The 2010 Annual Conference of the European Respiratory Society has been a major success for APEPTICO Forschung und Entwicklung GmbH

The beneficial pharmacodynamic effects APEPTICO's development compound AP301, also known as "TIP Peptide", have been subject of three presentations:

The E-Communication "The TNF-derived TIP peptide reduces Listeriolysin-induced ENaC dysfunction in human airway epithelial cells" summarised our findings that the main virulence factor of *L. monocytogenes*, listeriolysin (LLO), can contribute to the occurrence of permeability edema by reducing ENaC expression. However, APEPTICO's TIP peptide AP301 restores impaired ENaC-α expression and Sgk-1 activation in LLO-treated epithelial lung cells and activates Na+ uptake in LLO-treated airway epithelial cells.

In the oral presentation "Crucial role for protein kinase C-α and arginase activation in the induction of pulmonary endothelial hyperpermeability by pneumolysin" it was highlighted that the protein kinase C-α (PKC-α) is a potential upstream and arginase as a potential downstream therapeutic target during Gram+ infection-associated pulmonary hyperpermeability. It was summarised that APEPTICO's TIP peptide AP301 is able to blunt their activation by the toxin pneumolysin (PLY) secreted by *Streptococcus pneumoniae*. Death in these pneumonia patients correlates with the presence of the pore-forming toxin pneumolysin.

The poster presentation "Development of inhalation therapy for prevention and treatment of acute lung injury" summarised APEPTICO's therapeutic approach for the treatment of pulmonary oedema and acute lung injury (ALI)/acute respiratory distress syndrome (ARDS). A stable AP301 formulation has been developed that is transformed into an aerosol and effectively inhaled into the lower respiratory tract.

PLEASE find attached abstracts of all three presentations and PDF-copies of two posters.

Having demonstrated the therapeutic benefit of APEPTICO's compound AP301 in various animal models of pulmonary oedema (including ALI and ARDS) and microbial-induced pneumonia, currently APEPTICO concentrates on conduct of the "first-in-man" clinical study and to demonstrate the therapeutic effect of the lead compound AP301 in Influenza A (IAV) and respiratory syncytial virus (RSV) induced pneumonia.

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P2286

Development of inhalation therapy for prevention and treatment of acute lung injury

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In patients with Acute Lung Injury (ALI), mortality and the capacity of patients to resolve pulmonary oedema are inversely correlated. Alveolar liquid clearance is facilitated by ion gradients generated by sodium ion channels (ENaC) located at the apical side of type II alveolar cells.
We report the development of inhalation therapy that assists in alveolar liquid clearance by activating ENaC of alveolar cells.
Patch clamp measurement on human epithelial type II pulmonary cells demonstrated that synthetic peptides manufactured by Fmoc technology were capable of activating ENaC in a concentration-dependant manner. Formulation of peptide in WFI followed by nebulisation with Aeroneb ProX mesh-nebuliser, produced aerosol particles of diameters less than 4 µm, thus enabling the aerosol to penetrate the lower respiratory tract. Peptides activity was unaffected by the nebulisation procedure. Nebulised peptides were detected in lung tissue by using a combination of liquid chromatography (LC) and mass spectrometry (MS). With IRB approval, ALI was induced in pigs and 25 mg synthetic peptide was nebulised and inhaled into the lung of test animals followed by mechanical ventilation for five hours. Inhalation of synthetic peptide resulted in significant increase in paO_2 values and reduction of pulmonary oedema as measured by extravascular lung water content. Haemodynamic parameters remained unchanged during time of observation.
Our data demonstrate for the first time that small synthetic peptides can be inhaled into the lower respiratory tract as aerosol retaining biological activity to protect lung tissue from injury and to resolve pulmonary oedema.

