The impact of mucolytics on *Chlamydia* infection and the induced immune response

Ph.D. thesis

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1) Introduction

- **General description of the Chlamydiae phylum**

Chlamydiae are obligate Gram-negative intracellular pathogens with a biphasic development cycle. From amoebae to humans can be infected with these organisms. The *Chlamydiaceae* family is the best-studied group in the Chlamydiae phylum, and it comprises 11 species, that are pathogenic to animals including human. There are three human pathogens in the *Chlamydiaceae* family which are *Chlamydia trachomatis*, *Chlamydia psittaci* and *Chlamydia pneumoniae*.

- ***Chlamydia pneumoniae* infection**

Chlamydia pneumoniae (*C. pneumoniae*) is a common cause of acute respiratory infection, including community-acquired pneumonia (CAP), sinusitis, pharyngitis, bronchitis, and exacerbations of chronic bronchitis. *C. pneumoniae* is responsible for approximately 10% of all pneumonia cases. Seropositivity of the populations shows that approximately 50-70% of the community have been infected with *C. pneumoniae* in a lifetime. Moreover, *C. pneumoniae* infection has been associated with atherosclerosis. During the invasion of the upper and lower respiratory tract, phlegm is produced. To facilitate the removal of the phlegm, mucolytics are often prescribed by physicians.

- **Development cycle of Chlamydia and the induced immune response**

The members of the *Chlamydiaceae* family exist in two forms which are the elementary body (EB) and the reticular body (RB). These forms are morphologically and functionally different from each other. EBs are spore like forms, and responsible for the infection of the cells. After EBs enter in the cell, they differentiate to RBs. This form is the metabolically active form of the bacterium. After 8-10 division cycles RBs are differentiated to EBs, and the EBs exit the cell via extrusion or budding in order to begin a new infection cycle.
The defence against chlamydial infection takes place on three platforms simultaneously. One element is the so-called cell-autonomous immunity, the second is the apoptosis and the last is the activation of the immune system. One of the key elements in the cell autonomous immunity is indoleamine 2,3 dioxygenase (IDO) is an IFN-γ inducible enzyme that has two isoforms, IDO-1 and IDO-2. This enzyme is responsible of the degradation of tryptophan in the cell. This amino acid is crucial for the replicating bacteria. Another key element is the programmed cell death. A main event in the cascade is the activation of caspase 3,6,7 which leads to the apoptosis of the cell. It is known that the members of the *Chlamydiaceae* try to evade host cell apoptosis by the stimulation of Raf/MEK/ERK pathways thereby inducing Bag1. Bag1 binds to Bcl-2, thus sustaining its anti-apoptotic function. In the activation of the immune system several cytokines play a pivotal role in the defence of *C. pneumoniae* infection. IL-12 is a heterodimeric cytokine, which is produced by dendritic cells (DC), activated macrophages, and neutrophil granulocytes. IL-12 has a role in the activation of natural killer (NK) cells, as well as the differentiation of naive T-cells to $CD4^+$ cells. Moreover, it prompts the production of IFN-γ, and together with IFN-γ they promote the maturation of DCs. IL-17 is a proinflammatory cytokine family, which consists of 6 members IL-17(A-F). It has been described that IL-17A mediates neutrophil infiltration during chlamydial lung infection by promoting chemokines expression. IFN-γ plays a crucial role both in innate and adaptive immunity (e.g. stimulates TNF production of macrophages, upregulates MHC expression thereby aiding the antigen presentation, facilitates phagosome-lysosome fusion, and induces several IFN-γ inducible GTPases). Surfactant proteins (SP) consist of four proteins (SP A-D), from which SP-A and SP-D have a role in the defence of the lungs. SP-A and SP-D act as an opsonin, facilitate uptake of the bacteria by the alveolar macrophages and can aggregate bacteria.

- **Characteristics of mucolytics**

N-acetyl-cysteine (NAC) is a thiol group-containing commonly used agent in the healthcare profession. It has multiple therapeutic uses in psychiatry including bipolar disorder, major depressive disorder, schizophrenia and is useful in the case of an acetaminophen overdose. However, most often NAC is used as a mucolytic. Mechanism of action in the body occur in three ways: either thiol group can reduce disulphide bonds, therefore decreasing the viscosity of the mucus in the lungs or NAC can be the substrate of glutathione synthesis or it can directly neutralize oxidants. In Germany, NAC was the second most popular drug as mucolytics with 23.5% of the OTC expectorant market. Under the basis data that the National Health Insurance Fund of Hungary has provided us, little over 1 million units of NAC was sold in 2016 in Hungary, making NAC the most used mucolytics in Hungary. The direct antimicrobial role of NAC is not well defined, but there are data regarding its inhibitory effect on biofilm formation. NAC displayed a direct antimicrobial effect against extracellular pathogens, but the concentrations applied by different authors varied greatly (0.003–80 mg/ ml). In the case of tuberculosis, NAC was found to play a part in inhibiting the growth of intracellular *Mycobacterium tuberculosis* through bacteriostatic mechanisms.

Ambroxol (Ax) is widely used in the treatment of respiratory infections, chronic bronchitis, and neonatal respiratory distress syndrome due to its mucus viscosity-altering effect and has been shown to be a relatively safe drug. Furthermore, Ax shows beneficial effects on the course of Parkinson's disease by modifying glucocerebrosidase chaperon activity. Ax exhibits pro-inflammatory properties by elevating IL-10, IL-12, and IFN-γ expression, due to its aromatic moiety, Ax exhibits oxidant scavenger functions. Moreover, it has been reported that Ax elevates SP production and can reverse the LPS-stimulated induction of the extracellular signal-regulated kinase (ERK) 1/2 pathway. In Germany, Ax was the most popular drug as mucolytics with 24 % of the OTC expectorant market. Under the basis of data that the National Health Insurance Fund of Hungary has provided us, little over 605000 units of Ax was sold in 2016 in Hungary, making Ax the second most used mucolytics in Hungary. In addition, Ax is used for the symptomatic treatment of sore throat during viral infections, as it shows local anaesthetic effects.

