P_LIQ-BORD > PALV at the border of the alveolus (Eq. 2). That is, there is a pressure barrier ΔPb = PLIQ-BORD - PLIQ > 0 that traps liquid in flooded alveoli. Combining Eqs. 1 and 2,

\[ \Delta P_b = P_{LIQ\cdotBORD} - P_{LIQ} = T \left( 1/r_{INT\cdotBORD} - 1/r_{DUCT} + 2/r_M \right). \]  

Lowering T should reduce the pressure barrier both directly, as ΔPb is proportional to T, and indirectly, as a decrease in T causes a decrease in rDUCT (37). Lowering T should thus increase the likelihood of flooded alveolar clearance and, by reducing stress concentrations between adjacent alveoli, indirectly lessen ventilation injury.

**METHODS**

In the isolated rat lung, we infuse proteinaceous solution into surface alveoli to generate a local model of alveolar edema. We assess the effects of SRB, RWT, and sulforhodamine G (SRG) on alveolar interfacial tension.

**Isolated lung preparation.** We handle all animals in accord with a protocol approved by the Stevens Institute of Technology Institutional Animal Care and Use Committee. We anesthetize male, Sprague-Dawley rats (n = 60, 200–425 g) with 1.5–4.0% isoflurane (Penn Veterinary Supply, Lancaster, PA) in oxygen, inject 1,500 U/kg heparin (Penn Veterinary Supply) by cardiac puncture through the chest wall and withdraw ≤10 ml blood. We follow with a tracheotomy and cannulate the trachea. We excise the heart and lungs and position them with the costal surface upward (39). We inflate the lungs to P_ALV (equal, in the isolated lungs, to transpulmonary pressure) of 30 cm H₂O and then deflate them to a constant P_ALV of 5 cm H₂O.

In a subset of experiments in which we assess ventilation injury to the alveolar-capillary barrier, we perfuse the lungs to deliver dye to the capillaries and quantify dye leakage out of the capillaries into the alveolar liquid phase. We prepare the isolated lungs as above, but additionally cannulate the pulmonary artery (PA) and left atrium (LA). We connect the PA and LA cannulas to a perfusion circuit through which we pump, at 12 ml/min and 37°C, 10 ml of autologous blood plus 18 ml of 5% fatty acid-bound bovine serum albumin (Sigma Aldrich, St. Louis, MO) in normal saline. (Note, we do not use any fatty acid-free albumin in this study, whether bovine or human and whether added to the vasculature or the alveolus.) We maintain LA pressure at 3 cm H₂O; PA pressure is 10–12 cm H₂O.

**Local edema model.** We generate a local model of alveolar edema, as described previously (11, 38). With a glass pipette (3–5 μm tip ID), we micropuncture a surface alveolus and infuse model edema liquid. For T determination experiments, we infuse ~1.2 μl; for ventilation injury assessment experiments, we inject ~300 nl. After some alveoli clear, we are left with a region of heterogeneous alveolar flooding in which menisci are present at the mouths of the flooded alveoli (11, 34, 38).

Our base flooding solution is normal saline with 4.6% bovine serum albumin and either 31 μM fluorescein (Cole Parmer, Vernon Hills, IL) or 25 μM 2,7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) (Life Technologies, Grand Island, NY), included for alveolar liquid-phase visualization and verified not to alter surface tension (11). We additionally test solutions with alternative, 0–28% concentrations of bovine serum albumin, a solution of 4.6% human serum albumin (Sigma Aldrich), solutions in which we substitute dextran (Sigma Aldrich) or fibrinogen (Sigma Aldrich) for albumin, or blood plasma that we separate from heparinized rat blood by centrifugation at 5,000 g for 5 min. We determine total protein content of the blood plasma by Bradford assay. We test subsets of the preceding solutions in the absence and presence of the dye SRB (Sigma Aldrich) at 10⁻⁴ to 10² μM and of the dyes RWT (Cole Parmer) and SRG (Sigma Aldrich) at 1 μM to determine rhodamine dye effects on surface tension. We note, for in situ surface-tension determinations reported in the presence of 1 μM SRB or SRG, the stated concentration is a nominal concentration, whereas the actual concentration is 0.9 μM. All other concentrations are as stated.

