AP301: Development of inhalation medicine from scientific discovery to clinical proof-of-concept for treatment of life-threatening pulmonary oedema

Bernhard Fischer1, Susan Tzotzos1, Hendrik Fischer1, Helmut Pietschmann1, Waheed Shabbir1, Rosa Lemmens-Gruber2 & Rudolf Lucas3

1APEPTCO GmbH, Mariahilferstr. 136, Top 1.15, Vienna, 1150, Austria
2Department of Pharmacology & Toxicology, University Vienna, Vienna, 1190, Austria
3Medical College of Georgia, Georgia Regents University, Augusta, 30912-2500, USA

Summary
Pulmonary oedema is a life-threatening complication in patients with a variety of underlying critical diseases. Today, no medicinal product has been authorised directly activating alveolar liquid clearance. Based on the TNF loop structure as determined by x-ray crystallography, a family of AP301 peptides has been designed. Assessment of human epithelial cells A549 and H441 as well as primary isolates from mouse, rat, pig and dog demonstrated that AP301 binds to and activates the amiloride-sensitive epithelial sodium ion channel ("ENaC") of pulmonary cells. The interaction of AP301 and ENaC leads to enhanced Na⁺ ion transport, a Na⁺ ion gradient and a directed osmotic force across the pulmonary epithelial cell layer. Water molecules follow this osmotic gradient -- thus clearing oedema fluid from the lung. The oedema clearing potential of AP301 has been studied in great detail in various animal models of non-cardiogenic and cardiogenic pulmonary oedema, including models of hydrostatic and permeability oedema, Acute Lung Injury / ARDS, pneumonia, sepsis and lung transplantation. In addition to non-clinical pharmacodynamics studies, the complete battery of safety and toxicity studies has been conducted in mice, rats, pigs and dogs prior clinical assessment. To target the AP301 peptide into the pulmonary air space, AP301 peptide has been specifically formulated. Liquid preparation of AP301 is converted into an aerosol of fine particles by a mesh-type nebuliser. The AP301 aerosol is dispensed directly in the circuit of mechanically ventilated ICU-patients thus delivering a constant stream of AP301 droplets into the lung.

Safety and tolerability of orally inhaled AP301 has been demonstrated in a Phase I clinical study in 48 volunteers in 2011. From 2012 to 2014, an interventional, randomized, double-blind, placebo-controlled, phase IIa study has been conducted. The clinical study demonstrated that oral inhalation of AP301 by mechanically ventilated ICU-patients with lung oedema led to an earlier onset and more effective oedema clearance, improvement of Murray Lung Injury Score and increase in ventilator free days in comparison to placebo treatment, respectively.

Introduction
Pulmonary oedema is a life-threatening complication in patients with a variety of underlying critical diseases. Today, no medicinal product has been authorised to directly activate alveolar liquid clearance (ALC). While the cardiogenic lung oedema is associated with Acute Decompensated Heart Failure (ADHF), non-cardiogenic lung oedema is associated with critical diseases such as concomitant existing pneumonia, aspiration of gastric content, inhalation injury, near drowning, concomitant existing sepsis, multiple trauma, multiple blood transfusion, burns, acute pancreatitis, drug overdose. Both direct and indirect lung injury may lead to Acute Lung Injury (ALI) / Acute Respiratory Distress Syndrome (ARDS) in which the presence of a massive pulmonary permeability oedema is a major characteristic element. The current paradigm for liquid homeostasis in the adult mammalian lung is that passive apical uptake of Na⁺ via epithelial amiloride sensitive sodium ion channel (ENaC) and amiloride-insensitive CNG channels creates the major driving force for reabsorption of water through the alveolar epithelium. Water follows the osmotic gradient created by Na⁺ ion movement, resulting in fluid removal from the alveoli and subsequent extrusion into the interstitial space. Disruption of these processes occurs in pathologies in which permeability of the alveolar epithelium and pulmonary capillary endothelium is increased, leading to excessive accumulation of alveolar liquid fluid (ALF) and oedema. Human TNF-α plays a dual role in ENaC regulation. Activation of TNF receptor 1 mediates transcriptional inhibition of all three ENaC subunits (α, β, γ) and induces PKC-dependent post-translational inhibition of ENaC-α subunit expression in vitro and in damaged lungs in vivo. By contrast, TNF increases oedema reabsorption in a rat pneumonia model and stimulates Na⁺ uptake in A549 cells in a catecholamine-independent manner. These activities are mediated by the lectin-like domain ("TIP") of the cytokine, which is spatially distinct from the receptor binding sites. Based on the TNF lectin-like domain as determined by x-ray crystallography, a small 17-amino acid circular peptide ("AP301") has been designed to target ENαC and to activate Na⁺ ion movement through epithelial cells. The aim of the “AP301 project” was and still is to translate scientific discovery results into a brand-new inhalation medicine for direct treatment of patients with life-threatening pulmonary oedema.