2192

The TNF-derived TIP peptide reduces Listeriolysin-induced ENaC dysfunction in human airway epithelial cells

J. Hamacher, G. Yang, R. White, M. Leustik, A. Oseghale, B. Fischer, T. Chakraborty, R. Lucas
(Augusta, United States Of America; Giessen, Germany; Vienna, Austria)

Rationale: Pulmonary permeability edema is a major complication of listeriosis and involves a reduced alveolar Na^+ uptake capacity.
Methods: We have investigated the effect of the main virulence factor of *L. monocytogenes*, i.e. listeriolysin (LLO), on the expression of the crucial α subunit of ENaC, as well as on two positive and one negative regulator of ENaC expression, i.e. the Serum and Glucocorticoid dependent Kinase 1 (Sgk-1), protein kinase B (Akt-1), and Protein Kinase C-α in the human H441 cell line. We have also assessed the effects of LLO on amiloride-sensitive Na^+ currents in H441 cells, using patch-clamp. Finally, we have tested whether the TNF-derived TIP peptide, shown to activate Na^+ uptake in airway epithelial cells, can interfere with the LLO-induced effects.
Results: ENaC-α protein expression in H441 cells is significantly decreased from 2h post LLO-treatment on, an effect blunted by the protein kinase C-α inhibitor Ro-32-5032. LLO causes a significant reduction of phospho-Sgk-1-T256 and phospho-Akt-1-S473 expression, required for full activation of the kinases. The TNF-derived TIP peptide restores ENaC-α expression, as well as Sgk-1, but not Akt-1 activation in LLO-treated cells and significantly increases amiloride-sensitive Na^+ uptake in H441 cells in the presence of LLO.
Conclusion: These results indicate that LLO can contribute to the occurrence of permeability edema by reducing ENaC expression. The TIP peptide restores impaired ENaC-α expression and Sgk-1 activation in LLO-treated H441 cells and activates Na^+ uptake in LLO-treated airway epithelial cells.

2108

Crucial role for protein kinase C-α and arginase activation in the induction of pulmonary endothelial hyperpermeability by pneumolysin

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Rationale: Infections with *Streptococcus pneumoniae* can cause pulmonary endothelial hyperpermeability. Death in these pneumonia patients correlates with the presence of the pore-forming toxin pneumolysin (PLY). This study aims to clarify mechanisms of PLY-induced endothelial dysfunction, in order to identify novel therapeutic targets and treatment options.
Methods: We assessed PLY-induced endothelial hyperpermeability with transendothelial resistance measurements (ECIS) of monolayers of human lung microvascular endothelial cells (HL-MVEC) *in vitro*, or with an acute lung injury model *in vivo*, upon intratracheal PLY-instillation in mice.
Results: PLY induces a dose-dependent hyperpermeability in HL-MVEC *in vitro*, preceded by a disturbance of the RhoA/Rac1 balance and an increased myosin light chain (MLC) phosphorylation. The PKC-α inhibitor Ro-32-5032 blunts the permeability increasing effect of PLY and the toxin moreover induces a time-dependent activation of PKC-α. PLY leads to an increased arginase activity in HL-MVEC, which can cause eNOS uncoupling. Moreover, the arginase inhibitor BEC reduces PLY-mediated hyperpermeability. Intratracheal PLY instillation causes a PKC-α-dependent endothelial hyperpermeability in mice within 6h. The TNF-derived TIP peptide is able to inhibit PLY-induced PKC-α activation, as well as the resulting endothelial hyperpermeability *in vitro* and *in vivo*.
Conclusion: These results identify PKC-α as a potential upstream and arginase as a potential downstream therapeutic target during G^+ infection-associated pulmonary hyperpermeability and moreover indicate that the TIP peptide is able to blunt their activation by PLY.

The TNF-derived TIP peptide Reduces Listeriolysin-induced ENaC Dysfunction in Human Airway Epithelial Cells

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BACKGROUND

Listeriosis can result in lethal pulmonary complications (1), characterized by reduced sodium uptake capacity and alveolar epithelial/capillary endothelial hyperpermeability. Reduced sodium uptake capacity is often linked to reduced expression and function of the epithelial sodium channel (ENaC), as can be found in acute lung injury, severe pneumonia and ARDS (2).

We have investigated the effect of the pore-forming exotoxin Listeriolysin (LLO), the main virulence factor of *L. monocytogenes*, on the expression of the crucial α subunit of ENaC, as well as on two positive and one negative regulator of ENaC expression, i.e. the Serum and Glucocorticoid dependent Kinase 1 (Sgk-1), protein kinase B (Akt-1) and protein kinase C-α (PKC-α), respectively, in the human alveolar epithelial H441 cell line.