2) Aims and objectives

1. Investigating the effect of NAC on *C. pneumoniae* replication in *in vitro;*
2. Examining the effect of short term and long-term treatment of NAC on *C. pneumoniae* replication in *in vivo* in mice;
3. Determining the mechanism of NAC on *C. pneumoniae* replication;
4. Finding a replaceable mucolytics of NAC;
5. Investigating the effect of higher concentration of Ax on *C. pneumoniae* replication in *in vivo* in mice;
6. Examining the potential effect of Ax on the gene expression profile in known anti-chlamydial gene *in vivo* in mice;
7. Verifying the impact of Ax on IFN-γ and IL-6 production at the protein level *in vivo* in mice;
8. Analysing the effect of SP-A and SP-D protein on *C. pneumoniae* attachment and replication on mouse cell line;
9. Proving the effect of Ax treatment on ERK pathway regulation

3) Material and methods

- **Propagation of *C. pneumoniae***

In our experiments, the CWL029 strain of *C. pneumoniae* from the ATCC was used. *C. pneumoniae* was propagated in HEp-2 cells, as described previously. The titre of the infectious EBs was determined by an indirect immunofluorescence assay. Serial dilutions of the EB preparation were inoculated onto McCoy cells, and after being cultured for 48 h, the cells were fixed with acetone and stained with a monoclonal anti-Chlamydia lipopolysaccharide antibody and FITC-labelled anti-mouse IgG. The number of *C. pneumoniae* inclusions was counted under a UV microscope, and the titre was expressed as inclusion-forming units/ml.

- ***In vitro* effect of NAC and Ax**

McCoy cultures were grown in 24-well tissue culture plates containing a 13 mm cover glass in minimum essential medium Eagle with Earle's salts, supplemented with 10% vol/vol foetal calf serum, 0.5% wt/vol glucose, l-glutamine 0.3 mg/ml, 4 mM HEPES and 25 µg/ml gentamicin. Five parallel wells of semi-confluent cultures were infected with 2×10^3 /well *C. pneumoniae* or treated simultaneously with NAC (0.01–10 mg/ml) or Ax (0.002–0.05 mg/ml) at the time of infection. Separate cells were infected with *C. pneumoniae* pretreated (1 h) with different concentrations of NAC. Subsequently, *C. pneumoniae* EBs were pretreated with NAC or Ax by continuous shaking in the presence of NAC (0.1 mg/ml) or Ax (0.05 mg/ml) at room temperature, and after 1 h these drugs were washed out using a culture medium and centrifugation at 13800 *g* for 15 min or left unwashed. NAC- or Ax-treated *C. pneumoniae* or non-treated *C. pneumoniae* were inoculated onto cells on cover glasses in 24-well plates and centrifuged at 800 *g* for 1 h. After incubation for 48 h, the cells on the cover glasses were fixed with acetone and stained as described above in the 'Propagation of *C. pneumoniae*' section to visualize the inclusions of *C. pneumoniae*. To detect the effect of NAC or Ax on the attachment of chlamydial EBs to host cells, McCoy or A549 cells were infected with NAC- or Ax-treated *C. pneumoniae* EBs and after a 1 h incubation period and centrifugation the cells were washed and fixed and the bound EBs were stained by indirect immunofluorescence, as described above. Fluorescence signals were analysed via Olympus UV microscopy. The immunofluorescence of non-infected or *C. pneumoniae*-infected cells, or cells infected with drug-treated *C. pneumoniae* was analysed quantitatively by ImageQuantTL 8.1 software as follows: 6–6 equally sized circular areas covering the cells were randomly selected on each image, and then the background signals of the selected areas were eliminated by a threshold set-up and the fluorescence intensity/pixel values of the randomly selected cells were quantified.

- **Mice and infection conditions**

Pathogen-free 6-week-old female BALB/c mice were obtained from Charles River Laboratories (Hungary). The mice were maintained under standard husbandry conditions at the animal facility of the Department of Medical Microbiology and Immunobiology, University of Szeged, and were provided with food and water ad libitum. Before infection, the mice were mildly sedated with an intraperitoneal injection of 200 µl of sodium pentobarbital (7.5 mg/ml). They were then infected intranasally with 2×10^5 IFU *C. pneumoniae* in 20 µl sucrose/phosphate/glutamic acid (SPG) buffer. Four *in vivo* experiments were conducted: **I.** From the second day post-infection (*p.i.)* they were treated with 10 mg/kg NAC (Sigma) in a volume of 50 µl drinking water *per os* daily. Mice were anaesthetized and sacrificed on day 7. **II.** The above-described experiment was repeated, but mice were treated until 19 days *p.i.*, and sacrificed on 20 days *p.i.*. **III.** In a separate study, *C. pneumoniae*-infected mice were treated similarly as above with 1.25 mg/kg Ax (Sigma) and sacrificed 7 days after infection. **IV**. From the first day *p.i.*, the mice were administered daily with 5 mg/kg Ax. The mice were anaesthetized and euthanized 7 days *p.i.*.

In every experiment: behaviour and weight were monitored daily; control mice received the same amount of water via oral administration using a pipette to mimic the stress of the treatment process. The sera were harvested via cardiac puncture, lungs were removed and homogenized using acid-purified sea sand. Half of the homogenized tissue sample was processed for RNA extraction, whereas the other half was suspended in 1 ml of SPG for the detection of viable Chlamydia and to measure the cytokine levels.

This study was approved by the National Scientific Ethical Committee on Animal Experimentation of Hungary (August, 10 August 2016; III./3072/2016) and the Animal Welfare Committee of the University of Szeged. This study conformed to the Directive 2010/63/EU of the European Parliament.

- **Cultivation of *C. pneumoniae* from the lungs of mice**

After two freeze-thaw cycles, the homogenized lungs from individual mice were centrifuged (10 min, 400 *g*) and serial dilutions of the supernatants were inoculated onto McCoy cell monolayers. These samples were then centrifuged (1 h, 800 *g*), and after a 48 h culture, the cells were fixed with acetone and stained as described above in the 'Propagation of *C. pneumoniae*' section to visualize the inclusions of *C. pneumoniae*.