Experimental methods
AP301 peptide design: Using a 3D structural model for human TNF-α (PDB ID: 1A8M) as a template, 3D models of AP301 were built in DeepView by mutating the P100 and E116 residues to cysteine and the C101 to glycine, followed by removal of extra residues and energy minimization. Peptide synthesis: The AP301 peptide was synthesised by solid-phase peptide synthesis according to the fluorenylmethoxycarbonyl/t-butyl protection strategy.
on 2-chlorotritylchloride resin. Cyclisation in AP301 was achieved by oxidation of the terminal cysteine residues to form a disulphide bridge. The purity of the peptide was 96.3%. The average molecular mass was 1.923.1 Da. Isolation of alveolar type II cells: Dog, rat and pig alveolar epithelial type II cells were isolated as previously described\textsuperscript{10-11}. Enrichment for dog or rat alveolar type II cells was achieved by removal of macrophages and lymphocytes using differential adherence on bacteriological plates (37°C, 10% CO\textsubscript{2}) coated with dog IgG, pig or rat IgG, respectively. Cell culture: In addition to dog, pig and rat alveolar type II cells, the human alveolar carcinoma cell line A549 (ATCC no. CCL-185) was used for electrophysiological studies. Cells were grown in Dulbecco’s modified Eagle’s medium/nutrient mixture Ham’s F-12K supplemented with 10% foetal bovine serum and containing 1% penicillin-streptomycin as previously described\textsuperscript{12-13}. Electrophysiological experiments: Measurement of the amiloride-sensitive sodium ion current in enzymatically isolated alveolar epithelial type II cells from dog, pig and rat lungs was carried out using the patch clamp technique in whole cell mode\textsuperscript{14}. Use of nebuliser: The intended clinical route of administration of AP301 is pulmonary delivery by oral inhalation as aerosol. Prior to use, lyophilised AP301 is reconstituted with WFI. AP301 solution is converted into aerosol by a CE-marked mesh-type liquid nebuliser (Aeroneb® solo nebulizing system, Aerogen). Non-clinical toxicity and safety pharmacological studies: Mouse, rat and dog have been selected as relevant rodent and non-rodent animal species for the toxicity and safety pharmacology testing of AP301. Non-clinical toxicology and safety studies were conducted in compliance with the current ICH M3 (R2) guidance and were performed under GLP. Aerosol characterisation: The AP301 aerosol was characterised by following techniques: Nebulisation efficiency by mechanical breath simulator; Aerosol particle size by Next Generation Impactor technique and by laser diffraction measurements. Clinical trial: Following a phase I clinical study to assess safety and tolerability of orally inhaled AP301 (EUDRACT no. 2011-000223-38), a phase IIa clinical study (EUDRACT no. 2012-001863-64). Proof of concept study in male and female intensive care patients to investigate the clinical effect of repetitive orally inhaled doses of AP301 on alveolar liquid clearance in acute lung injury\textsuperscript{*} has been conducted to assess the effect of orally inhaled AP301 on alveolar liquid clearance in mechanically ventilated patients with ARDS within 7 days of treatment. This trial was an interventional, randomized, double-blind, placebo-controlled, parallel-group study, conducted at the Medical University Vienna.