We have also assessed the effects of LLO on amiloride-sensitive Na^+ currents in H441 cells, using voltage-clamped perforated patch-clamp.

Finally, we have investigated whether the TNF-derived TIP peptide, mimicking the lectin-like domain of the cytokine and previously shown to activate Na^+ uptake in airway epithelial cells (3-5), can interfere with the LLO-induced effects on sodium transport *in vitro* and *in vivo*.

AIMS

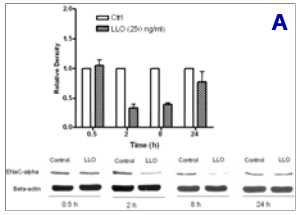
We want to answer the following questions:
1. Does LLO interfere with the expression and activity of ENaC and of its regulators?
2. Can the TNF-derived TIP peptide blunt LLO effects on ENaC function in H441 cells *in vitro* and in mouse lungs *in vivo*?

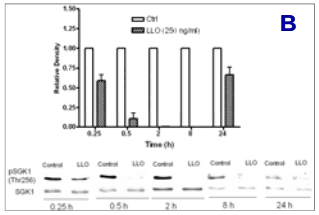
MATERIALS AND METHODS

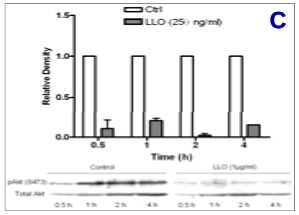
***In vitro* experiments.**
H441 cells (ATCC) derived from papillary adenocarcinoma of human lungs were cultured in RPMI-1640 medium with 10% fetal bovine serum. The protein levels of ENaC-α, Sgk-1, Akt-1 and PKC-α in H441 cells were determined by Western blotting. For patch clamp experiments, H441 cells were placed in normal Ringer's solution in a cell chamber and perforated whole cell currents were recorded in the presence of 200 mg/ml Amphotericin B in the pipette solution. Essentially LPS-free LLO preparations were generated and purified in Dr. Chakraborty's laboratory.

***In vivo* mouse model.**
6-8 wks old male C57BL6 mice were given 10 μg human TIP peptide/mouse at the time of intratracheal instillation of LLO (1 μg/mouse) or vehicle. Mouse lung wet-to dry ratio was assessed 6h after LLO instillation.

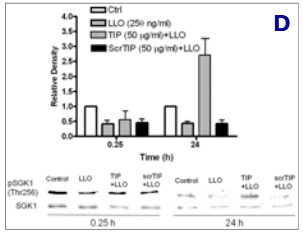
RESULTS

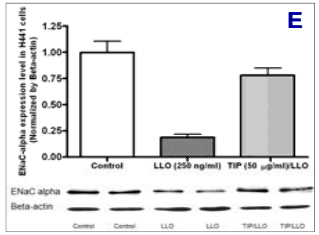


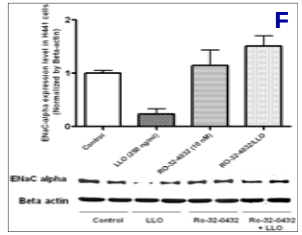




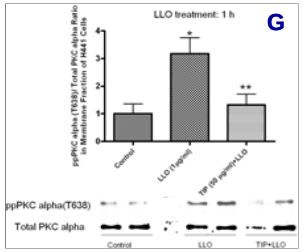
(A). LLO (250 ng/ml) significantly decreases ENaC-α protein expression in H441 cells after 2h of incubation ($p<0.05$) and this expression is partially restored at 24h post-treatment (n=3); **(B).** LLO does not significantly change p(S422)Sgk1 expression level (data not shown), but blunts p(T256)Sgk1 expression level, which is partially restored at 24h post-treatment (n=3) **(C).** LLO does not affect p(T308)Akt1 expression levels at different time points (data not shown), but significantly blunts p(S473)Akt1 expression in H441 cells (n=3; $p<0.05$)