**Surface-tension determination method.** We determine T in flooded alveoli of the excised lung, as described previously (11). Briefly, we ventilate the lungs twice between PALV of 5 and 15 cm H₂O and then hold the lungs at constant PALV of 5 or 15 cm H₂O. With the lungs statically inflated, we determine flooded alveolar liquid-phase pressure through a hyperosmolar saline solution-filled micropipette (2–4 μm tip ID) connected to a servo-nulling pressure-measurement system (Vista Electronics, Ramona, CA); alveolar air pressure with a transducer at the entrance to the trachea; and radius of curvature of the air-liquid interface in the flooded alveolus by confocal microscopy (SP5; Leica Microsystems, Buffalo Grove, IL). We then calculate T according to the Laplace relation.

We determine T in vitro according to the same principle (11). We place a 3-μl drop of test solution on a steel plate. We measure pressure within the test drop through a hyperosmolar saline-filled pipette connected to the servo-pressure system. With the pipette tip still in the fluid drop, we image the drop by confocal microscopy with an ×10 (0.3 N.A.) objective. Total imaging time is ~1 min. As we do for T determination in the alveolus (11), we use MATLAB (Mathworks, Natick, MA) to fit a sphere to the interface and determine interfacial radius. The coefficient of determination for the sphere fit is R² = 1.0 ± 0.0 (n = 16). We calculate T for the test-solution drop according to the Laplace relation.

**Injury assay method.** As a measure of stress concentration, and as described previously (38), we assess ventilation injury to the alveolar-capillary barrier in regions of heterogeneous alveolar flooding in which the barrier is initially intact. Briefly, in the isolated, perfused rat lung, we include 23 μM fluorescein in the perfusate. By micropuncture, we instill nonfluorescent liquid into surface alveoli. We instill either a small liquid volume, which spontaneously clears (38, 39), to obtain a micropunctured-but-aerated control region or a larger liquid volume to generate our local edema model. We image fluorescein fluorescence in the alveolar liquid at the start and end of a 5-min
baseline period at constant $P_{\text{ALV}}$, of 5 cm H$_2$O, apply five ventilation cycles with a specified end-expiratory pressure (PEEP) and tidal volume ($V_T$) to the lungs; and return the lungs to a constant PALV cycles with a specified positive end-expiratory pressure (PEEP) and return the lungs to a constant PALV.

We perform our analysis on the same alveoli at all time points. We track over time the fraction of these alveoli that are liquid flooded. We normalize each data point by the fraction of alveoli in the analysis region that are included in the analysis.

Inclusion of SRB in the flooding solution does not alter alveolar liquid-phase fluorescence intensity by capillary fluorescein intensity. We report as an injury score the increase above baseline normalized alveolar liquid-phase fluorescence intensity at 11 min postventilation. This injury score correlates with $T$ (38).

**Assessment of clearance.** To investigate whether liquid is trapped in discrete alveoli by a pressure barrier that is proportional to $T$, we generate our local edema model using solutions with different $P_{\text{ALV}}$, of 5 cm H$_2$O, image again at 1, 6, and 11 min postventilation. For imaging, as described previously (38), we excite fluorescein at 488 nm and collect fluorescence between 493 and 535 nm. With these settings, inclusion of SRB in the flooding solution does not alter alveolar liquid-phase fluorescence intensity (Fig. 2). At each time point, we normalize alveolar liquid-phase fluorescence intensity by capillary fluorescein intensity. We report as an injury score the increase above baseline normalized alveolar liquid-phase fluorescence intensity at 11 min postventilation. This injury score correlates with $T$ (38).

**RESULTS**

**Rhodamine dyes reduce surface tension.** In the isolated rat lung, we test rhodamine dye effects on $T$ in alveoli flooded with albumin solution, a model edema liquid. At $P_{\text{ALV}}$, of 15 cm H$_2$O, inclusion in the flooding solution of 1 $\mu$M SRB or RWT decreases meniscus radius without altering liquid-phase pressure (Table 1) and lowers $T$ by $\sim$27% (Fig. 3). Inclusion of 1 $\mu$M SRG does not alter $T$. At $P_{\text{ALV}}$, of 5 cm H$_2$O, given the low power of our $T$-determination method at low inflation pressure (11), no differences in meniscus radius, liquid-phase pressure, or $T$ are detectable between groups.