- **RNA extraction from the cells**

One-day-old semi-confluent McCoy cells in six-well plates were infected with *C. pneumoniae* using a multiplicity of infection (MOI) of 4. The cells were then left untreated, or NAC 0.1 mg/ml or Ax 0.05 mg/ml were added to the medium. Total RNA was extracted after a 1-day with Tri Reagent according to the manufacturer's protocol. RNA concentrations were measured at 260 nm via a NanoDrop spectrophotometer.

- **RNA extraction from the lungs of mice**

Half of the homogenized lung sample acquired in 'Mice and infection conditions' was processes with Tri Reagent and the extraction were conducted as described in 'RNA extraction from the cells.

- **cDNA synthesis**

After the RNA extraction protocol, 1 µg of total RNA was reverse transcribed using Maxima Reverse Transcriptase according to the manufacturer's protocol with random hexamer primer.

- **Quantitative PCR (qPCR) of the *Ido1, Ido2, Ifng Il12, Il23, Il17a, Il17f, Ifng, Sftpa, Sftpb, Sftpc, Sftpd, Actb***

qPCR was performed in a Bio-Rad CFX96 real-time system by using a SsoFast EvaGreen qPCR Supermix master mix and the murine-specific primer pairs. All of the primers were designed using PrimerQuest Tool software and synthesized by Integrated DNA Technologies, Inc. To check the amplification specificity, the qPCR was followed by a melting curve analysis. Threshold cycles (Ct) were calculated for the *Ido1, Ido2, Il12, Il23, Il17a, Il17f, Ifng, Sftpa, Sftpb, Sftpc, Sftpd* and *Actb* and the relative gene expressions were calculated by the ΔΔCt method. Student's t-test was used to compare the statistical differences of ΔCt values between the infected and control samples, or One-way analysis of variance with repeated measures (ANOVA RM) and planned comparisons were used to compare the statistical differences in the ΔCt values between the infected and control samples, as described previously, with a level of significance of $p < 0.05$.

- **Enzyme-linked immunosorbent assay (ELISA)**

Standard sandwich mouse IL-6 and mouse IFN-γ ELISA kits were used to determine the IL-6 and IFN-γ concentrations in the lung supernatants. Prior to use, the stored lung samples were thawed immediately, and the assay was performed according to the manufacturer's instructions at 10× dilution for IL-6 and at 50× dilution for IFN-γ. The dynamic range of the kits was between 10 and 1000 pg/ml. Plates were analysed using the Biochrom Anthos 2010 microplate reader. Samples were assayed in duplicate.

- **Western botting analysis of ERK pathway**

To investigate the levels of phosphorylation in RAF proto-oncogene serine/threonine-protein kinase (c-Raf), as well as dual specificity mitogen-activated protein kinase (MEK)1/2, ERK1/2, P90RSK1/2, and mitogen- and stress-activated protein kinase 1 (MSK-1) proteins, McCoy cells were grown in 6-well plates and treated with Ax (0.05 mg/ml) and/or infected with *C. pneumoniae* simultaneously at 0.01 MOI or left untreated and/or uninfected. After a 12-h incubation period, the cells were washed twice with ice-cold phosphate-buffered saline, scraped, and collected in a homogenization buffer (1×

radioimmunoprecipitation buffer supplemented with phosphatase inhibitors and a protease inhibitor cocktail). Cells were sonicated using an ultrasonic homogenizer (10 s, 4 °C), followed by centrifugation of the homogenate (14000× g, 10 min, 4 °C). The bicinchoninic acid assay was performed to determine the protein concentration in the homogenates[97]. Samples containing 20 µg of protein were boiled for 5 min. After preparation, the samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels. After separation, the proteins were transferred onto 0.45-µm pore-sized nitrocellulose membranes (35 V, 1.5 h, room temperature (RT)). Nonspecific binding was blocked via incubation (1 h, RT) in 0.05% (v/v) Tris-buffered saline–Tween-20 containing 1% bovine serum albumin (BSA; v/v) In all experiments, the membranes were cut horizontally according to the molecular weights of the investigated proteins. Membranes were incubated with specific primary antibodies against phosphorylated forms of Raf (1:1000), MEK (1:2000), p90 (1:1500), ERK (1:2000), and MSK (1:1000) as well as a housekeeping protein/loading control GAPDH (1:10,000) in 1% BSA solution containing 0.1% Tween-20 (overnight, 4 °C). After washing (Tris-buffered saline containing 0.05% Tween-20, Sigma), the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies for 45 min at RT. A chemiluminescence analysis was performed using the LumiGLO 20× reagent, followed by exposure of the membranes to X-ray films. All films were scanned (Epson Perfection V19 Scanner,(8-bit, 400 dpi), and the density of the protein bands was quantified using Quantity One software. The 90-kDa form of MSK-1 was used for the calculation of the protein contents of the bands according to the protocol.

- **Apoptosis assay and flow cytometry**

Caspase 3/7 activity assays were performed using the FAM FLICA™ Caspase-3/7 Kit combined with propidium iodide (PI). McCoy cells were divided into 4 experimental groups. Half of the McCoy cells were infected with 0.01 MOI *C. pneumoniae*, while the other half were left uninfected. Both the infected and uninfected groups were subdivided into those treated with 0.05 mg/ml Ax or left untreated. After 12 h of incubation, the apoptosis assay was performed according to the manufacturer's instructions, and the cells were analysed via flow cytometry. The assay discriminated between viable (caspase 3/7−/PI−), early apoptotic (caspase 3/7+/PI−), and late apoptotic (caspase 3/7+/PI+) cells. The fluorescence of the cell populations was analysed immediately using a BD fluorescence-activated cell sorting Aria Fusion flow cytometer

- ***In vitro* effects of SP-A and SP-D on *C. pneumoniae* proliferation and attachment**

McCoy cells were seeded (2.5 × 10^5 cells) to form a monolayer in 24-well plates and incubated overnight. *C. pneumoniae* (0.01 MOI) was diluted in 1 ml medium in the presence of either SP-A (1 µg/ml), SP-D (1 µg/ml) or neither. The mixtures were shaken at 37 °C for 1 h and then centrifuged at 800× *g* for 1 h; at 48 h *p.i.*, the cells were fixed using acetone and stained, as described in 'Propagation of *C. pneumoniae'*. To detect the effect of SP-A and SP-D proteins on the attachment of *C. pneumoniae* to J-774 cells, the experiment described above was repeated with the following modification. After 1 h of centrifugation, the cells were stained immediately, as described earlier.

