SCH79797 improves outcomes in experimental bacterial pneumonia by boosting neutrophil killing and direct antibiotic activity

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Objectives: The role of protease-activated receptor 1 (PAR1) in the pathogenesis of pneumonia and sepsis is ambiguous given the existing literature. As PAR1 is classically activated by the coagulation-based protease thrombin and leads to vascular leakage, our hypothesis was that PAR1 blockade with SCH79797 would be therapeutically beneficial in an experimental model of murine Gram-negative pneumonia.

Methods: In this study, we administered SCH79797 via the intrapulmonary route 6 h after the establishment of Escherichia coli pneumonia and observed a significant improvement in survival, lung injury, bacterial clearance and inflammation. We focused on neutrophils as a potential target of the PAR1 antagonist, since they are the predominant inflammatory cell type in the infected lung.

Results: Neutrophils appear to express PAR1 at low levels and the PAR1 antagonist SCH79797 enhanced neutrophil killing. Part of this effect may be explained by alterations in the generation of reactive oxygen species (ROS). SCH79797 also led to robust neutrophil extracellular trap (NET) generation and cathelicidin-related antimicrobial peptide (CRAMP) release by neutrophils. Surprisingly, SCH79797 also had a potent, direct antibiotic effect with disruption of the E. coli cell membrane. However, the newer-generation PAR1 antagonist, vorapaxar (SCH530348), had no appreciable effect on neutrophil activity or direct bacterial killing, which suggests the effects seen with SCH79797 may be PAR1 independent.

Conclusions: In summary, we observed that intrapulmonary treatment with SCH79797 has significant therapeutic effects in a model of E. coli pneumonia that appear to be due, in part, to both neutrophil-stimulating and direct antibacterial effects of SCH79797.

Introduction

Severe pneumonia is the most common cause of respiratory failure and sepsis among critically ill patients with a mortality rate approaching 40%–50% in the most severe cases. Other than timely antibiotic therapy and supportive care, there have been no proven pharmacological therapies for this condition. Given the rapid emergence of antibacterial resistance among pathogenic bacteria, the ability to effectively treat severe bacterial pneumonia and sepsis has been significantly impaired. This has been particularly observed among enteric Gram-negative organisms (Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae), which are frequent causes of severe pneumonia in the ICU. Therefore, novel therapies are needed to aid in the management of this complex syndrome.

Protease-activated receptors (PARs) are G protein-coupled, seven-transmembrane receptors that have a unique mechanism of activation that involves cleavage of the N-terminal region by coagulation-based serine proteases. PAR1 was the initial PAR discovered and is activated by a variety of enzymes, though thrombin is the principal agonist under physiological conditions. PAR1 is expressed by a variety of cell types including endothelial cells, epithelial cells and bone marrow-derived haematopoietic cells. Classically, PAR1 activation on endothelial cells by thrombin triggers a vascular disruptive phenotype, so that at areas of inflammation and coagulation there is fluid extravasation into organs and into third spaces (e.g. peritoneal cavity). In this study, we sought to determine the role of intrapulmonary PAR1 antagonism in a murine model of Gram-negative pneumonia using a well described and widely employed antagonist, SCH79797. We focused on the potential effects of SCH79797 on neutrophils, since these are the predominant inflammatory cells present in acute bacterial pneumonia.
The literature in this area is somewhat limited with conflicting results but suggests that there may be time-dependent roles of PAR1 antagonism in experimental models of sepsis with early blockade leading to beneficial outcomes. Kaneider et al.\textsuperscript{10} published a report demonstrating that early activation of PAR1 in a sepsis model was harmful, whereas late activation is beneficial via a PAR2-dependent mechanism. The authors utilized unique cell-penetrating peptides, pepducins, that specifically targeted the third intracellular loop of PAR1 to dissect the time-dependent effects of PAR1 activation in sepsis.\textsuperscript{10} In 2012, Schouten et al.\textsuperscript{11} used a pneumococcal pneumonia model in mice to show that PAR1 impairs host defence against infection. They presented data demonstrating that PAR1 \textsuperscript{-/-} mice had improved survival early after infection, lower bacterial burden and less inflammatory cell influx than WT mice.\textsuperscript{11} And, in 2013, Khoufache et al.\textsuperscript{1} examined the effect of PAR1 antagonism and deficiency in a murine model of influenza A pneumonia. They reported that treatment with a PAR1 antagonist improved outcomes in mice with influenza infection.\textsuperscript{12} However, Camerer et al.\textsuperscript{13} published an article in 2006 reporting that PAR1 \textsuperscript{-/-} mice did not have improved survival or inflammatory indices in an experimental model of sepsis using intraperitoneal endotoxin.