That SRB and RWT, rhodamine dyes with iminium cations, lower $T$ and that SRG, lacking an iminium cation, does not suggest that the iminium cation is responsible for the ability of SRB and RWT to lower $T$. We obtain SRB and RWT from different suppliers, yet both lower $T$. Thus it is likely that the dyes themselves, not any impurities, are responsible for the observed surface activity. We further test two alternative SRB forms, a 75% pure sodium salt of SRB and a 95% pure acid form of SRB. At $P_{\text{ALV}}$, of 15 cm H$_2$O, 1 $\mu$M of either SRB form lowers $T$ of 4.6% albumin solution to the same degree ($n = 4$/group, N.S.).

In alveoli flooded with 4.6% albumin solution, we investigate at $P_{\text{ALV}}$, of 15 cm H$_2$O the effect of SRB concentration on $T$ (Fig. 4). We find that SRB concentrations ranging from 1 nM to 1 $\mu$M lower $T$ by at least 23%. Below and above this concentration range, SRB fails to lower $T$.

Table 1. SRB/RWT effects on flooded alveolar meniscus radius and liquid-phase pressure at two alveolar air pressures

<table>
<thead>
<tr>
<th>$P_{\text{ALV}},$ cm H$_2$O</th>
<th>$r_m, \mu$m</th>
<th>$P_{\text{ALV}},$ cm H$_2$O</th>
<th>+ SRB or RWT</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>16.6 ± 4.9 ($n = 43$)</td>
<td>1.8 ± 0.6 ($n = 43$)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>18.8 ± 3.1 ($n = 46$)</td>
<td>1.9 ± 1.0 ($n = 46$)</td>
<td></td>
</tr>
</tbody>
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Values are means ± SD. Base flooding solution is 2.7–12% albumin in normal saline, plus 31 $\mu$M fluorescein. Sulforhodamine B (SRB) concentration is 1 nM–1 $\mu$M. Rhodamine WT (RWT) concentration is 1 $\mu$M. Rhodamine dye decreases flooded alveolar meniscus radius $r_m$ at alveolar air pressure ($P_{\text{ALV}}$) of 15 cm H$_2$O: $^*P < 0.001$ vs. same conditions in the absence of any rhodamine dye. Rhodamine dye does not alter liquid-phase pressure $P_{\text{Liq}}$. 

**Fig. 2.** Presence of sulforhodamine B (SRB) does not alter fluorescence intensity of fluorescein. Baseline images show isolated, perfused lung regions prepared for ventilation-injury assay. Perfusate is labeled with 23 $\mu$M fluorescein. Alveoli are flooded with 5% albumin in normal saline without or with 1 $\mu$M SRB. Inclusion of SRB does not alter alveolar liquid-phase fluorescence in green channel.

**Fig. 3.** Rhodamine dye effects on surface tension in alveoli flooded with albumin solution. Flooding solution is albumin in normal saline plus 31 $\mu$M fluorescein and rhodamine dye—rhodamine WT (RWT), SRB, or sulforhodamine G (SRG)—as specified. Inflation increases surface tension: $^*P < 0.001$ vs. transpulmonary pressure, $P_{\text{ALV}}$, of 5 cm H$_2$O data point in same group. Inclusion of RWT or SRB in flooding solution lowers surface tension at high lung inflation: $^#P < 0.01$ vs. 4.6% albumin in normal saline at $P_{\text{ALV}}$, of 15 cm H$_2$O.
To assess SRB efficacy over a range of albumin concentrations encompassing those present in ARDS, we test the lowest and highest effective SRB concentrations, 1 nM and 1 μM, in conjunction albumin concentrations of 0–28% (Fig. 5). In alveoli flooded with 4.6–12.0% albumin solution, both 1 nM and 1 μM SRB effectively lower $T$. In alveoli flooded with 2.7% albumin solution, 1 μM SRB lowers $T$ but 1 nM SRB loses efficacy. In alveoli flooded with 28% albumin solution, both 1 nM and 1 μM SRB lose efficacy. In the absence of albumin, SRB fails to lower $T$.

Given that albumin is necessary to facilitate the surface activity of SRB, we investigate whether SRB is surface active in the presence of human albumin. We find that SRB lowers $T$ as effectively in human albumin solution as in bovine (Fig. 6).