Fluorescence signals were analysed using the Olympus IX83 Live Cell Imaging system and scanR High-Content Screening Station microscopy. The immunofluorescence of cells infected with non-treated *C. pneumoniae*, SP-A-pretreated *C. pneumoniae*, or SP-D-pretreated *C. pneumoniae* was analysed quantitatively using ImageQuantTL 8.1 as described in '*In vitro* effect of NAC and Ax'

- **Statistical analysis**

Welch's t-test or one-way ANOVA RM with planned comparisons was performed using GraphPad Prism 8.0.1 software. Data were expressed as the mean ± standard deviation (SD). A value of $p < 0.05$ was considered significant.

4) Results

- **NAC increases the *in vitro* replication of *C. pneumoniae***

Initially, we wanted to check the potential anti-chlamydial effect of NAC under *in vitro* conditions. During our experiments, different concentrations of NAC were applied using two approaches. First, a decreasing concentration of NAC was mixed directly with *C. pneumoniae*, and the host cells were immediately infected. Among the concentrations applied, doses of 10 mg/ml and 2 mg/ml NAC were toxic to the cells. Surprisingly, 0.1 mg/ml NAC resulted in a nearly sixfold increase in the number of *C. pneumoniae* inclusions in McCoy cells as compared to the number observed after infection with untreated *C. pneumoniae*. Accordingly, we chose this concentration in later studies. In the second approach, *C. pneumoniae* was preincubated with NAC at the respective three concentrations by shaking the mixture of *C. pneumoniae* and NAC continuously for 1 h before infecting the cells. Based on our experiment there was no significant difference in the infectivity of *C. pneumoniae* pretreated for 1 h with NAC and *C. pneumoniae* treated with 0.1 mg/ml NAC immediately before inoculation of the cells ($p > 0.05$).

- **NAC increases the binding of *C. pneumoniae* to the host cell**

To investigate whether NAC is able to influence the attachment of *C. pneumoniae*, McCoy and the more relevant A549 epithelial cells of human respiratory origin were infected with NAC-treated *C. pneumoniae* or with untreated *C. pneumoniae*. NAC-treated *C. pneumoniae* produced a significantly higher fluorescence intensity as compared to the untreated *C. pneumoniae* in McCoy cells. A similar effect was observed in the A549 cells. The control cells did not display any fluorescence**.** Subsequent experiments indicated that the removal of NAC with the culture medium from *C. pneumoniae* did not significantly modify the number of replicating *C. pneumoniae* as compared to the outcome of the infection with unwashed *C. pneumoniae*. Adding NAC to the cells 6 or 24 h after *C. pneumoniae* infection did not change the number of inclusions formed by *C. pneumoniae*. Furthermore, preincubation of the host cells with NAC for 48 h before infection with *C. pneumoniae* did not modify the replication of *C. pneumoniae*.

- **Exposure to NAC not only increases the chlamydial lung burden but also prolongs the infection in mice**

Here, we investigated whether NAC might aggravate the chlamydial infection in the short term. Twenty mice were infected with *C. pneumoniae*, and 10 were treated with 10 mg/kg NAC *per os* at the same concentration as that applied for human respiratory infections as a mucolytic drug for 6 days. On the seventh day, the mice were sacrificed; the lungs were removed for the determination of recoverable *C. pneumoniae*. We found that after 6 days of exposure to NAC the severity of the *C. pneumoniae* infection increased. The number of recoverable chlamydial inclusions was approximately three times higher in the NAC-treated group than in the *C. pneumoniae*-infected mice without NAC treatment. Next, we investigated whether NAC prolongs the clearance of *C. pneumoniae* from the lungs. Sixteen mice were infected with *C. pneumoniae,* and eight mice were treated with NAC for 19 days. On the twentieth day, the mice were sacrificed, and their lungs were removed for the detection of viable *C. pneumoniae*. All of the mice were *C. pneumoniae* culture-positive in the NAC-treated group and the number of *C. pneumoniae* inclusions was 2.5 times higher than that in the control group. Moreover, in the control group, two mice became culture-negative (the sensitivity of our method was <40 IFU/lung), suggesting recovery from the disease. The data for these mice were not included in.

- **Ax does not increase the number of infective *C. pneumoniae***

To look for a better alternative to NAC, the effect of Ax on *C. pneumoniae* replication was tested in *in vitro* and *in vivo* systems. Using concentrations of 0.002 and 0.01 mg/ml, Ax did not cause any significant changes in bacterial replication in McCoy cells, but 0.05 mg/ml Ax had a strong antimicrobial effect, and this reduced the number of *C. pneumoniae* to approximately one-fifth of that observed with untreated cells. None of the applied concentrations of Ax influenced host cell viability. Increasing the dose to 0.25 mg/ml Ax proved to be toxic to the cells. We then wanted to determine the reason for the

antimicrobial activity of Ax. An identical experiment to that performed with NAC was conducted. Ax-treated and untreated *C. pneumoniae* were inoculated onto McCoy and A549 cells, and the cells were stained using the immunofluorescence method. An altered immunofluorescence was not observed in this case, and we inferred that the Ax treatment did not modify the number of *C. pneumoniae* EBs attached to the cell membrane. It is well documented that IDO1,2 shows antimicrobial activity that metabolizes the tryptophan, which is essential in chlamydial replication. We then tested whether Ax might influence the expression of IDO1,2. Ax caused a 2.5-fold increase in IDO-2 expression in *C. pneumoniae*-infected cells as compared to the expression in untreated cells. In our experiments, NAC treatment did not influence the expression of IDO1,2 in *C. pneumoniae*-infected cells. To further investigate the role of Ax during *C. pneumoniae* infection, mice were infected with *C. pneumoniae* and from the second day were treated with tap water-diluted Ax at the same concentration as it is applied for human respiratory infections when it is used as a mucolytic drug (1.25 mg/kg). The control mice were given tap water. During the 7-day period, the behaviour (activity and appetite) and weight of the animals did not change significantly between groups. The mice were sacrificed 7 days after the infection and the number of recoverable *C. pneumoniae* was counted from the lungs by indirect immunofluorescence. As result of our experiment the number of recoverable *C. pneumoniae* did not change significantly in the Ax-treated and untreated *C. pneumoniae*-infected group of mice.