In terms of neutrophils and PAR1 specifically, there are reports indicating that neutrophils express PAR1 and other PARs,\textsuperscript{14} and that PAR1 antagonism has been shown to influence neutrophil recruitment in an experimental pneumonia model.\textsuperscript{15,16} However, the literature is sparse in terms of describing the effects of PAR1 antagonism on neutrophil function directly.

Our hypothesis was that PAR1 antagonism with SCH79797 would be protective in murine \textit{E. coli} pneumonia and sepsis and modulate neutrophil activity in the lung. The results demonstrated that SCH79797 did improve survival, lung injury and bacterial clearance in our model of bacterial pneumonia through neutrophil boosting of bacterial killing as well as a direct antibacterial effect. However, these results were not seen with the newer-generation PAR1 antagonist vorapaxar (SCH530348), suggesting that SCH79797 may be acting through both PAR1-dependent and PAR1-independent effects, which has been previously reported.\textsuperscript{17}

\section*{Methods}

\subsection*{Murine \textit{E. coli} pneumonia model and treatment with SCH79797}

All mice used in this study were male C57BL/6J mice (Jackson Labs) between 12 and 15 weeks of age. Mice were housed under standard conditions in a clean facility at the University of California, San Diego (UCSD) approved by the Association for Accreditation and Certification of Laboratory Animal Care (AAALAC) and all experiments were approved by the UCSD Institutional Animal Care and Use Committee (IACUC).

\textit{E. coli} strain K1 was used in all in vivo and in vitro models in this study (originally isolated from the blood of a patient with biliary sepsis; provided by Xiao Su, MD, PhD, Institut Pasteur de Shanghai). Intratracheal (IT) instillation of \textit{E. coli} (1 million cfu) into mice was accomplished using a previously described protocol of direct visual instillation.\textsuperscript{18} SCH79797 (Tocris Bioscience) was administered IT as a treatment for \textit{E. coli} pneumonia at 6 h post-infection. See Supplementary Methods (available as Supplementary data at JAC Online) for more details.

\subsection*{Bronchoalveolar lavage and lung injury analyses}

Bronchoalveolar lavage (BAL) was done using a previously published protocol.\textsuperscript{19,20} Lungs were harvested and processed in a standard fashion and lung injury was scored using an established method.\textsuperscript{19,20} See Supplementary Methods for details.

\subsection*{Mouse neutrophil isolation}

Mouse neutrophils were isolated from the bone marrow of tibias and femurs of adult, male C57BL/6J mice using a previously published protocol.\textsuperscript{21} The purity of the neutrophils was assessed by doing a cytospin of an aliquot of cells, fixing the cells on a slide and then doing a hematoxylin and eosin (H&E) stain (90\% purity confirmed by this method). See Supplementary Methods for details.

\subsection*{Mouse neutrophil quantitative real-time PCR and western blotting for PAR1 expression}

RNA and protein were isolated from mouse neutrophils using standard techniques. Quantitative real-time PCR (qPCR) was done to analyse gene expression of PAR1 in neutrophils and western blotting was performed to determine whole-cell PAR1 protein expression. Bone marrow-derived mesenchymal stem cells (MSCs) were used as a positive control, as they have been recently shown to express PAR1 by our group.\textsuperscript{22} See Supplementary Methods for full details of the methods used for these analyses.

\subsection*{Neutrophil bacterial killing and reactive oxygen species assays}

Neutrophil bacterial killing assays were done, using a previously published protocol, with SCH79797 to determine the effects of this compound on killing efficiency.\textsuperscript{13} Reactive oxygen species (ROS) production was measured as has been previously published by our group.\textsuperscript{7} See Supplementary Methods for details.

