CAL02, a novel antitoxin liposomal agent, in severe pneumococcal pneumonia: a first-in-human, double-blind, placebo-controlled, randomised trial

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Summary

Background Severe community-acquired pneumonia caused by *Streptococcus pneumoniae* is associated with high morbidity and mortality rates. CAL02, a novel antitoxin agent with an unprecedented mode of action, consists of liposomes that capture bacterial toxins known to dysregulate inflammation, cause organ damage, and impede immune defence. We aimed to assess the safety of CAL02 as an add-on therapy to antibiotics.

Methods This randomised, double-blind, multicentre, placebo-controlled trial was done in ten intensive care units (ICUs) in France and Belgium (but only six units enrolled patients), in patients with severe community-acquired pneumococcal pneumonia who required ICU admission and had been identified as being infected with *S pneumoniae*. We randomly assigned participants in two stages—the first stage randomly assigned six patients (1:1) to either low-dose CAL02 or placebo, and the second stage randomly assigned 18 patients (14:4) to either high-dose CAL02 or placebo, and stratified in four blocks (4:1, 4:1, 3:1, and 3:1), in addition to standard of care. Block randomisation was done with a computer-generated random number list. Participants, investigators, other site study personnel, the sponsor, and the sponsor's designees involved in study management and monitoring were masked to the randomisation list and treatment assignment. Patients were treated with low-dose (4 mg/kg) or high-dose (16 mg/kg) CAL02 or placebo (saline), in addition to standard antibiotic therapy. Two intravenous doses of study treatment were infused, with a 24 h interval, at a concentration of 10 mg/mL, stepwise, over a maximum of 2 h on days 1 and 2. The primary objective of the study was to assess the safety and tolerability of low-dose and high-dose CAL02 in patients with severe community-acquired pneumonia treated with standard antibiotic therapy, and the primary analysis was done on the safety population (all patients who received at least one dose of the study treatment). Efficacy was a secondary outcome. This trial is registered with ClinicalTrials.gov, number NCT02583373.

Findings Between March 21, 2016, and Jan 13, 2018, we screened 280 patients with community-acquired pneumonia. 19 patients were enrolled and randomly assigned, resulting in 13 patients in the CAL02 groups (three assigned to low-dose CAL02 and ten assigned to high-dose CAL02) and six in the placebo group. One patient randomly assigned to placebo was allocated to the wrong treatment group and received high-dose CAL02 instead of placebo. Thus, 14 patients received CAL02 (three received low-dose CAL02 and 11 received high-dose CAL02) and five patients received placebo, constituting the safety population. At baseline, the mean APACHE II score for the total study population was 21·5 (SD 4·9; 95% CI 19·3–23·7) and 11 (58%) of 19 patients had septic shock. Adverse events occurred in 12 (86%) of 14 patients in the CAL02 treatment groups combined and all five (100%) patients in the placebo group. Serious adverse events occurred in four (29%) of 14 patients in the CAL02 treatment groups combined and two (40%) of five patients in the placebo group. One non-serious adverse event (mild increase in triglycerides) in a patient in the high-dose CAL02 group was reported as related to study drug. However, analysis of the changes in triglyceride levels in the CAL02 groups compared with the placebo group revealed no correlation with administration of CAL02. No adverse events were linked to local tolerability events. All patients, apart from one who died in the low CAL02 group (death not related to the study drug) achieved clinical cure at the test of cure visit between days 15 and 22. The sequential organ failure assessment score decreased by mean 65·0% (95% CI 50·7–79·4) in the combined CAL02 groups compared with 29·2% (12·8–45·5) in the placebo group between baseline and day 8.

Interpretation The nature of adverse events was consistent with the profile of the study population and CAL02 showed a promising safety profile and tolerability. However, the difference between high-dose and low-dose CAL02 could not be assessed in this study. Efficacy was in line with the expected benefits of neutralising toxins. The results of this study support further clinical development of CAL02 and provide a solid basis for a larger clinical study.

Funding Combioxin.

Research in context

Evidence before this study

Liposomes have long been used as a vehicle to deliver drugs, including antibiotics. However, we searched PubMed with no restrictions on publication date or language with the search terms "liposome" AND "virulence factor" and found no clinical human studies. By including any other type of publication regarding humans, our search retrieved 49 articles, of which only four described liposomal approaches against bacterial virulence factors—one was an in-vitro study showing that CAL02 reduced the interleukin-8 response of human nasopharyngeal epithelial cells to a range of *Streptococcus pneumoniae* isolates and three were reviews of preclinical results and their possible clinical applications.

Added value of this study

To our knowledge, this is the first clinical study to investigate the safety and efficacy of a potential liposome treatment directed against bacterial virulence factors. Our study was done in patients with severe community-acquired pneumococcal pneumonia. All patients received antibiotic treatment according to local standard of care guidelines. CAL02, which is composed only of lipids naturally present in the human body, showed a good safety profile and the potential to improve inflammatory and clinical outcomes of severe community-acquired pneumococcal pneumonia versus antibiotics alone. The results of this study pave the way for further clinical development of CAL02, not only in severe community-acquired pneumococcal pneumonia, but also in other severe infections.

Implications of all the available evidence

Even with the best available therapy, many patients die from the complications of severe community-acquired pneumococcal pneumonia that are caused by bacterial virulence factors. New therapeutic strategies to overcome insufficient clinical responses and increasing resistance to antibiotics are urgently needed. CAL02 could be developed as a first-in-class anti-infective drug that acts regardless of any drug resistance by neutralising bacterial virulence factors, to prevent bacterial spread in the tissues, worsening of clinical status, and fatal organ damage.

Introduction

Bacterial toxins cause highly damaging immune responses, and have a multifactorial role in the dramatic evolution of infections, causing tissue and organ damage and disruption of tissue barriers, facilitating bacterial dissemination, attacking the host's first line of immune defence against the pathogen, and prompting proinflammatory cascades.[1] Toxin-mediated complications can lead to long-term or fatal complications, including organ failure and septic shock.[2–4]

CAL02 is a novel antitoxin agent with an unprecedented mechanism of action.[5–8] CAL02 consists of a mixture of liposomes that create artificially large and stable liquid-ordered lipid microdomains that function as docking sites for a large range of bacterial toxins.[6,9] Therefore, CAL02 acts as a toxin trap and, by neutralising toxins that have a substantial upstream role in the progress and severity of infections, has the potential to provide protection against toxin-mediated organ damage and inflammation. The mechanism of action of CAL02 is complementary to that of antibiotics. Preclinical data show that when combined with antibiotics, CAL02 substantially improves survival outcomes in mice with severe pneumonia and bacteraemia.[6] In this first-in-human trial, two doses of CAL02 were used, in addition to standard of care, and compared with placebo in patients in the intensive care unit (ICU) with severe community-acquired pneumonia caused by *Streptococcus pneumoniae*.