- **Ax treatment at elevated concentration decreased *C. pneumoniae* proliferation in mouse lungs**

Subsequently, our aim was to determine whether a relatively higher concentration of Ax would influence the chlamydia replication. Therefore, the mice were infected with *C. pneumoniae* (2×10^5 IFU/mouse); half of the mice were treated daily from day 1 *p.i.* with a 4× higher concentration of Ax (5 mg/kg) than the dose used as a mucolytic in common respiratory infections, as we used in our earlier experiment. Seven days *p.i.*, the mice were euthanized, and the lungs were removed for detection of viable *C. pneumoniae*. A significant difference was found between the number of recoverable *C. pneumoniae* between the Ax-treated and untreated groups. The mean numbers of viable *C. pneumoniae* IFUs in the Ax-treated and untreated groups were 3.1×10^4 and 7.3×10^4 IFU/lung, respectively. The mice in the Ax-treated group exhibited less severe symptoms than those in the control group.

- **Ax treatment altered the gene expression and protein level of IFN-γ in the lungs of *C. pneumoniae*-infected mice**

We investigated whether gene expression was altered during Ax treatment, concerning the cytokine profile in *C. pneumoniae* infection. We found that the relative expression of IL-12, IL-23, IL-17A, and IFN-γ was significantly higher in the *C. pneumoniae*-infected/Ax-treated group than in the *C. pneumoniae*-infected group without treatment (2.22-, 3.07-, 2.46-, and 2.27-fold, respectively).

We previously reported that Ax treatment elevated the expression of the known anti-chlamydial enzyme IDO-2 in the A549 cell line; however, this elevation was not significant when a human equivalent mg/kg dose was administered to *C. pneumoniae*-infected mice. Therefore, we determined whether an elevated dose of Ax increased IDO-1 and IDO-2 expression in mice. We observed no significant difference in expression levels between *C. pneumoniae*-infected mice and *C. pneumoniae*-infected/Ax-treated mice. However, in accordance with the findings of Virok et al., which showed that IDO-1 and IDO-2 are active in *C. pneumoniae*-infected mice, in contrast to earlier findings by others, we also found significant increases in both IDO-1 and IDO-2 expression levels during *C. pneumoniae* infection compared to those of the untreated/uninfected group (15.14- and 10.81-fold, respectively).

SPs play a crucial role in maintaining lung homeostasis; moreover, SP-D shows an anti-*C. trachomatis* activity in a genital mouse model. Therefore, we determined the relative expression of SPs in the lungs of the mice. We found that all SPs were significantly upregulated in the untreated/infected mice compared to that of the uninfected/untreated group (SP-A, 4.55-fold; SP-B, 5.72-fold; SP-C, 5.28-fold; SP-D, 4.26-fold), suggesting that these anti-chlamydial mechanisms occur naturally in *C. pneumoniae*-infected mouse lungs. Notably, we observed a significant elevation in SP-A expression levels of mice in the treated/infected group compared to those of the untreated/infected group (2.89-fold).

After determining that the relative mRNA expression of *Ifng* was significantly higher in Ax-treated/infected mice than in untreated/infected mice, we investigated the protein expression levels of IFN-γ. We measured the cytokine concentrations in lung supernatants and found that IFN-γ levels were significantly higher in the *C. pneumoniae*-infected/Ax-treated group than in the untreated/infected group ($p = 0.0041$). We also measured the level of the pro-inflammatory cytokine IL-6; however, we did not observe significant differences between the expression levels of *C. pneumoniae*-infected and *C. pneumoniae*-infected/Ax-treated groups.

- **SP treatment increased the attachment of *C. pneumoniae* to macrophages and decreased bacterial proliferation**

After showing that Ax treatment increased the relative expression of SPs in the lungs of *C. pneumoniae*-infected mice, we investigated the effect of anti-chlamydial SP proteins on *C. pneumoniae* replication and attachment to macrophages *in vitro*. We found that the pre-treatment of EBs with SP-A or SP-D significantly reduced the number of *C. pneumoniae* inclusions (0.18-fold) ($p = 0.021$) and (0.57-fold) ($p = 0.034$), respectively, in infected cells. This anti-chlamydial effect was more prominent in SP-A treatment than in SP-D treatment. These results correspond to the findings of Oberley *et al*., who showed that SP-A and SP-D can aggregate Chlamydia, thereby inhibiting the infection of cells. Next, we wanted to investigate *C. pneumoniae* EBs treated with SP-A or SP-D, are taken up with higher efficiency by J-774 murine macrophage cells. Consequently *C. pneumoniae* EBs were treated with SP-A or SP-D and then, the samples were inoculated onto J-774 cells. After 1 h of centrifugation, we stained the cells with Chlamydia-specific immunofluorescent antibodies, and it was found that the average pixel intensity of FITC was significantly elevated after treatment with both types of SPs compared to that seen in cells infected with untreated EBs. This indicated that a relatively larger number of *C. pneumoniae* cells were recognized by J-774 cells after SP treatment. This effect was more prominent in SP-A-treated *C. pneumoniae* than in SP-D-treated *C. pneumoniae* (SP-A 2.74-fold ($p = 0.014$)) and (SP-D 2.05-fold ($p = 0.024$). The results were in agreement with Oberley et al., who demonstrated that SP-A or SP-D treated Chlamydia are recognized by THP-1 human monocytic cells with higher efficiency then untreated bacteria.