\subsection*{Mouse neutrophil extracellular trap visualization and quantification}

In order to ascertain whether the increase in neutrophil killing of \textit{E. coli} by SCH79797 correlates with neutrophil extracellular trap (NET) formation, studies were done to visualize and quantify NETs using a protocol that our group has previously published.\textsuperscript{23} See Supplementary Methods for complete details. Vorapaxar was also tested to determine whether the effects seen were generalizable to the class of PAR1 antagonists. A protein kinase C (PKC) inhibitor was used in some conditions to determine whether this pathway is involved in NET formation, since PKCs have been previously reported to be required for phorbol myristate acetate (PMA)-induced NETs.\textsuperscript{24}

\subsection*{Mouse neutrophil cathelicidin-related antimicrobial peptide expression analysis}

Neutrophils were isolated as above and resuspended in Hank’s balanced salt solution (HBSS) with calcium and magnesium. Cells were plated at a concentration of 1 million cells per 250 \textmu L in a 24-well tissue culture plate in the presence of SCH79797 (10 \textmu M), PMA (25 nM) or \textit{E. coli} (3 million cfu). Stimulation was added in a 50 \textmu L volume so the final total volume was 300 \textmu L per well. Each condition was done in triplicate. The plate was then centrifuged at 1600 rpm for 5 min and then incubated at 37 \textdegree C for 3 h, after which 3 \textmu L of the conditioned medium from each sample was applied directly onto a nitrocellulose membrane and allowed to completely dry. The membrane was blocked with appropriate buffer (LiCor) and cathelicidin-related antimicrobial peptide (CRAMP) protein was then detected and visualized using standard techniques (rabbit anti-mouse CRAMP antibody,
When SCH79797 was administered via the IT route 6 h after establishment of murine E. coli pneumonia, PAR1 antagonism with SCH79797 improves survival in mice.25,26

### Results

#### Statistical analyses

Survival data was analysed using a log-rank test, while the majority of the other data is presented as mean ± SD for each group analysed. An unpaired, two-sided Student's t test was used for most comparisons between two sets of data. If multiple groups of data were compared simultaneously, an ANOVA was used. A P value <0.05 was used for statistical significance.

#### Assessment of direct antibacterial effect of SCH79797

To determine whether there was a direct antibacterial effect of SCH79797 that may, in part, account for the protective in vivo effects observed, we carried out two different assays to measure how SCH79797 influences E. coli growth in vitro: (i) a continuous assessment of E. coli growth kinetics over 24 h; and (ii) a killing assay to quantify growth or killing of E. coli after 6 h. Separate conditions using vorapaxar were included to ascertain whether the effects seen with SCH79797 were applicable to other PAR1 antagonists. In addition, we tested the killing activity of SCH79797 against Gram-positive bacteria using an isolate of MRSA (TCH1516).

See Supplementary Methods for details.

#### Determination of mechanism of SCH79797 antibacterial action

Bacterial cytological profiling was utilized to determine the antibiotic mechanism of action of SCH79797 as has been previously published by our group.25,26 Bacillus subtilis was used as a Gram-positive bacterium against which to test the activity of SCH79797 given our group’s experience with this strain for morphological studies. See Supplementary Methods for details. Of note, experiments in high-sucrose medium were done to discriminate between cell membrane and cell wall effects of SCH79797. Published images were taken using fluorescent microscopy.

#### Mouse neutrophils express PAR1 and SCH79797 boosts neutrophil killing activity

Bone marrow-derived neutrophils demonstrated evidence of PAR1 gene and protein, albeit at low levels when compared with bone marrow-derived MSCs (Figure 2a and b). Incubation of neutrophils with SCH79797 (10 μM) significantly increased bacterial killing of E. coli when assessed after 3 h of stimulation to allow for formation of NETs. The addition of DNase, to disrupt the NETs, eliminated the effect of SCH79797 on neutrophil killing (Figure 2c). Of note, SCH79797 also increased neutrophil killing of bacteria after only 30 min of stimulation, but this effect was not as pronounced and did not reach a statistical level of significance (data not shown).

SCH79797 stimulated several pathways by which neutrophils classically kill bacteria including increasing ROS activity (Figure 2d), NET formation as seen visually and quantitatively (Figure 2e and f) and CRAMP release (Figure 2g). SCH79797-induced NET formation was significantly reduced by pre-incubation with the conventional PKC inhibitor Go6976. PMA was used as a positive control for these mechanistic studies and SCH79797 boosted neutrophil killing activities in a manner similar to or greater than that measured with PMA. Importantly, vorapaxar did not result in any measurable increase in NET formation suggesting that the effect seen with SCH79797 is not generalizable among the class of PAR1 antagonists.