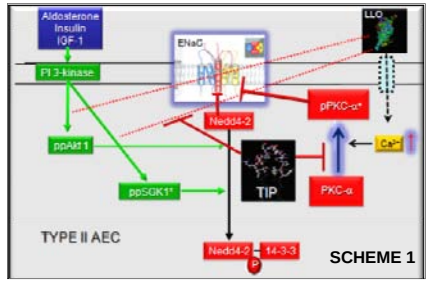


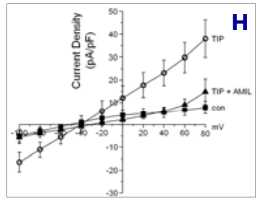


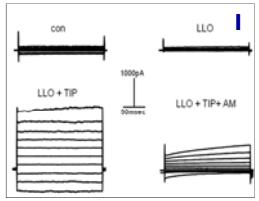
(D). The TNF-derived TIP peptide (50 μg/ml), when applied 30 min prior to LLO (250 ng/ml), restores LLO-blunted p(T256)Sgk1 expression after 24h of incubation and even increases its expression significantly over the basal level. (n=3) ;. **(E).** A 30 min pretreatment with the TNF-derived TIP peptide (50 μg/ml) partially restores LLO-blunted ENaC-α subunit expression after 2h of incubation (n=3) ; **(F).** The pretreatment with the PKC inhibitor RO-32-4032 (10 nM) restores ENaC-α subunit expression at 2h post LLO-treatment (1 μg/ml; n=3; $p<0.05$);



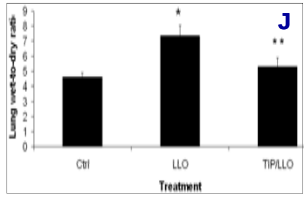
(G). TIP peptide significantly decreases LLO-induced PKC-α activation in H441 cells, as demonstrated by a significant increase of the ratio of pT638-PKC-α over total PKC-α within 1 h in the membrane fraction of the H441 cells (n=3), *: $p<0.05$ *vs.* ctrl; **: $p<0.05$ vs. LLO.







(H). The TIP peptide (50 μg/ml) increases amiloride-sensitive inward sodium current in H441 cells (n=7; representative current trace); **(I).** The TIP peptide blunts LLO (250 ng/ml)-induced inhibition of inward sodium current and amiloride (1 μM) can inhibit this effect in H441 cells (n=7; representative current trace);



(J). The TIP peptide (10 μg /mouse) inhibits the LLO (1 μg/mouse)-induced increase in lung wet-to-dry ratio in C57BL/6 mice after 6h treatment (n=7), *: $p<0.05$ vs. ctrl; **: $p<0.05$ vs. LLO.

SUMMARY AND CONCLUSIONS (see also scheme 1)

1. LLO activates PKC-α activity in H441 cells within 1h.
2. LLO decreases ENaC-α protein expression in H441 cells within 2h.
3. LLO reduces Sgk1 phosphorylation at residue T256 and Akt1 phosphorylation at residue S473.
4. The TNF-derived TIP peptide significantly blunts the LLO-induced PKC-α activation within 1h and restores ENaC-α expression in LLO-treated H441 cells.
5. The TIP peptide restores the LLO-induced decrease of amiloride-sensitive sodium uptake in H441 cells.
6. The TIP peptide inhibits LLO-induced pulmonary edema formation in LLO-treated C57BL6 mice.

These findings indicate that the lectin-like domain of TNF can counteract the detrimental effects of the G^+ exotoxin LLO on alveolar liquid clearance, thus indicating a therapeutic potential for the TNF-derived TIP peptide, mimicking this domain, in acute lung injury.

REFERENCES: (1) Ananthraman *et al. Respiration.* 1983; 44(2):153-157; (2) Eaton *et al. Annu Rev Physiol*, 2008; (3) Lucas *et al., Science* 1994, 263(5148): 814-817; (4) Elia *et al., AJRCCM* 2003; 168(9):1043-1050. (5) Hamacher *et al., Crit. Care Med.* 2010 ,38(3):871-878.

This project was supported by Award Number R01HL094609 from the NIH National Heart, Lung, And Blood Institute.