Community-acquired pneumonia is the fourth most common cause of death globally and *S pneumoniae* the most frequent pathogenic cause.[10,11] Community-acquired pneumonia is associated with a high economic burden mainly because of prolonged hospital stays and the fact that more than 20% of patients admitted to hospital will require ICU management.[12] Despite adequate antibiotic therapy, mortality for community-acquired pneumonia is 5–15%, rising to 40% in patients with severe community-acquired pneumonia who are admitted to the ICU, and the median length of stay in the ICU is 13 days.[13–15] Even when antibiotics effectively clear the pathogen, bacterial toxins such as pneumolysin cause widespread damage and can lead to fatal complications.[13,16,17] These toxins are released in massive numbers when a patient's condition worsens, when bacterial loads peak, and, particularly, after bacterial lysis caused by antibiotics.[2,5,18] The primary objective of this first-in-human study was to assess the safety of CAL02 as an add-on therapy to antibiotics.

Methods

Study design and participants

This first-in-human, double-blind, dose-escalation, placebo-controlled, randomised trial was done in ten ICUs (but only six enrolled patients) in France and Belgium, in patients with severe community-acquired pneumococcal pneumonia who required ICU admission and had been identified as being infected with *S pneumoniae*. Full details of the study design are provided in the appendix.

See Online for appendix

Men and women aged 18–80 years, with bodyweight 40–140 kg, were eligible for inclusion in the study if they had severe community-acquired pneumococcal pneumonia on the basis of the presence of at least one major severity criterion, defined by need for invasive

mechanical ventilation or vasopressors, or at least three of the following minor standard severity criteria: respiratory rate of 30 breaths per min or greater, partial pressure of oxygen in the blood to fraction of inspired oxygen (PaO_2/FiO_2) ratio 250 mm Hg or less, multilobar infiltrates; confusion or disorientation, blood urea concentration greater than 7 mM (>40 mg/dL), leukopenia (white blood cell count <4000 cells per µL), thrombocytopenia (platelet count <100 000 cells per µL), hypothermia (core temperature <36°C), and systolic blood pressure less than 90 mm Hg or mean arterial pressure less than 70 mm Hg and received 40 mL/kg or greater of fluid resuscitation over at least 2 h. Patients were excluded when more than 12 h had elapsed since diagnosis of severe community-acquired pneumococcal pneumonia and when more than 24 h or 60 h had elapsed between intravenous or oral antibiotic treatment, respectively, and administration of study treatment. Patients with acute physiology and chronic health evaluation (APACHE) II score greater than 30, with sequential organ failure assessment (SOFA) score greater than 12, with the inability to maintain a mean arterial pressure of at least 50 mm Hg despite administration of vasopressors and fluids, or with a likelihood of death within 72 h, as well as nursing and pregnant women, were also excluded. Immunosuppressant therapy and chronic use of high-dose steroids were also exclusion factors. Full inclusion and exclusion criteria are presented in the appendix.

Signed informed consent from patients, or provided by a relative or legal representative, was a requisite for enrolment. In some instances, the principal investigator, together with an independent medical doctor at the site, consented for the patient if deemed urgent and approved by local laws. Informed consent for patients who were enrolled by such emergency consent was subsequently obtained from the patients themselves, with the exception of two patients who died.

Ethics approval was obtained from the institutional review board at each site. The study followed the Declaration of Helsinki and all principles of good clinical practice.

Randomisation and masking

We planned to randomise 24 patients in two stages—the first stage comprised six patients randomly assigned (1:1) to either low-dose CAL02 or placebo, and a second stage comprised the remaining 18 patients randomly assigned (14:4) to either high-dose CAL02 or placebo, and stratified in four blocks (4:1, 4:1, 3:1, and 3:1). Block randomisation using a computer-generated random number list was prepared by 4Clinics (Waterloo, Belgium) for the study globally. The list was delivered to each site's unblinded study drug preparator in a sealed envelope to maintain allocation concealment. At the time of a participant's randomisation, an electronic data capture system generated a randomisation number that was communicated to site staff via the system. The randomisation number was provided to the study drug preparator, who checked the treatment arm assigned to this number and prepared the appropriate intravenous infusion solution to be administered to the patient. Participants, investigators, other site study personnel, the sponsor, and the sponsor's designees involved in study management and monitoring were masked to the randomisation list and treatment assignment. To ensure masking, all infusion material used to administer the study treatment (such as lines, infusion bags, and infusion pump device) were opaque or adequately covered to conceal the colour of the liquid inside.

Procedures

Patients were treated with low-dose (4 mg/kg) or high-dose (16 mg/kg) CAL02 or placebo (saline), in addition to standard antibiotic therapy. Two intravenous doses of study treatment were infused, with a 24 h interval, at a concentration of 10 mg/mL, stepwise, over a maximum of 2 h on days 1 and 2. Antibiotic therapy was administered to all patients, according to local guidelines, as per national recommendations for community-acquired pneumonia and following local stewardship.

As part of the baseline assessments, a urine antigen test for *S pneumoniae* identification was done in all patients. Additionally, respiratory samples or sputum and blood were obtained to confirm the microbiological diagnosis, and at least one respiratory sample was collected, when possible, between days 6 and 29 to assess the microbiological outcome of pneumonia. We calculated APACHE-II, SOFA, and CURB-65 scores at baseline. The SOFA score was also measured daily while the patient was in the ICU, or if the patient was discharged from the ICU, on days 2, 3, 4, 5, and 29 (or end of study).

Patients were assessed for clinical response to treatment daily in the ICU, on days 2, 3, 4 and 5, at the end of antibiotic treatment, at test-of-cure visits on day 8 (early test of cure) and between days 15 and 22 (test of cure), and on day 29 or at the end of the study. The total study duration was 30 days (1 day for screening and 29 days for treatment and follow-up).

Outcomes

The primary objective of the study was to assess the safety and tolerability of low-dose and high-dose CAL02 in patients with severe community-acquired pneumonia treated with standard antibiotic therapy. The secondary objectives were to evaluate the clinical and microbiological efficacy of CAL02, the pharmacodynamic characteristics of CAL02, and the most appropriate dose of CAL02 for future studies.

The primary efficacy outcome was clinical cure at the test of cure visit between days 15 and 22, as confirmed by the investigator and determined as follows: cure (complete resolution of pneumonia signs and symptoms at baseline, with no new symptoms or complications attributable to the pneumonia, with standard laboratory data back to normal, with fever subsided, and with an improved or a

clean x-ray, making antibiotic treatment unnecessary); failure (persistence or progression of baseline signs and symptoms of pneumonia; or baseline radiographic abnormalities after at least 2 days of treatment; or development of new pulmonary or extrapulmonary clinical findings consistent with active infection, or development of new pulmonary or extrapulmonary infection requiring antimicrobial therapy other than or in addition to the study drug; or death due to pneumonia); or unknown (extenuating circumstances precluding classification as cure or failure). All criteria had to be fulfilled to determine clinical cure.