**Rhodamine dyes lessen ventilation injury.** To investigate whether a rhodamine dye can directly lessen aerated alveolar overexpansion in a region with heterogeneous flooding, we use our ventilation-injury assay (38). With fluorescein in the perfusate, we flood alveoli with 3% albumin solution omitting or including 1 μM SRB. We provide the lungs with five ventilation cycles, with a PEEP of 10 or 20 cm $H_2O$ and a $V_T$ of 6 or 12 ml/kg. We find that inclusion of SRB in the alveolar flooding solution can lessen ventilation injury (Fig. 7).

To investigate whether administration of a rhodamine dye promotes clearance of flooded alveoli, we flood alveoli with a solution that omits or includes RWT, ventilate the lungs, and track over time the percentage of alveoli that are flooded. We flood alveoli with 4% albumin solution containing 25 μM BCECF, which does not alter $T$ (11), or 0.8 μM RWT, which lowers $T$ by 25%. Over 100 ventilation cycles, we find greater clearance with RWT inclusion in the model edema liquid (Fig. 8). These data support our hypothesis that a rhodamine dye, by lowering $T$, can lessen the pressure barrier $\Delta P_B$ and facilitate alveolar clearance. With clearance, liquid initially concentrated in an alveolus is spread out across regional alveoli. In a region with discrete alveolar flooding, clearance might, by reducing stress concentrations between neighboring alveoli, indirectly lessen ventilation injury.

**Rhodamine-dye–albumin interaction.** To investigate how albumin facilitates rhodamine activity, we substitute alternates for albumin and determine $T$ in the absence and presence of SRB (Fig. 9A). To determine whether osmotic pressure enables albumin to facilitate SRB activity, we substitute dextran for albumin. In 4.6% 70-kDa dextran solution, 1 μM SRB loses efficacy. To test an alternative plasma protein, we substitute fibrinogen for albumin. In 4.6% fibrinogen solution, 1 μM SRB shows a tendency to but does not significantly lower $T$.

Given that it appears that SRB in fibrinogen solution might lower $T$ by an amount not detectable with our $T$-determination method (38), we repeat our investigation of SRB efficacy in fibrinogen solution with our injury assay. As injury is proportional to $T$ (38), this assay is an alternative indicator of surface tension. We first confirm, in control injury-assay experiments, that SRB is ineffective in saline solution and facilitated by albumin solution (Fig. 9B). We then find that fibrinogen fails to facilitate SRB surface activity. We interpret that the ability to facilitate rhodamine surface activity is specific to albumin solution.

Given the different effects of albumin and fibrinogen on SRB, we test SRB surface activity in blood plasma that has a total protein concentration of 3.9 ± 0.4% ($n = 6$, 3 replicates from each of 2 rats). We find SRB to be effective when included in the instilled plasma at a concentration of 1–100 nM but not at 1 μM (Fig. 10).

**Fig. 4. SRB concentration effect on surface tension in alveoli flooded with albumin solution.** Flooding solution is 4.6% albumin in normal saline plus 31 μM fluorescein and SRB as specified. Transpulmonary pressure is 15 cm $H_2O$. Inclusion of 1 nM–1 μM SRB in flooding solution lowers surface tension: *$P < 0.01$ vs. no SRB; #$P < 0.02$ vs. 1 nM and vs. 100 nM SRB.

**Fig. 5. Albumin concentration effect on surface tension in alveoli flooded with albumin solution.** Flooding solution is 4.6% albumin in normal saline plus 31 μM fluorescein and SRB as specified. Transpulmonary pressure is 15 cm $H_2O$. Inclusion of 1 nM–1 μM SRB in flooding solution lowers surface tension: *$P < 0.01$ vs. no SRB; #$P < 0.02$ vs. 1 nM and vs. 100 nM SRB.

**Fig. 6. SRB concentration effect on surface tension in alveoli flooded with albumin solution.** Flooding solution is 4.6% albumin in normal saline plus 31 μM fluorescein and SRB as specified. Transpulmonary pressure is 15 cm $H_2O$. Inclusion of 1 nM–1 μM SRB in flooding solution lowers surface tension: *$P < 0.01$ vs. no SRB; #$P < 0.02$ vs. 1 nM and vs. 100 nM SRB.