#### SCH79797 reduces lung injury, bacterial burden and markers of inflammation

Treatment with 10 μM SCH79797 6 h after E. coli infection resulted in a significant reduction in alveolar oedema 24 h after infection as determined by scoring of lung histological sections (Figure 1b and c). In addition, BAL studies done 24 h after infection showed a significant reduction in bacterial burden and inflammatory cell influx and a trend towards lower neutrophil chemokine levels (Figure 1d–f). Also, there was a near-significant reduction in the quantity of alveolar total protein level with treatment with SCH79797 at 10 μM (Figure 1g), but not when higher doses of SCH79797 were used (data not shown).

#### SCH79797 induces NETs in human neutrophils and enhances their bacterial killing

To determine whether the findings in mouse neutrophils extend to human neutrophils, studies assessing the effects of SCH79797 on human neutrophil function were carried out, similar to those done with mouse neutrophils. Initially, it was determined that human neutrophils do express the transcript for PAR1 by qPCR (data not shown). Then, fluorescent microscopy demonstrated that SCH79797, at a dose of 10 μM, led to significant NET formation in human neutrophils (Figure 3a and b). As with the mouse neutrophil studies, the use of the PKC inhibitor Go6976 partially reduced the formation of NETs (Figure 3a). Quantification of NET formation also demonstrated a significant increase with SCH79797 at 10 μM that closely approximated the positive control, PMA (Figure 3b). However, vorapaxar did not result in any measurable increase in NET formation as was seen in the mouse neutrophil studies (Figure 3b). In addition, human neutrophil killing activity was significantly improved with SCH79797 (10 μM) and this effect was completely eliminated with the addition of DNase as seen in
The Bioscreen assay demonstrated that SCH79797, starting at 1 \( \mu \)M, exhibited direct antibacterial activity targeting the membrane. SCH79797 significantly improves survival, lung injury and inflammation in murine \textit{E. coli} pneumonia. SCH79797 at 10 \( \mu \)M was determined to be the most effective dose when given as a treatment 6 h after infection (\( n = 17–19 \) per group, \( P < 0.05 \) for 10 \( \mu \)M SCH79797, SCH 10, versus PBS; 100 \( \mu \)M SCH79797 (SCH 100) provided no survival advantage versus PBS). H&E staining of lungs demonstrated less lung injury in mice treated with SCH79797 10 \( \mu \)M (SCH) at 24 h post-infection (\( n = 3 \) lungs per group for lung injury score analysis, \( P < 0.05 \) for SCH versus PBS-treated groups, data as mean \( \pm \) SD). BAL at 24 h showed reduced inflammatory cell influx (d), reduced bacterial burden (e) and a trend towards a reduction in both inflammatory cytokine levels (f) and vascular permeability (g) with SCH treatment (\( n = 6 \) per group for all analyses, \( P < 0.05 \) and \( P = 0.16 \) for macrophage inflammatory protein-2 (MIP-2) concentrations and \( P = 0.055 \) for albumin concentrations, data as mean \( \pm \) SD). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

\textbf{SCH79797 exhibits direct antibacterial activity targeting the membrane}

The Bioscreen assay demonstrated that SCH79797, starting at 1 \( \mu \)M, inhibited \textit{E. coli} growth compared with the positive control of \textit{E. coli} alone in growth medium (Figure 4a). Concentrations of SCH79797 that were 10 \( \mu \)M and greater led to no apparent growth of \textit{E. coli} as detected by the Bioscreen assay. When separate analyses were carried out to quantify the bacterial counts after 6 h of incubation of SCH79797 with \textit{E. coli}, 10 \( \mu \)M SCH79797 led to significant suppression of growth, while 100 \( \mu \)M led to pronounced bacterial killing (Figure 4b). However, vorapaxar did not significantly reduce bacterial growth when tested at 10 and 100 \( \mu \)M. These comparisons were made in reference to the initial inoculum of \textit{E. coli} plated per well.