Development of inhalation therapy for prevention and treatment of Acute Lung Injury

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Aims

Pulmonary oedema is a major complication of Acute Lung Injury (ALI), severe pneumonia and Acute Respiratory Disease Syndrome (ARDS). In patients with ALI/ARDS, mortality and the capacity of patients to resolve pulmonary oedema are inversely correlated; failure of lungs to rapidly clear oedema fluid is associated with higher morbidity and mortality. Resolution of alveolar oedema depends on the active removal of salt and water from the distal air spaces of the lung across the distal lung epithelial barrier. Alveolar liquid clearance is facilitated by sodium gradients generated by the epithelial sodium channel on the apical side and by the Na+-K+-ATPase on the basolateral side of type II alveolar epithelial cells.

We report the development of inhalation therapy that assists in alveolar liquid clearance by activating ENaC of alveolar cells.

Methods

The lectin-like domain of human TNF-α was used as template for the design of a series of novel cyclic peptides. All peptides were synthesised by solid phase Fmoc strategy.

Activation by synthetic peptides of ENaC of epithelial A549 lung cells was analysed *in vitro* by patch clamp technique in the whole-cell mode 24 to 48 h after plating of cells. For evaluation of ion selectivity, ENaC was blocked by 10 to 100 µM amiloride before the addition of peptide. Subsequent addition of 10 mM tetraethylammonium chloride (TEA) indicated whether any observed increases in the current were due to potassium current.

Prior to nebulisation, synthetic peptides were dissolved in Water for Injection (WFI). Nebulisation was accomplished with the Aeroneb Solo nebulizer. Aerosol particles analysis was performed by particle size measurements with the Next Generation Impactor (NGI) and by laser diffraction (HELOS). Pulmonary deposition was analysed by a breath simulator.

Acute lung injury in the pig model was achieved by surfactant-depletion by bronchoalveolar lavage (LAV). Spirometry and hemodynamic monitoring were perfomed by Datex Ohmeda S5 (Datex Ohmeda GmnH, Duisburg, Germany). Extravascular lung water content (EVLW) and cardiac output (CO) were assessed by PiCCO® system (PiCCO®, Pulsion Medical Systems, München, Germany). Arterial and central venous blood gas (ABG, CVBG) analyses were performed with the Rapidlab 248 device (Bayer Healthcare, Leverkusen, Germany). Multiple inert gas elimination technique by micropore membrane inlet mass spectrometry - (MMIMS - MIGET) technology was used for for pulmonary shunt measurement and analysis of the ventilation/perfusion (V/Q) distribution (Oscillogy LLC, Folsom, PA, USA).

Conclusions

A series of synthetic peptides were designed and chemically manufactured. A patch clamp measurement on human epithelial type II pulmonary cells demonstrated that these synthetic peptides were capable of activating ENaC in a concentration-dependant manner.

Formulation of peptide in WFI followed by nebulisation with Aeroneb ProX mesh-nebuliser, produced aerosol particles of diameters less than 4 µm, thus enabling the aerosol to penetrate the lower respiratory tract. Peptide activity was unaffected by the nebulisation procedure. Nebulised peptides were detected in lung tissue by using a combination of liquid chromatography (LC) and mass spectrometry (MS).

With IRB approval, Acute Lung Injury (ALI) was induced in pigs and 1mg/kg synthetic peptide was nebulised and inhaled into the lung of test animals followed by mechanical ventilation for five hours. Inhalation of synthetic peptide resulted in significant increase in paO2 values and reduction of pulmonary oedema as measured by extravascular lung water content. Haemodynamic parameters remained unchanged during time of observation.

Our data demonstrate for the first time that small synthetic peptides can be inhaled into the lower respiratory tract as aerosol retaining biological activity to protect lung tissue from injury and to resolve pulmonary oedema *in vivo*.

Results 1

The lectin-like domain of human TNF-α was used as template for the design of a series of novel cyclic peptides (Fig. 1).

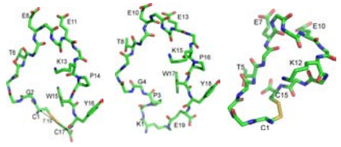

Fig. 1. Structural models of various synthetic peptides.

The activation by synthetic peptides of ENaC was analysed by patch-clamp measurements of human epithelial A549 cells at holding potentials ranging from –100 to +100 mV. Fig. 2 (A) shows control results; Fig. 2 (B) shows current-voltage relationship in the presence of 120 nM synthetic peptide "#1"; Fig. 2 (C) shows the IV-curves for control (open circles), in presence of 10 mM amiloride (open triangles), which blocks the sodium current, and with 60 nM synthetic peptide "#1".