**Fig. 7. SRB concentration effect on surface tension in alveoli flooded with albumin solution.** Flooding solution is 4.6% albumin in normal saline plus 31 μM fluorescein and SRB as specified. Transpulmonary pressure is 15 cm $H_2O$. Inclusion of 1 nM–1 μM SRB in flooding solution lowers surface tension: *$P < 0.01$ vs. no SRB; #$P < 0.02$ vs. 1 nM and vs. 100 nM SRB.
To investigate whether SRB in albumin solution is directly surface active, we determine in vitro the surface tension of solutions containing albumin and/or SRB (Fig. 11). As expected, we find $T$ of a saline drop to be $73 \pm 1$ mN/m and unaltered by addition of 1 μM SRB (16, 22). Also as expected, $T$ of 5% albumin in normal saline is $45 \pm 4$ mN/m (35). Subsequent addition of 1 μM SRB to 5% albumin solution reduces $T$ to $24 \pm 3$ mN/m. We find that the combination of SRB and albumin is directly surface active.

**DISCUSSION**

Areas of heterogeneous alveolar flooding in the edematous lung are likely sites of ventilation-induced injury. In such areas, ventilation exacerbates stress concentrations and injuriously overexpands aerated alveoli to a degree that is proportional to surface tension (19, 38). We present, here, further analysis suggesting that the heterogeneous edema pattern itself is attributable to surface tension. That is, liquid is trapped in discrete alveoli by a pressure barrier, $\Delta P_B$, that is proportional to $T$. Thus lowering $T$ in regions of heterogeneous alveolar flooding should 1) directly lessen ventilation-induced overexpansion injury of aerated alveoli and 2) by lowering $\Delta P_B$, facilitate clearance of flooded alveoli, reduce stress concentrations, and indirectly lessen ventilation injury. We demonstrate experimentally that the rhodamine dyes SRB and RWT, in the presence of albumin, lower $T$, lessen ventilation injury, and promote alveolar clearance.

**Pressure barrier and alveolar clearance.** From the assumption of an inflection point in the curvature of the flooded alveolar interface, we identify a pressure barrier $\Delta P_B$ as responsible for trapping liquid in discrete alveoli (Fig. 1). Despite the hydrostatic pressure difference between liquid at the center and border of the flooded alveolus, alveolar liquid is static in a statically inflated isolated lung and quasistatic during ventilation. That is, the pressure barrier does not cause liquid to flow inward from the edge of the flooded alveolus. In the adjacent aerated alveolus, where interfacial curvature also varies from concave along the septum separating the aerated from the flooded alveolus to saddle shaped at the end of the same septum, there is a similar pressure barrier, yet fluid is likewise static. We speculate that liquid-phase thickness may vary inversely with pressure such that net force on a given fluid element is zero (Fig. 12). Alternatively, the hydrostatic pressure difference may be opposed by a different stress, for example, osmotic pressure. The mechanism responsible for the quasistatic condition of liquid in the flooded alveolus remains to be determined.

When we generate our local edema model, we flood numerous alveoli, and some clear immediately (11, 34, 38). After initial clearance, however, we are left with a region in which ~42% of surface alveoli are stably flooded (11, 38). As lung inflation increases $T$ and thus $\Delta P_B$, which is proportional to $T$, lung inflation to near total lung capacity generally fails to clear flooded alveoli. However, an individual flooded alveolus will occasionally clear spontaneously. When one happens to observe such clearance through the microscope, the alveolus appears to pop open. Instantaneously, the liquid is gone and the

**Fig. 6.** SRB surface activity in alveoli flooded with bovine or human albumin solution. Flooding solution is 4.6% bovine serum albumin (BSA) or human serum albumin (HSA) in normal saline plus 31 μM fluorescein and SRB as specified. Transpulmonary pressure is 15 cm H$_2$O. SRB lowers surface tension of serum albumin (HSA) in normal saline plus 31 μM fluorescein and SRB as specified. Presence of discrete flooding causes injury: all discrete flooding groups differ ($P < 0.001$, statistics not shown on graph) from control, aerated groups. SRB can lessen injury: $^*P < 0.02$ vs. same ventilation settings without SRB. Higher PEEP or $V_T$ increases injury; among discrete flooding groups, a group with a letter at its base differs ($P < 0.02$) from all other groups except those with the same letter above their error bars.
alveolus, which had been shrunken in its liquid-filled state (19), is enlarged. Upon clearance, the liquid that had been trapped in the alveolus presumably distributes across regional alveoli. The volume of released liquid is small enough, relative to local surface area, that clearance does not noticeably alter regional liquid-phase thickness.