Secondary efficacy outcomes were clinical cure at the early test of cure visit (day 8) and the late test of cure visit (on day 29 or end of study); all-cause mortality on days 8, 15, and 29 (or end of study); SOFA score, and PaO_2/FiO_2 during ICU stay; 28-day ventilation-free days; chest x-ray at screening and subsequently as medically required (at least one assessment until cure of pneumonia or end of study); times to end of invasive and non-invasive ventilation, clinical cure, discharge from ICU, discharge from hospital, recurrence of pneumonia or re-infection, and death; entry symptoms, physical examination, and chest x-ray while in the ICU and at the time of hospital discharge or death; lung fluid bacterial load from respiratory samples following bronchoalveolar lavage or mini-bronchoalveolar lavage or endotracheal aspirate, protected brush specimen, or sputum during the study; and use of rescue antibiotics (addition or change of antibiotic treatments due to the occurrence of antibiotic resistance after microbiology results at baseline or insufficient efficacy during the study). Exploratory efficacy outcomes were the analysis of inflammatory biomarkers in blood samples on days 1 (pre-dose), 2, 3 and 5, including, but not limited to C-reactive protein and procalcitonin.

Safety assessments were done from the time of signing informed consent to the end of the study, or follow-up when appropriate, and included frequency, duration, severity, and outcome of treatment-emergent adverse events; signs of infusion-related reactions, including clinical signs and symptoms, changes in diastolic or systolic blood pressure, heart rate, oxygen saturation, and skin or local reactions at the infusion site; changes from screening in clinical signs and scores, vital signs (systolic and diastolic blood pressure, heart rate, core temperature during the 12 h after infusion), changes in one-lead or 12-lead electrocardiogram (ECG), and changes from screening in safety laboratory analyses (haematology, clinical chemistry, and urinalysis). Detailed safety evaluation criteria are described in the appendix (pp 2–3). Treatment emergent adverse events were adverse events that started or increased in severity at the time of, or after administration of the first dose of, study medication.

Statistical analysis

As this was a first-in-human study, no formal sample size calculation was done, and only descriptive or exploratory analyses were undertaken. Categorical variables are presented as mean (SD) or median (range), by treatment arm and by visit. Normally distributed continuous variables are expressed in the main text as means (SDs or 95% CIs). For continuous variables with an asymmetrical distribution, data are summarised with the median (range). Full details of the statistical methods and analyses were defined in the statistical analysis plan before database lock and unblinding.

The primary safety analysis was done on the safety population (all patients who received partial or complete study treatment). The efficacy analyses were done on the following populations: the intention-to-treat population (all randomly assigned patients), the modified intention-to-treat population (all randomly assigned patients who received at least one dose of study drug), the as-treated population (intention-to-treat population analysed according to the actual treatment received), the clinically evaluable population (all patients in the modified intention-to-treat population who met the inclusion criteria, received full-course treatment as per random assignment and antibiotic standard of care treatment for at least 5 days, and completed the main visits).

Data management and statistical analyses were done with SAS version 9.4 and R version 3.4.1.

An Independent Data Monitoring Committee (IDMC) was formed before the start of the study to review any relevant safety information, with a focus on events occurring immediately after the start of study drug administration (the first 72 h). The IDMC was composed of four international experts, who were also clinicians with experience in the diagnosis and management of severe pneumonia and practical experience in clinical studies, with a good understanding of the issues and limitations of such studies. The IDMC complied with a charter documenting their main responsibility, the timelines and format of the data to be assessed, including methodological aspects for their analysis of the safety data, and the process by which decisions were made, in agreement with the Guideline on Data Monitoring Committees (EMEA/CHMP/EWP/5872/03).[19] Members of the IDMC were not involved in the conduct of the trial.

As planned in the protocol, the IDMC gathered twice—first, to review the blinded data of patients in the first stage of the trial (six patients recruited and randomly assigned 1:1 to low-dose CAL02 or placebo), and second, to review the blinded data of the five patients in the first randomisation block of the second stage of the trial (randomly assigned 4:1 to high-dose CAL02 or placebo). After the first safety evaluation, the IDMC completed a dose escalation decision form, listing their recommendations with regards to the continuation of the trial to the second stage. After the second safety evaluation, the IDMC completed a dose continuation form, listing their recommendations with regard to the continuation of