Results

AP301 mimics the lectin-like domain of human TNF, located in triplicate as a loop structure at the apex of the native TNF homotrimeric molecule\textsuperscript{15}. The lectin-like domain of human TNF comprises residues C101-E116 in each of the polypeptide chains A, B and C. Addition of a cysteine residue at C1 and replacements of C101 by a glycine and E116 by a cysteine residue C17 resulted the 17-mer AP301-peptide. Formation of a disulphide bond between the cysteine residues, C1 and C17 resulted in the cyclic peptide AP301. The aim of cyclisation was to preserve as much as possible the 3D loop structure of the lectin-like domain in order to maintain its biological function. The aim of mimicking the lectin-like domain of TNF has been successfully achieved. The biological activity of AP301 was tested electrophysiologically in whole-cell patch clamp experiments using the mammalian lung adenocarcinoma line, A549, as well as freshly-isolated type II alveolar cells from dog, pig and rat and more recently HEK and CHO cells heterologously expressing human ENaC subunits\textsuperscript{12,13,16}. Addition of AP301 to patch-clamp cell assays results in an immediate influx of Na\textsuperscript{+} ions into the cells and addition of amiloride eliminates this effect. Converting the AP301 solution into an aerosol by mesh type nebuliser had no effect on the ENaC-activating effect of AP301, showing that the AP301 peptide is robust and withstands mechanical forces.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{(A) hole cell recordings from A549 cells clamped at holding potentials (Eh) ranging from -100 mV to +100 mV used to plot IV curves for the control (open circles), in presence of 60 nM AP301 (filled squares) and after addition of 10 \textmu M amiloride, which blocks the Na\textsuperscript{+} current in control as well as in the presence of AP301. Mean values ± SE are given for five experiments. (B) Amiloride-sensitive AP301-induced current activation in transiently and endogenously expressed ENaC. AP301 activated the Na\textsuperscript{+} current in transiently expressed ENaC in HEK-293 and CHO cells with similar efficiency and potency (EC\textsubscript{50} in HEK: 54.7 ± 2.2 nM, n=7; in CHO: 58.1 ± 1.9 nM, n=9) to endogenously expressed ENaC in A549 cells (EC\textsubscript{50} 54.7 ± 1.0 nM, n=11). The respective control currents were 78.2 ±5.9 pA in HEK-293, 109.7 ±11.0 pA in CHO and 118.5 ±8.3 pA in A549 cells.}
\end{figure}
Reconstituted AP301 at three different concentrations (25 mg/ml, 5 mg/ml and 1 mg/ml) was converted into aerosol by a mesh-type liquid nebuliser. There was a close correlation between results of NGI and laser diffraction measurements. Breath simulator studies predicted that on average 70% of the nominal nebuliser filling dose of AP301 aerosol reaches the mouth of subjects when using AP301 concentration of 25 mg/ml.

Table 1 Results of NGI analysis of aerosolised AP301 solution produced by Aeroneb Solo nebuliser.

<table>
<thead>
<tr>
<th>AP301 (mg/mL)</th>
<th>MMAD(μm)</th>
<th>FPF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.86</td>
<td>49.1</td>
</tr>
<tr>
<td>5</td>
<td>5.77</td>
<td>37.7</td>
</tr>
<tr>
<td>25</td>
<td>5.32</td>
<td>46.2</td>
</tr>
</tbody>
</table>

Figure 2 Particle size distribution of nebulised AP301 solution (25 mg/mL) produced by Aeroneb Solo device as determined by HELOS laser diffractometer.

In non-clinical pharmacodynamics animal models, such as hydrostatic lung oedema, permeability lung oedema, lung oedema caused by pneumonia, sepsis and influenza A virus infection, pulmonary administration of AP301-peptide resulted efficient alveolar liquid clearance (ALC). In a model of acute lung injury in intubated and mechanically ventilated pigs, AP301 aerosol was dispensed directly into the breathing circuit. Treatment resulted in a sustained improvement of the lung function according to the surrogate parameters extravascular lung water index (EVLW), oxygenation index and pulmonary shunt fraction.

In regulatory toxicology and safety pharmacology studies, AP301 aerosol was applied by inhalation. In rat studies, the AP301 aerosol was delivered through the nose. In dog studies, the aerosol was delivered via a face mask. In all animal studies, the FPF was approx. 60% (Anderson Cascade Impactor). In rat and dog studies, the daily achieved AP301 exposure doses were approx. 25 mg/kg b.w. resulting total exposure doses of approx. 350 mg/kg b.w. following 14 days of AP301 inhalation.