Using bacterial cytological profiling, it was determined that SCH79797 used at 25 \( \mu \)M (5 \( \times \) the calculated MIC) resulted in substantial \textit{E. coli} lysis and exposure of DNA to the membrane-impermeable dye SYTOX Green (Figure 4c–f). This was seen at both the 30 min and 2 h timepoints. The rapid permeabilization of \textit{E. coli} at 30 min suggests a membrane-active effect, but this finding could also be due to a potent inhibition of cell wall biosynthesis (Figure S1A and B, available as Supplementary data at JAC Online).

To distinguish between a membrane-active or cell wall-inhibitory effect of SCH79797, \textit{E. coli} was treated with SCH79797, D-cycloserine or polymyxin B under osmotic stabilization conditions with 0.5 M sucrose (Figure 4g and h and Figure S1E and F). In the absence of sucrose, SCH79797, the cell wall biosynthesis inhibitor D-cycloserine and the membrane-active polymyxin B cause cellular permeability at 30 min and cellular debris can be seen alongside cells with minimal shape defects at 2 h (Figure 4d and f and Figure S1A–D). However, at 2 h of treatment in the presence of sucrose, cells treated with D-cycloserine formed rounded shapes with minimal membrane permeabilization (Figure S1E), whereas cells treated with SCH79797 and polymyxin B maintained a shape typical of cells grown in sucrose but were still permeabilized (Figure 4h and Figure S1F). The morphological similarity between cells treated with SCH79797 and polymyxin B, especially under osmotically stabilizing conditions, indicates that SCH79797 acts as a membrane-active compound and not a cell wall biosynthesis inhibitor.

In testing the activity of SCH79797 against Gram-positive bacteria, it was observed that SCH79797 led to significant bacterial growth when tested at 10 and 100 \( \mu \)M (Figure 4b). However, vorapaxar did not significantly reduce bacterial growth compared with the positive control of 0.5 M sucrose, SCH79797, the cell wall biosynthesis inhibitor D-cycloserine and the membrane-active polymyxin B under osmotic stabilization conditions with 0.5 M sucrose (Figure 4g and h and Figure S1E and F). The morphological similarity between cells treated with SCH79797 and polymyxin B, especially under osmotically stabilizing conditions, indicates that SCH79797 acts as a membrane-active compound and not a cell wall biosynthesis inhibitor.
membrane disruption of *B. subtilis* as early as 30 min after incubation (Figure S2A-D). Furthermore, SCH79797 showed significant growth suppression of MRSA at a dose of 1 μM and substantial bactericidal activity at 10 μM (Figure S3).

**Discussion**

PARs are G protein-coupled receptors that have a unique mechanism of activation and are predominantly cleaved and activated by coagulation-based serine proteases. PAR1 is the principal target of thrombin and sits at the fulcrum of inflammation and coagulation in response to infection. The role of PAR1 in experimental models of infection has been investigated to some extent, but there is still uncertainty as to whether activation is beneficial or harmful in this setting. We undertook the current study to determine whether intrapulmonary PAR1 antagonism with SCH79797 would have protective effects in an experimental model of *E. coli* pneumonia in mice. Our data yielded the following main results: (i) SCH79797 significantly improved survival, reduced lung injury and inflammation, and enhanced bacterial clearance when given as a treatment; (ii) neutrophils express low levels of PAR1 and SCH79797 boosts killing of *E. coli* by enhancing ROS production, NET formation and by several mechanisms. Neutrophils (PMNs) express low levels of the PAR1 gene and protein as evidenced by qPCR (a; n = 3 per group, **P < 0.01 for PMN versus MSCs and #P < 0.01 for negative control (neg ctrl) versus PMN and neg ctrl versus MSCs, data as mean ± SD) and western blotting (b). SCH79797 at 10 μM (SCH) increased mouse neutrophil killing efficiency (c) and ROS production (d) significantly, while the addition of DNase eliminated the enhanced bacterial killing seen with SCH79797 (n = 6 per group for killing and n = 3 per group for ROS assay, **P < 0.01 for PMN + SCH versus unstimulated (unstim) PMN, #P < 0.01 for PMN + SCH + DNase versus PMN + SCH and ⋄P < 0.01 for PMN + PMA versus unstim PMN, data as mean ± SD). Fluorescent imaging demonstrated qualitatively more NET formation with incubation of SCH79797 at 10 μM and this effect was reduced with the PKC inhibitor Go6976 at 10 μM (e). Quantitative analysis of NET formation showed a significant increase with 25 nM PMA and 10 μM SCH79797 and this effect was significantly reduced with pre-incubation with the PKC inhibitor Go6976. Vorapaxar 10 μM (Vorapax), however, did not result in any appreciable increase in NET formation compared with unstimulated neutrophils (f; n = 4–8 per group, **P < 0.01 for PMA and SCH versus unstim, ⋄P < 0.01 for Go6976 + PMA versus PMA and, ⋄P < 0.01 for Go6976 + SCH versus SCH, data as mean ± SD). SCH79797 at 10 μM significantly increased the release of the antimicrobial peptide CRAMP from neutrophils at a level that approximates observed that there was no effect on NET formation or direct effects against *E. coli* as early as 30 min after incubation (Figure S2A–D). Furthermore, SCH79797 showed significant growth suppression of MRSA at a dose of 1 μM and substantial bactericidal activity at 10 μM (Figure S3).
bacterial killing. These results suggest that the in vivo effects obtained with SCH79797 are probably a combination of PAR1-dependent and -independent effects.