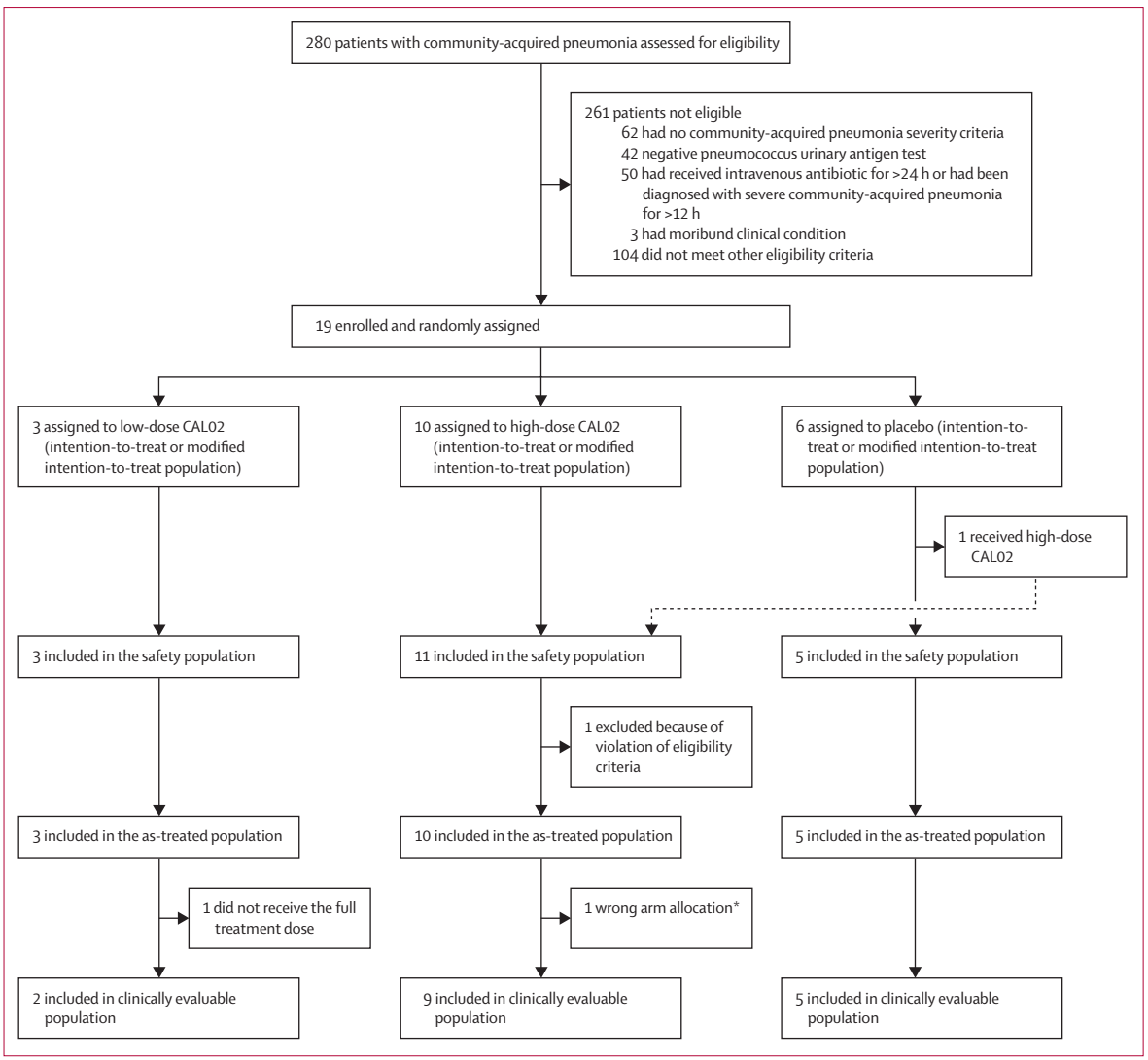

Figure 1: **Trial profile**
*This patient was originally randomly assigned to placebo.

the second stage of the trial. The study was halted during both IDMC evaluations.

This study is registered with ClinicalTrials.gov, number NCT02583373.

Role of the funding source

The funder of the study was responsible for the study design, data collection, data analysis, and writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between March 21, 2016, and Jan 13, 2018, we screened 280 patients with community-acquired pneumonia. Recruitment was stopped on the expiry date of the study drug, at which point a total of 19 patients had been enrolled and randomly assigned and no safety issues had been raised. The patients were randomly assigned according to a predefined sequence of randomisation blocks (appendix), resulting in 13 patients in the CAL02 groups (three assigned to low-dose CAL02 and ten assigned to high-dose CAL02) and six in the placebo group (figure 1).

One patient randomly assigned to placebo was allocated to the wrong treatment group by the site's study drug preparator and received high-dose CAL02 instead of placebo. Thus, 14 patients received CAL02 (three received low-dose CAL02 and 11 received high-dose CAL02) and five patients received placebo, constituting the safety population. The intention-to-treat and modified intention-to-treat populations were identical because all randomised patients received at least one dose of study treatment. One patient in the high-dose CAL02 group

was excluded because of violation of a major eligibility criterion (the patient was outside the time window relative to severity onset). As one of the six patients assigned to placebo received high-dose CAL02, we used the as-treated population for our efficacy analyses. One patient in the low-dose CAL02 group received only one dose of study treatment and was thus excluded from the clinically evaluable population. All surviving patients completed follow-up visits to the end of the study.

Baseline characteristics and comorbidities are presented in table 1. For the diagnosis of severe community-acquired pneumonia, at least one major severity criteria was met in all three (100%) patients in the low-dose CAL02 group, in seven (64%) of 11 patients in the high-dose CAL02 group (the remaining four [36%] patients had three or more minor severity criteria), and in three (60%) of five patients in the placebo group (the remaining two patients [40%] had three or more minor severity criteria; table 1). At baseline, the total study population had a mean APACHE II score of 21·5 (SD 4·9; 95% CI 19·3–23·7), SOFA score of 7·7 (3·3), and PaO_2/FiO_2 of 110·0 (64–350). 11 (58%) of 19 patients were in septic shock (three in the low-dose CAL02 group, seven in the high-dose CAL02 group, and two in the placebo group). No patients were receiving renal replacement therapy at baseline. Baseline characteristics were similar in the modified intention-to-treat population (data not shown), as-treated population (appendix), and clinically evaluable population (data not shown).

All enrolled patients had a positive antigen urinary test for *S pneumoniae*. The infection was confirmed in respiratory samples or blood cultures in 11 (58%) of 19 patients. At baseline, blood cultures were positive for *S pneumoniae* in two (67%) of three patients in the low-dose CAL02 group and three (27%) of 11 patients in the high-dose CAL02-group. No positive blood cultures were reported at baseline in the placebo group. Meticillin-susceptible *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* were detected at baseline in addition to *S pneumoniae* in respiratory samples, but not in blood cultures. No meticillin-resistant *S aureus* strains were documented in patients at baseline. *S aureus* was detected in three patients (one in the low-dose CAL02 group and two in the high-dose CAL02 group) in respiratory samples (sputum or endotracheal aspirate). For two of these patients (one in the low-dose CAL02 group and one in the high-dose CAL02 group), *S aureus* was not considered for treatment by the treating physician and therefore no specific anti-staphylococcal antibiotic was added to treat this pathogen. For the third patient (in the high-dose CAL02 group), the treating physician initiated treatment for both *S aureus* and *S pneumoniae*—notably, augmentin (penicillin and clavulanic acid) and a quinolone. *E coli* and *K pneumoniae* were co-detected in a mini-bronchoalveolar lavage sample in one patient in the low-dose CAL02 group and were not considered for treatment by the treating physician. The clinical outcome for these four patients was cure at test of cure. No blood cultures positive for any pathogen were reported after baseline, no rescue antibiotics were used, and we observed no differences in microbiological outcomes (appendix). No patients were vaccinated against pneumococcus. One patients in the placebo group was vaccinated against influenza. One patient in the low-dose CAL02 group had a viral influenza co-infection.

All patients received appropriate standard antibiotic treatment according to susceptibility in a timely fashion on the basis of final bacteria antibiotic susceptibility, and following local guidelines (appendix). The median time to first dose of study drug since start of intravenous antibiotic treatment was 5 h 10 min (2 h 35 min to 9 h 5 min) in the low-dose CAL02 group, 10 h 25 min (8 h 25 min to 18 h 29 min) in the high-dose CAL02 group,