- **Ax treatment did not induce apoptosis via the caspase-dependent pathway but decreased ERK 1/2 activation in *C. pneumoniae*-infected cells**

It has been shown that *C. pneumoniae* can prevent the occurrence of host cell apoptosis; therefore, we investigated whether Ax treatment affected host cell death during *C. pneumoniae* infection. According to Galle *et al*., the assessment of apoptosis in *C. pneumoniae*-infected cells via annexin V staining is not accurate due to the *C. pneumoniae*-induced externalization of phosphatidylserine on the host cell membrane, which provides a binding site for annexin V and creates a false-positive apoptosis signal. Therefore, we determined caspase 3/7 activity using flow cytometry. The analysis revealed that caspase 3/7 activity did not differ significantly between the treated/infected group and the untreated/infected group.

C. pneumoniae activates the ERK 1/2 pathway to acquire several essential molecules from host cells and avoid host cell apoptosis. Therefore, we investigated the effect of Ax treatment on the MAPK/ERK activity in *C. pneumoniae*-infected cells. Western blotting analysis showed that the ERK-1 (1.49-fold; $p < 0.05$) and ERK-2 (2.04-fold; $p < 0.05$) protein expression levels were significantly increased in untreated/infected cells compared to those in untreated/uninfected cells. We also observed that *C. pneumoniae* infection significantly increased the MSK-1 levels (2.25-fold) in untreated/infected cells compared to those in untreated/uninfected cells. MSK-1 is responsible for the activation of nuclear factor kappa-enhancer of B-cells and induction of early genes such as c-fos, junB, and mkp-1. The Ax treatment of *C. pneumoniae*-infected cells significantly decreased the levels of c-RAF (0.42-fold), ERK 1 (0.16-fold), ERK 2 (0.15-fold), P90RSK1 (0.61-fold), P90RSK2 (0.67-fold), and MSK-1 (0.19-fold) ($p < 0.05$) compared to those of the untreated/infected group.

5) Discussion

NAC is a multifaceted drug that is used in the treatment of different diseases, mainly as a mucolytic agent. It is relatively inexpensive and commercially available as an OTC medicine. It has been shown to increase the level of GSH, the body's major antioxidant, by increasing glutathione S-transferase activity. It is a powerful antioxidant and has the potential to treat diseases characterized by the generation of free oxygen radicals. For instance, NAC is a therapeutic option in chronic obstructive pulmonary disease. In an open-label study of 1392 patients, NAC reduced the viscosity of expectorated sputum, reduced cough severity and improved the ease of expectoration in patients after 2 months of treatment. Furthermore, NAC dramatically attenuated influenza symptoms in a group of patients as compared with a placebo-treated group. In our study, we found that instead of decreasing bacterial replication, NAC actually increased the number of replicating *C. pneumoniae* in both *in vitro* and *in vivo* infections.

Based on our *in vitro* experiment, we disclosed that NAC increased the attachment of the pathogens to the host cells. Lazarev et al. investigated the role of intracellular GSH in *C. trachomatis* infection, and they found that the treatment of cells with buthionine sulfoximine, which causes the irreversible inhibition of GSH biosynthesis or hydrogen peroxide-induced oxidation of GSH, decreased the number of *C. trachomatis* inclusions. In contrast with this finding, the treatment of EBs with NAC increased the number of chlamydial inclusions. However, the researcher did not aim to examine the underling mechanism of this phenomenon. Moreover, the researchers concluded that GSH plays a crucial role in chlamydial replication. However, in their experiments, NAC was used as a GSH precursor, and they did not attempt to investigate the anti-chlamydial effect of NAC. However, it is well known that the infectivity of Chlamydia species depends on the reduced status of the cell membrane protein OmcB. In agreement with our hypothesis, NAC treatment of the EBs increases the attachment directly, probably by reducing OmcB, which could not have been from an increased level of GSH, because NAC was removed from the culture medium in some of our experiments.

According to our *in vivo* results, NAC significantly increased *C. pneumoniae* replication both in early phase of acute infection and late phase of acute infection and in chronic infection. During the experiment the mice showed more severe signs of infection (e.g. changes in weight, posture, behaviour) in NAC treated group compared to untreated group, which indicates that the infection was more severe.

Aside from the negative effect of NAC on acute *C. pneumoniae* infection, we need to take into account another possible effect of this drug. *C. trachomatis*, which belongs to the family *Chlamydiaceae*, is one of the most common sexually transmitted pathogens. Unfortunately, the infection is asymptomatic in up to 70 % of the cases. The severe consequences of chronic *C. trachomati*s infection are ectopic pregnancy, infertility, or a pelvic inflammatory disease. In the worst-case scenario, NAC applied simultaneously in the treatment of ongoing respiratory diseases may not only increase the growth of *C. pneumoniae* but stimulate the growth of *C. trachomatis* as well. In order to circumvent NAC's aggravating effect on *C. pneumoniae* infection, we looked for a better mucolytic agent that does not increase the severity of the respiratory disease. In our *in vitro* study, Ax displayed significant anti-chlamydial activity that was not associated with decreased binding of the pathogen to the host cells. The complete antimicrobial mechanism of Ax was not analysed, but Ax treatment increased the expression of the anti-chlamydial IDO-2, which may in part cause a reduction in the number of pathogens. In our first *in vivo* experiment, mice were infected with *C. pneumoniae* and then treated with Ax. Using a human equivalent Ax/body weight dose we found no significant difference in the number of recoverable *C. pneumoniae* between the treated and the untreated groups. Yang et al. found that Ax 10 mg/kg/day (which is eight times higher than the normal dose in a human) significantly reduced the mortality of mice infected with a lethal dose of H3N2 influenza virus.

C. pneumoniae is a common respiratory pathogen and unfortunately it is not always diagnosed correctly, and if a doctor suggests NAC as a mucolytic agent, it might worsen and delay the patient's recovery. Based on the prevalence of *C. pneumoniae*, many patients could suffer prolonged respiratory disease because of NAC. Overall, on the basis of our results, we can state that NAC can aggravate and prolong the infection caused by *C. pneumoniae* in an animal model. This information will be useful for physicians who recommend NAC as a mucolytic drug in respiratory diseases with a non-identified

aetiology. In the case of a *C. pneumoniae* infection, a correct laboratory diagnosis is imperative, because in its absence the use of NAC may worsen the patient's chance of recovery. It is important that clinical studies to prove our results are implemented.