To correlate animal toxicity and safety data to human equivalent dose, scaling between animal species and man based on orally delivered AP301 concentration per lung weight, rather than per body surface area or body weight. Animal NOAEL values were converted into MRSD (Maximum Recommended Starting Dose) values taking account of species specific pulmonary deposition factors and appropriate safety factors: Animal NOAEL values were converted into estimated lung exposures by multiplying it with species-specific lung weight factors. Then, estimated dose level per lung weight was corrected for the FPF in order to calculate the delivered dose per lung weight. The human equivalence lung dose was then obtained by dividing the delivered dose per animal lung weight by the human lung weight factor. For generating the MRSD (AP301 per kg human lung weight), HELD values were divided by safety factors (SF) of 50.

In a phase I clinical study, six dose levels of AP301 between 4 mg and 120 mg were orally inhaled by healthy volunteers. The randomized, double-blind, placebo-controlled parallel-group first-in-men “dose escalation study in healthy male subjects to investigate safety, tolerability and systemic exposure of orally inhaled single doses of AP301” has been performed at the Medical University / General Hospital in Vienna. In total 48 subjects inhaled either aerosolized AP301 or placebo solution from an adapted mesh type nebuliser with mouthpiece. The local and systemic tolerability profile of AP301-peptide was excellent. No serious or unexpected adverse events were reported. No significant changes in exhaled NO lung function and safety laboratory parameters were observed. Inhalation of AP301 showed no meaningful effects on vital signs (blood pressure, pulse rate, body temperature). Systemic absorption and distribution of AP301, as determined by HPLC–MS/MS, was minimal.

Between Q3/2012 and Q1/2014, an interventional, randomized, placebo-controlled, parallel-group “proof of concept study in male and female intensive care patients to investigate the clinical effect of repetitive orally inhaled doses of
AP301 on alveolar liquid clearance in acute lung injury has been performed. In this study, 40 mechanically ventilated, critical ill intensive care patients with pulmonary oedema received two daily doses of either AP301 aerosol or placebo for up to 7 days. Patients were stratified into two groups regarding a SOFA (Sequential Organ Failure Assessment) score ≤ or > 10. Primary efficacy endpoint was the amount of extravascular lung water (EVLW) between day 0 and day 7 of treatment. EVLW was measured twice daily using PiCCO technique. The clinical study demonstrated that oral inhalation of AP301 by mechanically ventilated ICU-patients with lung oedema led to an earlier onset and more effective oedema clearance, improvement of Murray Lung Injury Score and increase in ventilator free days in comparison to placebo treatment, respectively.

**Conclusion**

Alveolar liquid clearance (ALC) is critical to preventing excess fluid accumulation in the alveoli, which compromises gas exchange. Dysfunctional ALC correlates with morbidity and mortality in ICU patients. ALC is mediated mainly by vectorial Na$^+$ transport through the apical epithelial sodium channel (ENaC). The AP301-peptide has been developed from discovery through pre-clinical, phase I and phase IIa “proof-of-concept” clinical studies. The final goal of the project is to develop a therapeutic molecule that activates ALC in mechanically ventilated patients with pulmonary permeability oedema. Currently, no approved treatment exists.

Performing a battery of pharmaceutical and technical studies, non-clinical pharmacodynamics, cell-based and animal studies, followed by regulatory toxicology and safety pharmacology studies, allowed the initiation of clinical studies in volunteers and patients. Following a successful phase I clinical study in 2012, a recent European study compared placebo treatment with AP301 inhalation in patients with pulmonary permeability oedema and ARDS and found that AP301 elicited earlier and more pronounced clearance of pulmonary oedema. Our clinical data support the novel therapeutic use of AP301 aerosols in patients with life-threatening pulmonary permeability oedema and ARDS.

Future developments have been initiated to assess the potential of AP301 in related life-threatening conditions, such as high altitude pulmonary oedema (HAPE) and primary graft dysfunction (PGD) in patients following lung transplantation.
References