	Low-dose CAL02 (n=3)	High-dose CAL02 (n=11)	CAL02 combined (n=14)	Placebo (n=5)
Demographic characteristics				
Age (years)	51·1 (13·23)	59·9 (17·49)	58·0 (16·62)	62·6 (14·85)
Sex				
Male	2 (67%)	7 (64%)	9 (64%)	5 (100%)
Female	1 (33%)	4 (36%)	5 (36%)	0
Body-mass index	20·6 (20–22)	25·2 (19–37)	22·8 (19–37)	29·2 (22–40)
Clinical characteristics				
CURB-65	3·3 (0·58)	3·6 (0·92)	3·6 (0·85)	3·2 (0·84)
Acute Physiology and Chronic Health Evaluation II score	25·3 (5·03)	22·4 (3·98)	23·0 (4·21)	17·4 (4·67)
Sequential Organ Failure Assessment score	11·0 (1·00)	7·1 (3·83)	7·9 (3·77)	7·0 (1·00)
Severe community-acquired pneumococcal pneumonia criteria				
Major	3 (100%)	7 (64%)	10 (71%)	3 (60%)
Minor	0	4 (36%)	4 (29%)	2 (40%)
Invasive mechanical ventilation	3 (100%)	3 (27%)	6 (43%)	2 (40%)
Septic shock	3 (100%)	6 (55%)	9 (64%)	2 (40%)
Septic shock and invasive mechanical ventilation	3 (100%)	2 (18%)	5 (36%)	1 (20%)
Acute respiratory distress syndrome	3 (100%)	8 (73%)	11 (79%)	4 (80%)
Bacteraemia	2 (67%)	3 (27%)	5 (36%)	0
PaO_2/FiO_2 ratio	73·0 (64–86)	134·0 (71–350)	107·0 (64–350)	113·0 (67–222)
C-reactive protein (mg/L)	322·3 (117·72)	295·0 (149·80)	300·9 (139·75)	300·6 (198·09)
Procalcitonin (ng/mL)	20·0 (18–22)	28·0 (1–118)	21 (1–118)	7·0 (1–37)
Comorbidities				
Renal failure	2 (67%)	5 (45%)	7 (50%)	2 (40%)
Cardiac disorders	0	6 (55%)	6 (43%)	3 (60%)
Hepatic disorders	0	3 (27%)	3 (21%)	2 (40%)
Thrombocytopenia	2 (67%)	3 (27%)	5 (36%)	1 (20%)

Data are mean (SD), n (%), or median (range). PaO_2/FiO_2=partial pressure of oxygen in the blood/fraction of inspired oxygen.

Table 1: **Baseline characteristics (safety population)**

	Low-dose CAL02 (n=3)	High-dose CAL02 (n=11)	Combined CAL02 (n=14)	Placebo (n=5)
Incidence	3 (100%)	9 (82%)	12 (86%)	5 (100%)
Number of treatment-emergent adverse events	18	67	85	39
Duration of treatment-emergent adverse events (days)	7·1 (4·5–9·7)	6·7 (4·7–8·7)	6·8 (5·2–8·4)	11·6 (8·1–15·2)
Severity of treatment-emergent adverse events				
Mild	7 (39%)	27 (40%)	34 (40%)	18 (46%)
Moderate	9 (50%)	27 (40%)	36 (42%)	16 (41%)
Severe	2 (11%)	13 (19%)	15 (18%)	5 (13%)
Treatment-emergent adverse events related to local tolerability at infusion site	0	0	0	0
Treatment-emergent adverse events suspected or related to study drug	0	1 (9%)	1 (7%)	0
Patient with at least one serious treatment-emergent adverse event	1 (33%)	3 (27%)	4 (29%)	2 (40%)
Number of serious treatment-emergent adverse events	1	10	11	5
Serious treatment-emergent adverse events related to study drug	0	0	0	0
Treatment-emergent adverse events by preferred term (in three or more patients)				
Anaemia	1 (33%)	2 (18%)	3 (21%)	0
Thrombocytopenia	1 (33%)	2 (18%)	3 (21%)	0
Atrial fibrillation	0	1 (9%)	1 (7%)	2 (40%)
Constipation	0	2 (18%)	2 (14%)	2 (40%)
Cholestasis	1 (33%)	2 (18%)	3 (21%)	1 (20%)
Hepatocellular injury	1 (33%)	3 (27%)	4 (29%)	1 (20%)
Blood phosphorus decreased	2 (67%)	0	2 (14%)	2 (40%)
Pleural effusion	1 (33%)	4 (36%)	5 (36%)	0
Hyperglycaemia	0	2 (18%)	2 (14%)	2 (40%)
Hypernatraemia	0	1 (9%)	1 (7%)	2 (40%)
Hypoglycaemia	1 (33%)	2 (18%)	3 (21%)	0
Hypokalaemia	2 (67%)	0	2 (14%)	2 (40%)
Hyponatraemia	1 (33%)	0	1 (7%)	2 (40%)
Renal failure	0	2 (18%)	2 (14%)	1 (20%)
Hypotension	0	1 (9%)	1 (7%)	2 (40%)

Data are n (%), n, or mean (95% CI).

Table 2: **Treatment-emergent adverse events (safety population)**

and 11 h 45 min (4 h 35 min to 14 h 55 min) in the placebo group. 11 (61%) of 18 patients (two [67%] of three in the low-dose CAL02 group, five [50%] of ten in the high-dose CAL02 group, and four [80%] of five in the placebo group) received at least one dose of systemic corticosteroids during the study.

12 (86%) of 14 patients in the CAL02 groups combined and all five (100%) patients in the placebo group had treatment-emergent adverse events, with 124 treatment-emergent adverse events in total (85 in the CAL02 groups combined and 39 in the placebo group). The severity and profile of these treatment-emergent adverse events were similar between groups (table 2). The mean duration of treatment-emergent adverse events was 6·8 days (95% CI 5·2–8·4) in the combined CAL02 groups and 11·6 (95% CI 8·1–15·2) in the placebo group. 57 (67%) of 85 treatment-emergent adverse events in the CAL02 groups combined and 32 (82%) of 39 treatment-emergent adverse events in the placebo group were resolved at the end of the study. Four (29%) of 14 patients in the CAL02 groups combined (one event in one of three patients in the low-dose CAL02 group and ten events in three of 11 patients in the high-dose CAL02 group) had serious adverse events and two (40%) of five patients in the placebo group had serious adverse events (five events in two of five patients; appendix). No serious adverse events were reported as related to the study drug. One patient in the high-dose CAL02 group received renal replacement therapy (dialysis) starting on day 4 after worsening of pre-existing acute renal failure.

The most frequently reported adverse events were linked to underlying diseases (table 2). An increase in transaminases was reported in five patients (four in the combined CAL02 groups and one in the placebo group). This increase was present before study drug dosing in all cases and no Hy's law cases were identified in a detailed examination of liver function (appendix). A one-time mild increase in triglycerides (from 1·04 mmol/L at baseline to 2·17 mmol/L 24 h later) in one patient in the high-dose CAL02 group was reported as related to the study drug. However, analysis of the changes in triglyceride levels in the CAL02 groups compared with the placebo group revealed no correlation with administration of CAL02 (appendix) and no increase in cholesterol was detected. No adverse events were linked to local tolerability events (itching, pain, swelling, redness, bruising, or erythema). We found no marked differences between the treatment groups in terms of vital signs (heart rate, systolic and diastolic blood pressure, and body temperature) recorded 2 h and 12 h after both study drug administrations, which were well tolerated without safety signals (appendix). We observed no changes in one-lead or 12-lead ECGs after study drug administration. We detected no effects of study drug after assessment of QT intervals (QTc) using Bazett's and Fridericia's formulas (appendix). Laboratory data revealed no differences between the treatment groups. No safety concerns were raised by the IDMC.