Ax, a metabolite of bromhexine, is considered a relatively safe OTC drug. It is primarily recommended as a secretory agent for the treatment of various respiratory diseases that are associated with extensive mucus production. Ax treatment increases surfactant synthesis and facilitates their secretion from type II pneumocytes. Furthermore, Ax exhibits voltage-dependent sodium channel inhibitory properties, which can be beneficial in cases of diseases associated with sore throat by incorporating Ax in lozenges. Ax can also be used in the treatment of Gaucher disease and is considered a supplemental medication for treating Parkinson's disease, as it increases glucocerebrosidase activity. Additionally, a recently published study suggested that when Ax is administered together with antibiotics, a relatively higher antibiotic concentration can be obtained in the lungs due to its secretion-supporting function. Several studies have shown that Ax exhibits antimicrobial characteristics. Ax treatment reverses the resistance of *Candida albicans* to fluconazole. Moreover, it inhibits the mucoid conversion of *Pseudomonas aeruginosa*, facilitates the bactericidal activity of ciprofloxacin against biofilms, and exhibits synergy with vancomycin for the elimination of catheter-related *Staphylococcus epidermidis* biofilms both *in vitro* and *in vivo*. Furthermore, Ax impedes the rhinovirus infection in primary cultures of human tracheal epithelial cells. Ax also shows antibiofilm properties; the biofilm formed by *Pseudomonas aeruginosa* treated with Ax for 7 days is thinner and more fragmented than that formed by untreated cells.

It is known that Ax has a well-balanced and favourable risk-benefit profile; therefore, we investigated the effect of a four-fold increase in Ax dose on *C. pneumoniae* infection. In our *in vivo* study, we found that 5mg/kg Ax significantly decreased the number of viable *C. pneumoniae* IFUs in the lungs of mice. We found several significantly elevated cytokine gene expressions, which has a crucial role in the defence of *C. pneumoniae* infection. IL-17A is associated with a neutrophil influx in the lungs; mice infected with *C. pneumoniae* and treated with anti-IL-17A antibodies have a relatively higher *C. pneumoniae* burden. IL-23 has been described to be essential for inducing the *C. pneumoniae*-specific Th17 response. In our study, Ax treatment in *C. pneumoniae*-infected mice significantly increased the relative expression levels of IFN-γ, IL-12, IL-17A, and IL-23 compared to those of the untreated/infected mice; additionally, the IFN-γ levels were higher in the lungs of the Ax-treated *C. pneumoniae*-infected mice than in infected/untreated mice. These results suggest that Ax treatment may enhance inflammation in the lungs and promote the anti-chlamydial response, thus resulting in a reduction in bacterial burden.

Furthermore, additional factors may contribute to a decreased number of recoverable *C. pneumoniae* inclusions. SP-A and SP-D are known to play an immunological role in maintaining lung homeostasis. It has been shown that these proteins are able to aggregate *C. trachomatis* and can facilitate the uptake of bacteria by human macrophages. Our qPCR results suggest elevated SP-A levels that might also contribute to the improved elimination of bacteria in Ax-treated mice. Our *in vitro* results are in agreement with this phenomenon, as we found that the pretreatment of *C. pneumoniae* with SP-A or SP-D decreased the number of *C. pneumoniae* inclusion in the McCoy cell line, probably due the aggregating effect of SP-A and SP-D, thus resulting in a smaller proportion of *C. pneumoniae* infected cells. According to our results, SP-A and SP-D increased *C. pneumoniae* attachment to mouse macrophage cells, similar to the findings in human macrophage cells.

It is known that *C. pneumoniae* inhibit cell apoptosis in the early phase of the infection, however, in the late phase of infection pro-apoptotic process will dominate. Indeed, our research group has been established also that pro and anti-apoptotic processes simultaneously emerge in the Chlamydia infected cells. In our earlier experiment murine epithelial cells were infected with *C. trachomatis* and were treated with IFN-γ, afterwards, transcriptome analysis was carried out with DNA chip analysis. The experiment revealed that both positive and negative regulation of apoptosis were highly enriched, which overall resulted lack of apoptosis in *C. trachomatis* infected and IFN-γ treated cells. Our results are in agreement with these findings, as we could not detect elevated caspase 3/7 activity at 12 h *p.i.*. Additionally, *C. pneumoniae* stimulates the ERK 1/2 pathway activity to prevent caspase-independent

apoptosis. Similarly, we found that *C. pneumoniae* infection significantly enhanced the ERK 1/2 pathway activity compared to that in uninfected/untreated cells. Notably, we observed a novel possible mechanism via which *C. pneumoniae* prevents host cell apoptosis: *C. pneumoniae* significantly elevates the levels of phosphorylated MSK-1, which can also promote BCL-2-associated agonist of cell death (BAD) phosphorylation and lead to the repression of BAD-induced apoptosis. Contrary, when the cells were treated with Ax in *C. pneumoniae*-treated cells, MAPK/ERK activity was significantly reduced. This result suggests that the anti-chlamydial activity of Ax may be partially attributed to the reduced MAPK/ERK 1/2 activity. The MAPK/ERK 1/2 pathway also plays a crucial role in the nourishment of proliferating bacteria, and inhibition of this process is fatal to the pathogen; it has been shown that MAPK inhibitors can inhibit *C. pneumoniae* infection. The level of the P90RSK1/2 protein was also found to be significantly decreased; this protein is crucial for the phosphorylation of several antiapoptotic proteins such as death-associated protein kinase 1 and BAD-1. Additionally, the levels of phosphorylated MSK-1 decrease significantly, which are known to result in apoptosis. These findings suggest that Ax treatment may induce apoptosis in *C. pneumoniae*-infected cells, thereby inhibiting bacterial proliferation.