Addition of rhodamine dye to albumin-containing alveolar flooding solution promotes flooded alveolar clearance (Fig. 8). Rhodamine-induced clearance, like spontaneous alveolar clearance, is a rapid phenomenon. This clearance is distinct from the slower process of liquid reabsorption across the epithelium, which even when stimulated by a β-adrenergic agonist occurs over the course of hours (17). Given that just five ventilation cycles are sufficient to injure an area with heterogeneous alveolar flooding (38), the ability to clear alveoli rapidly is important.

Thus flooded alveoli occasionally clear spontaneously. When flooded alveoli clear, they do so instantaneously. And lowering T can promote flooded alveolar clearance. These observations suggest that the flooded alveolus is in a state of local, but not global, equilibrium.

Dye effects on surface tension. We find that SRB and RWT, but not SRG, all at 1 μM in 4.6–4.9% albumin solution lower T. Given the structural difference between SRB and RWT, each with an iminium cation, and SRG, without a cation, we attribute the T-lowering ability of SRB and RWT to the presence of the iminium cation. We then focus our investigations on SRB and the conditions under which it is effective, as SRB could potentially be used as a clinical therapeutic agent (see RW T and SR B toxicity, below). We test alternative SRB concentrations in 4.6% albumin solution. We test the minimum and maximum effective SRB concentrations in alternative albumin concentrations. Although we expect that RWT would behave similarly to SRB, we have not tested RWT under alternative conditions. We have not tested SRG, either, under alternative conditions. We note, however, it is the ability of SRB and RWT to lower T of the native surfactant-containing alveolar liquid phase, not the inability of SRG to do so, that is unexpected.

We have determined the effect on in situ T of various fluorescent dyes. We have compared T in the presence of nonrhodamine dyes, at 95 nM-40 μM, to that in the absence of any dye to verify that we use dyes at low enough concentrations that they do not detectably alter T (11). SRG, like all of the nonrhodamine dyes that we have tested, carries only negative charges. We interpret that the rhodamine dye structure and iminium cation, together, are required to lower surface tension.

Effective albumin concentration range. We find SRB to lower T when instilled in combination with 2.7–12.0% albumin solution. Following alveolar instillation, we require ~20 min to determine T. Because of diffusive albumin efflux during this time, actual tested albumin concentrations are lower than injected concentrations. In ARDS, edema liquid albumin concentration averages ~3% (7, 15). Thus SRB is effective at the albumin concentration present in clinical ARDS.

We find 1 μM SRB to lower T in combination with 2.7% albumin solution but not rat blood plasma (Figs. 5 and 10). As total protein concentration in the rat plasma with which we flood alveoli is 3.9% and albumin accounts for ~52% of all proteins in rat plasma (6), the albumin concentration in rat plasma may be close to that of our 1.8% albumin solution, in which we find SRB ineffective (Fig. 5). Why 1 nM SRB, which is not effective in 1.8 or 2.7% albumin solution, is effective in rat plasma remains to be determined.

Rhodamine dye-albumin interaction. We find that SRB and RWT require the presence of albumin to lower T, whether in saline solution in vitro or with the additional presence of lung surfactant in situ. SRB is known to bind to albumin (12, 33). SRB also interacts with and increases the surface activity of the surfactant sodium dodecyl sulfate (SDS) (22), suggesting that SRB might interact with lung surfactant phospholipids.

Amphiphilic SRB is, on its own, surface active. Computational modeling suggests that the xanthene rings of SRB, despite the iminium cation that they support, constitute the hydrophobic moiety of SRB and that, when SRB adsorbs, the xanthene rings align with the interface (22). However, SRB on its own is only surface active at >1.3 mM (22), a concentration...
SRB binds by hydrophobic interaction to the Sudlow site I of fatty acid-free albumin at a stoichiometry of ~1:1, with the hydrophobic xanthene rings of SRB likely situated in the albumin-binding cavity (12). We combine ≤1 μM SRB with 5% (0.7 mM) fatty acid-bound albumin. Given the low SRB concentration relative to that of albumin, we expect that a significant fraction of SRB in our model is bound to albumin.

How the presence of fatty acids affects the stoichiometry of SRB-albumin binding, however, must be directly tested.

In vitro experiments, we find surface tension of a solution containing both SRB and albumin to be significantly lower, 24 mN/m, than that of a solution containing SRB or albumin alone (Fig. 11). As a comparison, adsorption of surfactant to a static interface lowers T to 26 mN/m (8, 28). It is possible that the T-lowering effect of SRB in situ in the lungs is attributable to direct SRB-albumin surface activity.