Considering the small size of the study population, efficacy parameters were meant to be only descriptive. Efficacy endpoints for the as-treated analysis set are described here (table 3; for clinically evaluable and modified intention-to-treat analysis sets, see appendix). All surviving patients achieved clinical cure at test of cure (between days 15–22), and cure at early test of cure (day 8) was achieved in five (56%) of nine patients in the high-dose CAL02 group, one (20%) of five patients in the placebo group, and no patients in the low-dose CAL02 group. One death occurred in each group, representing an overall mortality rate of 16% (three of 19 patients; table 3). None of these deaths were judged as related to the study drug by the investigator, the pharmacovigilance team, and the IDMC. One patient in the low-dose CAL02

	Low-dose CAL02 (n=3)	High-dose CAL02 (n=10)	Placebo (n=5)
Cured at early test of cure (day 8)	0	5 (56%)*	1 (20%)
Cured at test of cure (between days 15–22)	2 (100%)*	10 (100%)	5 (100%)
Median time to cure (days)	15·0 (14 to 16)†	8·0 (6 to 16)	10·0 (7 to 14)
All-cause mortality	1 (33%)	1 (10%)	1 (20%)
Relative change in Sequential Organ Failure Assessment score from baseline to day 8	−65·9% (−34·7 to −97·1)	−64·7% (−46·3 to −83·1)	−29·2% (−12·8 to −45·5)
Relative change in Acute Physiology and Chronic Health Evaluation II score from baseline to day 8	−59·9% (−34·0 to −85·8)	−60·4% (−45·3 to −75·5)	−22·1% (−15·5 to −28·7)
Relative change in PaO_2/FiO_2 from baseline to day 8	153·1 %(116·2 to 189·9)	78·4% (7·4 to 149·3)	58·5% (−27·5 to 137·9)
Median duration of invasive mechanical ventilation (days)†	12·0 (5 to 19)†	4·5 (4 to 14)	12·0 (11 to 56)
28-day ventilation-free days (days)	16·5 (1·8 to 31·2)†	25·1 (22·0 to 28·2)†	17·8 (7·7 to 27·9)
Median duration of intensive care unit stay (days)	15·0 (9 to 21)†	5·0 (2 to 15)	12·0 (6 to 56)
Median duration of stay in hospital (days)	33·0 (12 to 54)†	13·0 (4 to 28)†	21·0 (6 to 56)

Data are n (%), median (range), or mean (95% CI). PaO_2/FiO_2=partial pressure of oxygen in the blood/fraction of inspired oxygen. *One patient was missing for the assessment (because of death). †One patient censored because of death.

Table 3: **Overview of primary and secondary efficacy endpoints in CAL02 and placebo treatment groups (as-treated population)**

group died on day 1 due to refractory septic shock that started before study drug administration. One patient in the high-dose CAL02 group with underlying cardiovascular disease died on day 13 due to respiratory acidosis after care limitation was required by the family following an episode of ventricular tachycardia. This patient was cured and discharged from the ICU at early test of cure. These two patients were bacteraemic at baseline. One patient in the placebo group died in the ICU on day 56 due to underlying chronic obstructive pulmonary disease.

Between baseline and day 8, APACHE II score decreased by a mean of 60·2% (95% CI 48·6–71·9) in the combined CAL02 groups compared with 22·1% (15·5–28·7) in the placebo group (table 3), and SOFA score decreased by a mean of 65·0% (50·7–79·4) in the combined CAL02 groups compared with 29·2% (12·8–45·5) in the placebo group (figure 2, table 3). The cardiovascular SOFA subscore appeared to drive this decrease, with a complete normalisation in both CAL02 groups by day 6 (figure 2). The mean 28-day ventilation-free days was 16·5 (1·8–31·2) in the low-dose CAL02 group and 25·1 (22·0–28·2) in the high-dose CAL02 group, compared with 17·8 (7·7–27·9) in the placebo group (table 3). The median duration of invasive mechanical ventilation was 12·0 days (range 5–21) in two patients in the low-dose CAL02 group, 4·5 days (4–14) in four patients in the high-dose CAL02 group, and 12·0 days (11–56) in three patients in the placebo group (table 3). Entry symptoms and chest x-rays were resolved in all patients at clinical cure. The median time to cure was 15·0 days (range 14–16) in the low-dose CAL02 group, 8·0 days (6–16) in the high-dose CAL02 group, and 10·0 days (7–14) in the placebo group (table 3). The duration of ICU stay was 15·0 days (range 9–21) in the low-dose CAL02 group, 5·0 days (2–15) in the high-dose CAL02 group, and 12·0 days (6–56) in the placebo group (table 3, appendix). In four patients undergoing invasive mechanical ventilation in the high-dose CAL02 group, the median ICU stay was 7·5 days (range 4–15), and in three patients undergoing invasive mechanical

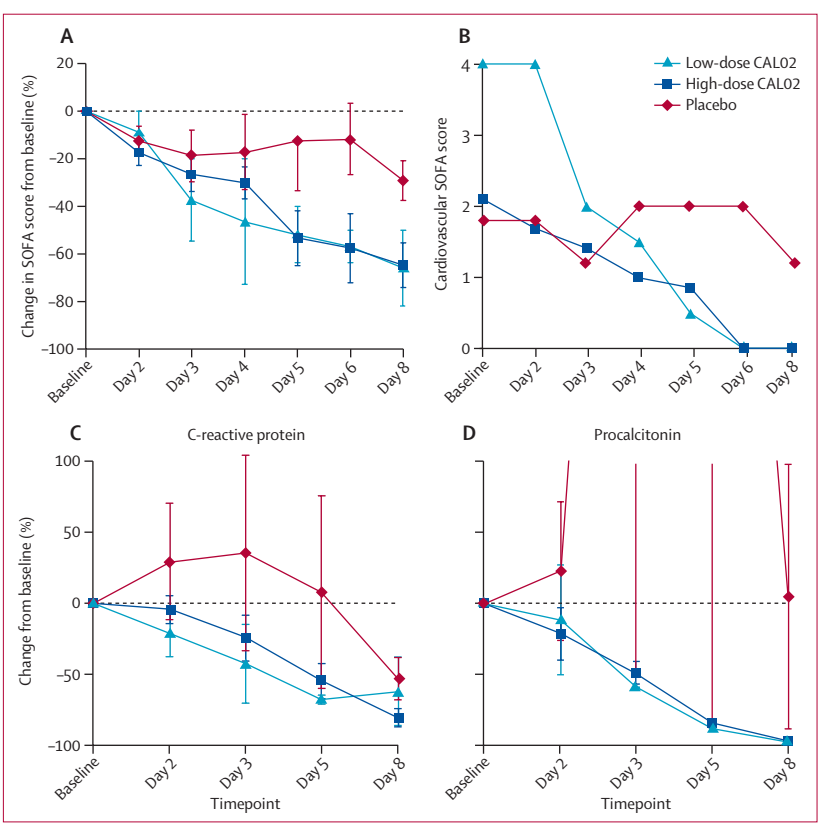

Figure 2: **Change in SOFA score, cardiovascular SOFA score, and C-reactive protein and procalcitonin from baseline to day 8**

Change in SOFA score (A), cardiovascular SOFA score (B), and C-reactive protein (C) and procalcitonin (D). Datapoints show means and error bars represent SE. Due to the scale of the graph, some datapoints in D cannot be shown—these data are 780% (SE −51 to 1611) and 676% (−82 to 1434). SOFA=Sequential Organ Failure Assessment.

ventilation in the placebo group, the median ICU stay was 16 days (12–56; all patients in the low-dose CAL02 group underwent invasive mechanical ventilation). The time courses of C-reactive protein and procalcitonin between baseline and day 8 showed rapid decreases in the CAL02 groups (figure 2).