In relation to the existing literature on PAR1 in experimental models of sepsis, our findings are most consistent with the findings reported by Khoufache et al. regarding the beneficial effects of SCH79797 administration in a model of influenza pneumonia. The publication by Schouten et al. is also congruent with our results as they report that PAR1−/− mice have improved outcomes in a model of pneumococcal pneumonia. Our study does provide some additional insights into the effects of SCH79797 on neutrophil function by demonstrating that SCH79797 enhances neutrophil killing of bacteria through several possible mechanisms, including ROS production, NET formation and CRAMP release. The induction of NET formation by SCH79797 was particularly robust and exceeded the quantity seen with the positive control PMA when using mouse neutrophils. NETs have been recognized in the literature as potent antimicrobial networks of chromatin and granule proteins since their initial description. NETs have been postulated to increase the concentration of antimicrobial factors, which aid in bacterial killing, and our finding of an increase in CRAMP release during the same approximate time period may synergize with the formation of NETs to result in enhanced killing. The parallel increase in ROS and NET formation is consistent with reports in the literature that suggest these two pathways are interlinked. Furthermore, the observed inhibition of NET formation by the conventional PKC inhibitor Go6976 is also consistent with previous studies demonstrating that PKC isoforms are implicated in the generation of NETs. Importantly, we were able to demonstrate that our findings using SCH79797 with mouse neutrophils did extend to human neutrophils, which also showed robust NET formation and enhanced killing with SCH79797. However, there is experimental literature demonstrating that NETs are pathogenic in models of lung injury and that targeting NETs may have therapeutic potential. In this study, the stimulation of NET formation by SCH79797 may have conflicting effects by having a beneficial effect in promoting bacterial clearance but potentially exacerbating the development of lung injury. The net effect of NET formation in vivo is probably dependent on how extensive the NETs are within the lung and the resultant balance of these competing effects.

In addition to the boosting the effect on neutrophil killing, we observed that SCH79797 had direct, potent antibacterial effects against E. coli. Using both the Bioscreen analysis system and direct killing assays, we were able to demonstrate that SCH79797 had bacteriostatic effects at a concentration of 10 μM and significant bactericidal activity at 100 μM. Using bacterial cytological profiling,
we then were able to ascertain that SCH79797 exhibits rapid permeabilization of bacteria to the DNA dye, SYTOX green, indicating membrane disruption. This is the first description of a PAR1 antagonist exhibiting antibiotic properties, to our knowledge. Furthermore, SCH79797 exerted significant antibacterial effects against Gram-positive bacteria as assessed by morphological (B. subtilis) and killing (MRSA) assays. Of note, the newer-generation PAR1 antagonist vorapaxar had no effect on bacterial growth and did not induce NET formation as mentioned above. These findings support the possibility that SCH79797 is exerting PAR1-independent effects on neutrophils and on bacteria directly and are consistent with prior reports of SCH79797 exhibiting PAR1-independent functions.17

Although we did identify two possible mechanisms to explain the therapeutic effect of the PAR1 antagonist, SCH79797, our study did not explore the effects of this compound on other cells known to express PAR1. This includes endothelial cells, epithelial cells and fibroblasts in the lung parenchyma and possibly other immune cells such as macrophages and dendritic cells. It is likely that SCH79797 also affects some of these cell types, which may explain the dose-dependent effects we observed in vivo. In this context, it is important to note that lower doses of SCH79797 led to a greater survival benefit and a greater reduction in vascular permeability, consistent with the fact that higher doses of SCH79797 can have deleterious effects on endothelial cells and other cell types.17,31 We focused on neutrophils primarily.