Alternatively, SRB and albumin may interact with lung surfactant in situ. SRB, which at a given concentration is less surface active than SDS, enhances the surface activity of SDS (22). Computational modeling suggests that SRB inserts into the outer layer of SDS micelles—the xanthene rings of SRB, again, likely most embedded (22). The SRB concentration at which this facilitation of SDS has been shown to occur, 9 mM, is far greater than the SRB concentrations that we examine. Furthermore, SRB-SDS interaction has been investigated in the absence of albumin, which we find essential in our model. Nonetheless, that SRB interacts with SDS suggests that SRB might, likewise, interact with lung surfactant. For example, SRB/albumin might interact with tubular myelin in the alveolar liquid subphase to promote phospholipid adsorption. Alternatively, whether SRB/albumin affects alveolar epithelial type II cell secretion or reuptake of surfactant has not been investigated.

How SRB, albumin, and lung surfactant interact is not known. As the xanthene rings of SRB embed in both albumin and in SDS micelles, it is unlikely that SRB links albumin to surfactant. In our experiments, when we introduce SRB- and albumin-containing solution into the alveolus, it is likely that SRB and albumin are already bound. Whether lung surfactant...
interacts with SRB or albumin, perhaps competing for SRB in a fashion that alters surface tension, remains to be determined.

**RWT and SRB toxicity.** SRB is approved as a food dye in Japan (32), and both SRB and RWT are used as ground water tracers (29). There is an extensive literature on the toxicity of these dyes. In briefly surveying this literature, below, we note that most toxicity studies have been performed using dye formulations of unknown purity. The exception, summarized at the end of this section, is a series of SRB toxicity tests performed by a European Commission committee using high-purity SRB (27).

Rhodamine WT has an LD$_{50}$ of $\geq 430$ mg/kg (5, 14). In limited in vivo testing of RWT, administration of 25–80% of the LD$_{50}$ over 1–5 days did not cause histopathological changes, increase the rate of micronuclei generation, or increase the rate of occurrence of sperm abnormalities (5, 14). Results of in vitro tests of RWT genotoxicity/mutagenicity, however, have been mixed. RWT tested negative for genotoxicity at 4 mM by mammalian cell sister chromatid exchange and at 12 mM by mammalian cell chromosome aberration test (5). However, RWT tested positive at $\sim 2–10$ μM and 4 mM by Ames test (3, 18) and at $\sim 2–10$ μM by mammalian cell chromosome aberration test (3). The purity of the dyes used in the above studies is not known, and it is possible that purified RWT would test negative for genotoxicity. On the basis of the existing literature, however, RWT does not appear suitable for use as a clinical agent.

SRB of unknown purity tested negative for genotoxicity at $\sim 2–10$ μM by Ames test (3); at 10 μM and 1.7 mM by rec assay (9, 31); and at $\sim 2–10$ μM by mammalian cell chromosome aberration test (3). The only study in which SRB tested positive for genotoxicity is one in which the tested SRB concentration is not stated (10). In a series of tests on the cytotoxicity of Japanese food dyes, toxicity of SRB at 1–2 mM, $\geq 1,000 \times$, the highest concentration at which we find SRB to lower $T$, was not found to be a concern (13, 26), except for the detection of low-level toxicity in cultured fetal rat cells at day 4 after plating that decreased by day 7 (25). Furthermore, following administration of 2,000 mg/kg SRB to pregnant rats, there was no evidence of DNA damage in cells biopsied from dams or embryos (32).

A 2008 report (27) by the Scientific Committee on Consumer Products of the European Commission presents SRB toxicity data from tests in which dye formulations of generally high purity were used. The LD$_{50}$ for SRB (unknown purity) was found to exceed 1,000 mg/kg. Administration of 1,000 mg/kg per day of $\geq 99\%$ purity SRB for 13 wk to mature rats produced no observable adverse effects. Administration of 1,000 mg/kg per day of $\geq 99\%$ purity SRB for 12 days to pregnant rats produced no observable adverse effects in dams or fetuses. One-time administration of 2,000 mg/kg of $> 90\%$ purity SRB to mice did not increase the rate of generation of micronuclei. A diet of 5% of $\geq 99\%$ purity SRB over 2 yr was not carcinogenic in rats. Finally, SRB of $\geq 99\%$ purity tested negative for genotoxicity at 9 μmol/plate by Ames test and at 9 mM by mammalian cell chromosome aberration test. SRB appears to be a suitable candidate for clinical administration.