Discussion

This study evaluated, for the first time to our knowledge, the safety and tolerability of a novel, broad-spectrum, anti-toxin agent—CAL02—in patients with severe community-acquired pneumococcal pneumonia in the intensive care unit treated with standard antibiotic therapy. All patients were diagnosed with pneumococcal community-acquired pneumonia. The disease severity of the study population corresponded to that expected from the inclusion and exclusion criteria (community-acquired pneumonia severity criteria, CURB-65, APACHE II, and SOFA scores). No differences in the baseline characteristics between treatment groups were considered to have had a substantial effect on safety and efficacy outcomes. At both low and high doses, CAL02 proved to be safe and well tolerated. The nature of treatment-emergent adverse events, including deaths, serious adverse events, and other relevant adverse events, was consistent with what was expected in the study population. A similar distribution of treatment-emergent adverse events and no difference in the frequency and severity of adverse events was observed between groups. Clinical safety laboratory tests revealed no other meaningful differences between groups. We observed no relevant treatment effects on vital signs, ECGs, or physical examination, and no deaths or serious adverse events were considered related to CAL02. Efficacy parameters (secondary outcomes) were descriptive only because of the small size of the study population. A trend to a faster resolution was observed in the high-dose CAL02 group for clinical cure at early test of cure, time to cure, change in SOFA score, inflammatory biomarkers, and ICU length of stay. The difference between high-dose and low-dose CAL02 could not be assessed in this study. Importantly, results in the as-treated population remained comparable to those in the modified intention-to-treat and clinically evaluable populations.

The overall mortality in this study (16%) was lower than that reported in previous studies in similar populations, although a broad mortality range has been observed, from 1% to more than 40%.[14,15] Multiple risk factors are associated with the outcome of severe pneumococcal pneumonia (eg, age, comorbidities, septic shock and invasive mechanical ventilation, alcohol abuse, chronic cardiac or pulmonary disease, renal failure, immunosuppression, and socioeconomic status) and some were excluded or we were unable to study them extensively in this study.

We chose the CAL02 dose range and regimen in this study to cover the efficacious dose range reported in preclinical stoichiometric and in-vivo efficacy studies, to offer a large safety margin according to preclinical toxicology studies, and to be within the range of doses of total liposomal lipids for approved intravenous liposome formulations. For several decades, liposomes have been used as biocompatible and biologically inert vehicles for drug delivery. Liposomes have been shown not to produce antigenic or pyrogenic reactions and have not raised concerns over possible long-term accumulation in the body.[20] The sole components of CAL02—cholesterol and sphingomyelin—are naturally-occurring lipids that are present in the outer leaflet of human cell membranes. Liposomal carriers have been well described in the literature and vary in terms of lipid composition, surface charge, size, and method of preparation.[21] Since their discovery in 1965 and the first market approval of a liposomal pharmaceutical product (Doxil, in 1995) more than ten liposomal formulations encapsulating active pharmaceutical ingredients have been approved for human use in Europe and the USA.[22] The spectrum of toxicities reported with these formulations has been consistently associated with the encapsulated drug rather than the liposomal carrier itself.[22] Although no adverse events have been reported as associated with empty liposomal carriers, hypersensitivity-related changes in blood pressure and ECG at the first exposure (also known as complement activation-related pseudoallergy) have been described for some liposomal formulations.[23,24] Several factors have been associated with the occurrence of complement activation-related pseudoallergy, including encapsulated drug, large liposome size, polydispersity, surface charge, and lipid composition.[24–26] CAL02, which consists of empty, neutral, small liposomes, has none of these risk factors. No systemic reaction suggestive of complement activation-related pseudoallergy nor relevant treatment effects in vital signs, ECG, or physical examination were reported in this trial.

Another crucial factor pertaining to the safety of CAL02 concerns the fate of the entrapped toxins once CAL02 is degraded in the liver.[20] Therefore, we paid particular attention to hepatocellular injury (increase in transaminases) in this study. We observed no differences between CAL02-treated and placebo-treated patients in terms of hepatocellular injury. In fact, for the formation of a membrane-insertable composite, bacterial toxins must undergo an irreversible conformational transformation. For example, pneumolysin, which has an integral role in disease and the development of severe and fatal complications and is nearly uniformly present in all *S pneumoniae* clinical isolates, is released by *S pneumoniae* as a soluble monomer. These monomers subsequently oligomerise and the toxins undergo a radical conformational helical-to-sheet conversion, which is essential for their irreversible transformation into membrane-insertable channels.[27,28] Therefore, toxins inserted in CAL02 surfaces differ substantially, in both structure and toxic capability, from the soluble monomers released by bacteria, which might explain why their possible release

during liposome degradation had no toxic consequences in organs where liposomes are metabolised. Additionally, we detected no increase in cholesterol in any of the treatment groups. A mild and transient increase in triglycerides was reported in one patient treated in the high-dose CAL02 group and was judged by the investigator to be related to study drug; however, analysis of triglycerides levels in all patients during the 15 days after treatment showed an overall and notably greater increase only in the placebo arm.

CAL02 contrasts with other antitoxins in clinical development, which consist of monoclonal antibodies with specific affinities to a given toxin. The breadth of action of CAL02, combined with the fact that it is not conducive to the emergence of new resistance, make this agent useful when time for intervention is critical. Moreover, CAL02 is active regardless of the resistance profile of the pathogen and of antibiotic class and susceptibility. CAL02 complements standard-of-care treatments by neutralising pathogenic components against which antibiotics have no activity.

Our study has some limitations. First, the small sample size precludes precise assessment of the clinical efficacy benefit of CAL02. Second, plasma pneumolysin determination could not be done as no recognised techniques for its determination are currently available. However, efficacy trends observed in this first-in-human study are in line with the mode of action of CAL02, which neutralises toxins directly responsible for tissue and organ damage.[1]

To our knowledge, these are the first clinical findings with a drug engineered to neutralise bacterial toxins. Our results show that CAL02 was safe and well tolerated. Data in this study show a potential benefit of CAL02 for parameters related to organ dysfunction and dysregulated inflammation. Therefore, these results suggest a new framework for the treatment of severe infection and provide a solid basis for a larger clinical study with CAL02.