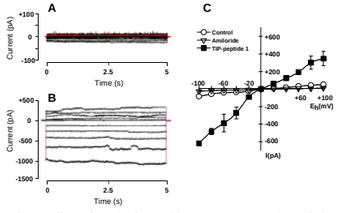

Fig. 2. Effect of synthetic peptide #1 on current-voltage (IV) relationship. Patch clamp holding potential: -100V to +100 V.

For all ENaC-activating peptides a maximal effect of Na-current activation could be observed at 120 nM (Fig. 3). Some of the peptides (No. 4, 5, 8, 9) were more active than reference peptide 1, but less active than TNF-α, whereas peptide 3 was as active as peptide 1.

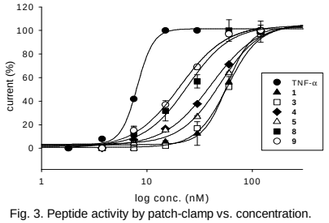

Fig. 3. Peptide activity by patch-clamp vs. concentration.

Fig. 4. Nebulisation of synthetic peptide.

Aqueous solutions of ENaC-activating peptides were converted into aerosol by a mesh-type nebuliser (Fig. 4). Peptide at a concentration of 25 mg/ml was nebulised and the aerosol was recovered by condensing it in a cool trap. Activity measurement demonstrated that the nebulisation process does not decrease biological activity synthetic peptides (Fig. 3).

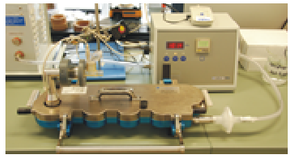

Fig. 5. Assessment of aerosol parameters by NGI.

Synthetic peptides were formulated as aqueous solution and a peptide-containing aerosol was generated by state-of-the-art mesh-type nebuliser. Aerosol particle sizes were analysed both by "New Generation Impactor" (NGI) (Fig. 5 and Fig. 6) and laser diffraction measurements. The fine particle fraction of nebulised synthetic peptide was approx. 50% (NGI results).

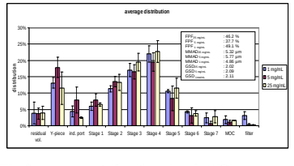

Fig. 6. Assessment of particle sizes by NGI.

Assessment of average particle size by laser diffraction produced similar results: The Mass Median Aerodynamic Diameter (MMAD) of the fine particle fraction was 5 µm and less.

Results 2

With approval of the State and Institutional Animal Care Committee, therapeutic experiments were performed in 25 - 27 kg german pigs using a bronchoalveolar lavage (LAV) model of ALI. ALI was defined as an oxygenation index (paO2/FiO2) < 300 according to the currently valid definition of the American - European Consensus Conference (AECC) from 1994. Non - protective ventilation (tidal volume: Vt = 10 ml/kg body weight, ZEEP, respiratory rate; RR = 25 - 35 /min, FiO2 1.0) were chosen to maintain stable ALI criteria.

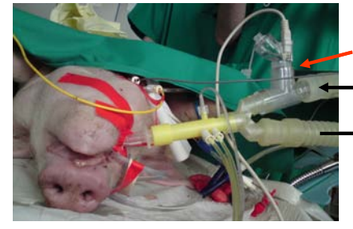

Fig. 7. Detail view of ventilated pig with nebuliser.

Two study groups of 8 animals each were employed: AP301 nebulisation (1mg/kg in 5ml WFI) (LAV +AP301); Control nebulisation (5ml WFI only) (LAV + CTRL). Primary endpoints were the oxygenation index (paO2/FiO2) and the EVLWI (via transpulmonary thermodilution using the PiCCO system). Apparently, inhalation of aerosol of synthetic peptide significantly increased the oxygenation index (Fig. 9) and reduced the content of extravascular lung water (Fig. 8).