Figure 4. SCH79797 has a direct antibiotic effect through disruption of the bacterial membrane. SCH79797 (SCH) significantly impaired the growth of E. coli over the course of 24 h starting at a concentration of 1 μM (a; n = 3 per condition, *P < 0.05 versus E. coli alone) and resulted in no bacterial growth when used between 10 and 100 μM (a; n = 3 per condition, **P < 0.01 versus E. coli). After 6 h, SCH79797 significantly reduced E. coli growth at a concentration of 10 μM, and, at 100 μM, SCH79797 resulted in approximately 95% killing of E. coli. Vorapaxar (Vorapax) at 10 and 100 μM did not significantly suppress E. coli growth (b; n = 3 per group, **P < 0.01 versus positive control (pos ctrl) DMSO condition, data as mean ± SD). The mechanism of SCH79797’s antibiotic effect was determined through cytological profiling of SCH79797 (c–h). ΔtolC E. coli cells were treated with 2% DMSO (c, e and g) or 25 μM (5 × MIC) SCH79797 (d, f and h). (c and d): 30 min post-treatment, LB medium. (e and f): 2 h post-treatment, LB medium. (g and h): 2 h post-treatment, LB medium with 0.5 M sucrose. Cell membranes are stained with FM4–64 (red). DNA is stained with the membrane-permeable dye DAPI (blue) and the membrane-impermeable dye SYTOX Green (green). The presence of SYTOX Green indicates cells with compromised membranes; thus, SYTOX Green is normally absent from untreated cells. Scale bars = 2 μm. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
because they are the predominant innate immune cells present in the alveolar space during the acute phase of pneumonia, comprising up to 90% of the total inflammatory cell component (assessed by cytospin and H&E staining of BAL fluid). Therefore, we anticipated that effects of SCH79797 on neutrophils would have a significant impact on lung injury and mortality in a pneumonia model. Understanding the relative contributions of the various effects of SCH79797 (neutrophil boosting, direct bacterial killing and reduction in vascular permeability) in the current model is difficult to ascertain specifically and the mortality benefit seen with SCH79797 treatment probably represents a summation of these effects.

In addition to the limitations outlined above, another important factor to consider that was not addressed in the current project is the role of other PARs in mediating neutrophil killing of bacteria and the pathogenesis of bacterial pneumonia. Our data (not shown) show that mouse neutrophils do appear to express mRNA for all PARs and have detectable protein expression for PAR1 and PAR2 under basal conditions. Therefore, it is likely highly that other PARs are playing a role in regulating neutrophil activity in response to bacterial infection and that different bacterial strains activate distinct PARs on neutrophils through the production of proteases. These areas will be the focus of future studies.

In conclusion, we have demonstrated that treatment with intrapulmonary PAR1 antagonist using SCH79797 exhibits significant protective effects in a mouse model of E. coli pneumonia. These effects can probably be explained, in part, through the observations that SCH79797 boosts neutrophil killing of bacteria and has direct antibiotic properties. Some of the effects observed with SCH79797 are probably PAR1 independent, because a newer-generation PAR1 antagonist vorapaxar did not produce similar results on neutrophil function or bacterial killing. SCH79797 and PAR1 antagonism more generally, may have therapeutic implications for patients with severe, antibiotic-resistant pneumonia, but understanding which effects are PAR1 dependent and elucidating the optimal, therapeutic concentration will be very important in any translational effort.

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**Transparency declarations**

None to declare.

**Author contributions**

All authors participated in planning experiments. N. G., R. C., R. L. and S. S. carried out experiments. N. G., R. L. and R. C. wrote the manuscript and all authors edited the manuscript. N. G., J. H. G. and V. N. funded the studies.

**Supplementary data**

Supplementary Methods and Figures S1 to S3 are available as Supplementary data at JAC Online.

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