Contributors

P-FL, BF, and AP designed the study. P-FL, BF, AP, SAdS, and FL analysed the data and wrote the manuscript. P-FL, BF, GC, P-FD, TD, and TB recruited participants and collected data. All authors reviewed the manuscript and approved the submission for publication.

Declaration of interests

P-FL reports personal fees from Combioxin, the funder of the trial, outside the submitted work. P-FD and TD's institutions received payments from Combioxin for the inclusion of patients in this trial. FL is a member of the board of directors of Combioxin, but has never received financial compensation from Combioxin for this position. AP works as an external Chief Medical Officer of Combioxin. BF reports personal fees as a member of the Advisory Board of Combioxin during the conduct of the study, and personal fees from Ferring, Asahi-Kasai, Aridis, Inotrem, and AM-Pharma outside the submitted work. All other authors declare no competing interests.

Data sharing

Combioxin is committed to safeguarding the privacy of research participants. De-identified participant-level data and accompanying research resources are available upon request and the data generated in this study will be shared at www.ClinicalTrials.gov. Distribution of data will require compliance with all applicable regulatory and ethical processes, including establishment and approval of an appropriate data sharing agreement.

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References

1 Los FC, Randis TM, Aroian RV, Ratner AJ. Role of pore-forming toxins in bacterial infectious diseases. *Microbiol Mol Biol Rev* 2013; **77:** 173–207.
2 Hirst RA, Kadioglu A, O'Callaghan C, Andrew PW. The role of pneumolysin in pneumococcal pneumonia and meningitis. *Clin Exp Immunol* 2004; **138:** 195–201.
3 Nau R, Eiffert H. Minimizing the release of proinflammatory and toxic bacterial products within the host: a promising approach to improve outcome in life-threatening infections. *FEMS Immunol Med Microbiol* 2005; **44:** 1–16.
4 Otto M. *Staphylococcus aureus* toxins. *Curr Opin Microbiol* 2014; **17:** 32–37.
5 Baumgartner D, Aebi S, Grandgirard D, et al. Clinical *Streptococcus pneumoniae* isolates induce differing CXCL8 responses from human nasopharyngeal epithelial cells which are reduced by liposomes. *BMC Microbiol* 2016; **16:** 154–60.
6 Henry BD, Neill DR, Becker KA, et al. Engineered liposomes sequester bacterial exotoxins and protect from severe invasive infections in mice. *Nat Biotechnol* 2015; **33:** 81–88.
7 Rello J, Bunsow E, Perez A. What if there were no new antibiotics? A look at alternatives. *Expert Rev Clin Pharmacol* 2016; **9:** 1547–55.
8 Wolfmeier H, Radecke J, Schoenauer R, et al. Active release of pneumolysin prepores and pores by mammalian cells undergoing a *Streptococcus pneumoniae* attack. *Biochim Biophys Acta* 2016; **1860:** 2498–509.
9 Simons K, Ikonen E. Functional rafts in cell membranes. *Nature* 1997; **387:** 569–72.
10 Blasi F, Mantero M, Santus P, Tarsia P. Understanding the burden of pneumococcal disease in adults. *Clin Microbiol Infect* 2012; **18** (suppl 5)**:** 7–14.
11 Capelastegui A, España PP, Bilbao A, et al. Etiology of community-acquired pneumonia in a population-based study: link between etiology and patients characteristics, process-of-care, clinical evolution and outcomes. *BMC Infect Dis* 2012; **12:** 134–43.
12 Jain S, Self WH, Wunderink RG, et al. Community-acquired pneumonia requiring hospitalization among US adults. *N Engl J Med* 2015; **373:** 415–27.
13 Torres A. Community-acquired pneumonia: changing paradigms about mortality. *Community Acquir Infect* 2014; **1:** 1–3.
14 Mongardon N, Max A, Bouglé A, et al. Epidemiology and outcome of severe pneumococcal pneumonia admitted to intensive care unit: a multicenter study. *Crit Care* 2012; **16:** R155.
15 Welte T, Torres A, Nathwani D. Clinical and economic burden of community-acquired pneumonia among adults in Europe. *Thorax* 2012; **67:** 71–79.
16 Musher DM, Thorner AR. Community-acquired pneumonia. *N Engl J Med* 2014; **371:** 1619–28.
17 Torres A, Peetermans WE, Viegi G, Blasi F. Risk factors for community-acquired pneumonia in adults in Europe: a literature review. *Thorax* 2013; **68:** 1057–65.
18 Lucas R, Czikora I, Sridhar S, et al. Mini-review: novel therapeutic strategies to blunt actions of pneumolysin in the lungs. *Toxins* 2013; **5:** 1244–60.
19 EMEA. Guideline on Data Monitoring Committees (EMEA/CHMP/EWP/5872/03). https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-data-monitoring-committees_en.pdf (accessed April 20, 2019).
20 Bitounis D, Fanciullino R, Iliadis A, Ciccolini J. Optimizing druggability through liposomal formulations: new approaches to an old concept. *ISRN Pharm* 2012; **2012:** 738432.
21 Chang HI, Yeh MK. Clinical development of liposome-based drugs: formulation, characterization, and therapeutic efficacy. *Int J Nanomedicine* 2012; **7:** 49–60.

22 Working PK, Dayan AD. Pharmacological-toxicological expert report. CAELYX. (Stealth liposomal doxorubicin HCl). *Hum Exp Toxicol* 1996; **15:** 751–85.

23 Soloman R, Gabizon AA. Clinical pharmacology of liposomal anthracyclines: focus on pegylated liposomal doxorubicin. *Clin Lymphoma Myeloma* 2008; **8:** 21–32.

24 Szebeni J, Muggia F, Gabizon A, Barenholz Y. Activation of complement by therapeutic liposomes and other lipid excipient-based therapeutic products: prediction and prevention. *Adv Drug Deliv Rev* 2011; **63:** 1020–30.

25 Cullis PR, Chonn A, Semple SC. Interactions of liposomes and lipid-based carrier systems with blood proteins: relation to clearance behaviour in vivo. *Adv Drug Deliv Rev* 1998; **32:** 3–17.

26 Szebeni J, Baranyi L, Savay S, et al. Liposome-induced pulmonary hypertension: properties and mechanism of a complement-mediated pseudoallergic reaction. *Am J Physiol Heart Circ Physiol* 2000; **279:** H1319–28.

27 Gilbert RJ. Pore-forming toxins. *Cell Mol Life Sci* 2002; **59:** 832–44.

28 Leung C, Dudkina NV, Lukoyanova N, et al. Stepwise visualization of membrane pore formation by suilysin, a bacterial cholesterol-dependent cytolysin. *Elife* 2014; **3:** e04247.