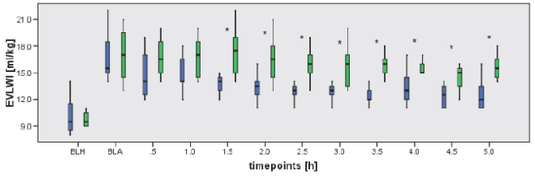

Fig. 8. (left) Flow chart of the EVLWI.
LAV + AP301 vs. LAV + CTRL.
BLH = baseline healthy, BLA= baseline ALI.
* p < 0.05, Mann - Withney - U - Test.

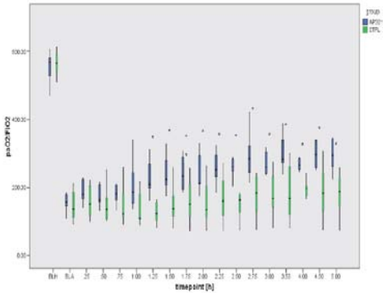

Fig. 9. (top) Flow chart of the paO2.
LAV + AP301 vs. LAV + CTRL.
BLH = baseline healthy, BLA = baseline ALI.
* p < 0.05, Mann - Withney - U - Test.

	Timepoint	MAP	CO	HR	SPAP	DPAP	PCWP	CVP
LAV +AP301	BLH	79.6 ± 18	3.40 ± 0.68	101.9 ± 21.7	22.9 ± 5.9	18.1 ± 4.9	12.5 ± 2.5	12.2 ± 1.5
	BLA	97.1 ± 20.1	4.58 ± 1.08	116.0 ± 33.4	32.4 ± 7.5	23.9 ± 6.9	13.5 ± 4.8	11.5 ± 1.5
	Nebulisation	100.1 ± 17.3		105.6 ± 20.8	33.0 ± 7.3	25.4 ± 5.4		11.2 ± 1.7
	1st hour	97.6 ± 14.3	3.88 ± 0.53	99.3 ± 15.2	31.6 ± 5.7	24.3 ± 2.8	11.6 ± 2.2	11.5 ± 2.0
	2nd - 5th hour	88 ± 6.7	3.74 ± 0.40	97.7 ± 9.4	31.1 ± 6.3	22.7 ± 4.2	11.2 ± 2.1	11.0 ± 2.5
LAV+CTRL	BLH	83.8 ± 19.0	3.59 ± 0.54	101.6 ± 21.4	27.1 ± 3.4	20.7 ± 3.9	14.0 ± 1.6	10.8 ± 3.0
	BLA	90.3 ± 18.6	3.90 ± 0.62	106.8 ± 12.6	40.0 ± 8.7	29.3 ± 6.6	11.8 ± 1.9	9.6 ± 2.9
	Nebulisation	91.5 ± 15.6		107.5 ± 12.3	42.1 ± 11.6	30.5 ± 7.4		9.9 ± 3.2
	1st hour	96.1 ± 14	4.75 ± 2.39	104.2 ± 17.1	39.1 ± 9.0	28.9 ± 5.9	13.1 ± 2.4	9.6 ± 3.0
	2nd - 5th hour	81.5 ± 11.3	3.82 ± 0.45	101.2 ± 11.8	36.8 ± 8.1	25.8 ± 5.4	12.1 ± 1.9	9.5 ± 3.0

Tab. 1. (top) Extended hemodynamic monitoring.
Means ± SD. BLH = baseline healthy, BLA = baseline ALI. MAP = mean arterial pressure, SPAP = systolic PA pressure, DPAP = diastolic PA pressure, PCWP = pulmonary capillary wedge pressure, CVP = central venous pressure [mmHg]. CO = cardiac output [ml/min]. HR = heart rate [/min].

Secondary endpoints Spirometry (tidal volume, plateau pressure, PEEP, respiratory rate, FiO2, I:E ratio, endtidal CO2) and extended hemodynamic data (mean arterial pressure, heart rate, cardiac output, PA pressures, pulmonary capillary wedge pressure) were assessed continuously. Results of the hemodynamic parameters are summarized in Table 1. No severe hemodynamic instability occurred during the experiments.

Summary

In a porcine lavage model of acute lung injury APEPTICO´s TNF - α derived TIP peptide AP301 was applied vs. a control nebulisation of WFI under comparable ventilator settings and hemodynamic conditions. A sustained improvement of the lung function was recorded following AP301 nebulisation according to the surrogate parameters oxygenation index, EVLWI and pulmonary shunt fraction.