**Surface tension-dependent injury.** Employing our ventilation-injury assay in the absence/presence of SRB (Fig. 7), we confirm our previous findings with the exogenous surfactant Survanta (38). Ventilation that is not injurious to the aerated lung causes injury to areas with heterogeneous alveolar flooding. Lowering $T$, whether with SRB or Survanta, lessens the degree of injury. We observe aerated alveoli adjacent to flooded alveoli to be significantly over expanded at $P_{A\ell}$ of both 5 and 15 cm H$_2$O (19), an observation that constitutes the basis of our theoretical injury model (38). We assume that septa between aerated and flooded alveoli, which bow into the flooded alveoli (19), are subjected to the greatest overdissension. As discussed previously (38), however, the relative degrees of overdissension of the various septa in the aerated alveolus remain to be quantified.

We find that SRB/RWT inclusion in the alveolar liquid phase lowers $T$ without altering $P_{L\ell}$ (Table 1). That is, SRB/RWT inclusion does not alter the hydrostatic pressure difference between capillary and alveolar liquid phase. In our injury assay, in the absence of a difference in driving pressure, we attribute reduced fluorescein entrance into the alveolar liquid in the presence of SRB to a $T$-dependent reduction in injury to the alveolar-capillary barrier.

We expect clearance of flooded alveoli to lessen ventilation injury indirectly by equating mechanics between adjacent alveoli. We do not know the shear stresses applied to the epithelium by alveolar clearance and cannot rule out that shear stresses imposed by fluid clearance might themselves be injurious. However, in contrast with ventilation-induced overdissension, which is imposed cyclically, any clearance-induced shear injury would be imposed only once. In our injury assay, as alveolar flooding is constant over five initial ventilation cycles (Fig. 8), imposed injury is not attributable to clearance but rather to alveolar overexpansion.

**Potential therapeutic use of SRB.** We find that instilled SRB concentrations of 1–100 nM effectively lower $T$ in both 4.6% albumin solution and in rat plasma (Figs. 4 and 10). Because of diffusive efflux in the 20 min required to determine $T$, as for albumin, the actual SRB concentration tested is less than the concentration instilled. Thus an actual alveolar SRB concentration of 1–10 nM would likely be an appropriate target clinical concentration.

Clinically, SRB could be delivered to the alveolus via the trachea or the vasculature. If delivered via the trachea, watersoluble SRB should diffuse toward the distal lung; concurrent tracheal instillation of exogenous surfactant might enhance SRB delivery by generating Marangoni flows and thus convective and diffusive transport. SRB would likely be instilled as a concentrated bolus in the trachea such that, once distributed throughout the lung edema liquid, it would be...
diluted to a concentration at which it would lower \( T \). How effectively SRB instilled in the trachea would disperse to the lung periphery requires investigation.

An alternative route of SRB delivery would be via the vasculature. Especially in permeability edema, when barrier permeability is sufficiently elevated to allow albumin entrance into the alveolus, even albumin-bound SRB should be able to diffuse out of the vasculature into the alveolar liquid phase. SRB delivery via the vasculature, which must also be investigated, holds potential as a new therapy for the treatment of ARDS.

**Conclusions.** We demonstrate that the rhodamine dyes SRB and RWT lower surface tension in lung regions flooded with albumin-containing solution. When the flooding pattern is heterogeneous, rhodamine dye administration facilitates clearance of liquid-flooded alveoli, which should, indirectly, lessen ventilation injury. We identify SRB, in particular, as a potential new therapeutic agent that might be used to treat ARDS and that might be administered via a new route, the vasculature. The mechanism through which these rhodamine dyes interact with albumin to lower surface tension remains to be determined.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: A.B.K., Y.W., and C.E.P. conception and design of research; A.B.K. and Y.W. performed experiments; A.B.K. and Y.W. analyzed data; A.B.K., Y.W., and C.E.P. interpreted results of experiments; A.B.K., Y.W., and C.E.P. prepared figures; A.B.K. and C.E.P. drafted manuscript; A.B.K., Y.W., and C.E.P. edited and revised manuscript; A.B.K., Y.W., and C.E.P. approved final version of manuscript.

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