The results of a phase 2 clinical trial showed that Ax is well-tolerated beyond its standard administration at 1.25 mg/kg. Given the coronavirus disease 2019 (COVID-19) pandemic, several studies have shown that Ax can be used as a supplemental medication for treating COVID-19. Notably, MAPK/ERK activation plays a pivotal role in additional bacterial infections, including *Coxiella burnetii* infections, which are similar to Chlamydia infections. Therefore, we think it may be worth to investigate the role of Ax in *C. burnetii* infections.

6) Considered novel findings:

1. NAC increases *C. pneumoniae* replication *in vitro*
2. NAC increases *C. pneumoniae* replication *in vivo* in mice and prolongs the clearance of the bacteria
3. NAC increases the attachment of EBs to the host cell membrane, which may lead to higher efficiency in the bacterial uptake in the cell, thus elevating the bacterial number.
4. Ax has anti-chlamydial activity *in vitro* partly due to the IDO-2 elevation
5. Ax does not have anti-chlamydial activity at the concentration used in human medicine *in vivo* in mice
6. Ax has anti-chlamydial activity at 4x higher concentration than it is used in human medicine *in vivo* in mice
7. Ax significantly elevates IL-12, IL-17A IL-23, IFN-γ and SP-A expression in lungs of *C. pneumoniae*-infected mice, which indicates higher inflammation, thus a better chlamydia clearance
8. Ax significantly elevates IFN-γ protein level in the supernatant of *C. pneumoniae*-infected mice lungs, which signals prompt cellular immune response
9. *C. pneumoniae* increases phosphorylated MSK-1 level, which results in anti-apoptotic processes
10. Ax decreases ERK1/2 activity, which lead to the apoptosis of the host cell, thus decreasing the number of replicating *C. pneumoniae*.

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My Ph.D. thesis is dedicated to my relatives, who can not be with us today: **Sarolta**, **János** and **Gabriella.**

8) Publications

Publications related to the subject of the Thesis:

1. N-acetyl-cysteine increases the replication of *Chlamydia pneumoniae* and prolongs the clearance of the pathogen from mice.

   David Kokai, Timea Mosolygo, Dezso Peter Virok, Valeria Endresz, Katalin Burian
   JOURNAL OF MEDICAL MICROBIOLOGY 67: 5 pp. 702-708. , 7 p (2018).
   doi: 10.1099/jmm.0.000716

   Q2 **IF_{2018}: 1.926**

2. Ambroxol Treatment Suppresses the Proliferation of *Chlamydia pneumoniae* in Murine Lungs

   Dávid Kókai, Dóra Paróczai, Dezso Peter Virok, Valéria Endrész, Renáta Gáspár, Tamás Csont, Renáta Bozó and Katalin Burián
   MICROORGANISMS 9: 4 p. 880 , 14 p. (2021)
   https://doi.org/microorganisms9040880

   Q2 **IF_{2020}: 4.128**

Cumulative impact factor: 6.054

Publications not related to the subject of the thesis:

1. Indoleamine 2,3-dioxygenase activity in *Chlamydia muridarum* and *Chlamydia pneumoniae* infected mouse lung tissues

   Dezső Virok, Tímea Raffai, **David Kokai**, Dóra Paróczai, Anita Bogdanov, Gábor Veres, László Vécsei, Szilard Poliska, László Tiszlavicz, Ferenc Somogyvári, Valéria Endrész, Katalin Burián
   FRONTIERS IN CELLULAR AND INFECTION MICROBIOLOGY 9
   Paper: 192, 12 p. (2019)
   doi: 10.3389/fcimb.2019.00192

   D1 IF_{2019}: 4.123

3. Aerodynamic properties and in silico deposition of isoniazid loaded chitosan/thiolated chitosan and hyaluronic acid hybrid nanoplex DPIs as a potential TB treatment

   Mahwash Mukhtar, Edina Pallagi, Ildikó Csóka, Edit Benke, Árpád Farkas, Mahira Zeeshan, Katalin Burián, **Dávid Kókai**, Rita Ambrus
   INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES 165 pp. 3007-3019. , 13 p. (2020)
   https://doi.org/10.1016/j.ijbiomac.2020.10.192

   Q1 IF_{2020}:6.953

2. Chlamydia pneumoniae Influence on Cytokine Production in Steroid-Resistant and Steroid-Sensitive Asthmatics

   Paróczai, Dóra; Mosolygó, Tímea; **Kókai, Dávid**; Endrész, Valéria; Virók, Dezső Péter; Somfay, Attila; Burián, Katalin
   PATHOGENS 9: 2 Paper: 112, 13 p. (2020)
   doi: 10.3390/pathogens9020112

   Q2 IF_{2020}: 3.492

4. Beneficial Immunomodulatory Effects of Fluticasone Propionate in *Chlamydia pneumoniae*-Infected Mice

   Dóra Paróczai, Anita Sejben, **Dávid Kókai**, Dezso P. Virok, Valéria Endrész and Katalin Burián
   PATHOGENS 10: 3 Paper: 338, 13 p. (2021)
   https://doi.org/10.3390/pathogens10030338

   Q2 IF_{2020}: 3.492

5. Freeze-dried vs spray-dried nanoplex DPIs based on chitosan and its derivatives conjugated with hyaluronic acid for tuberculosis: *In vitro* aerodynamic and in silico deposition profiles

   Mahwash Mukhtara, Zsolt Szakonyi, Árpád Farkas, Katalin Burian, **Dávid Kókai**, Rita Ambrus
   EUROPEAN POLYMER JOURNAL 160 Paper: 110775, 14 p. (2021)
   https://doi.org/10.1016/j.eurpolymj.2021.110775

   Q1 IF_{2020}: 4.598

6. Indoleamine 2,3-dioxygenase cannot inhibit Chlamydia trachomatis growth in HL-60 human neutrophil granulocytes

   Dezso Peter Virok, Ferenc Tömösi, Anita Varga-Bogdanov, Szilard Poliska, Bella Bruszel, Zsuzsanna Cseh, **David Kokai**, Dóra Paróczai, Valeria Endresz, Tamas Janaky, Katalin Burián

   FRONTIERS IN IMMUNOLOGY 12 Paper: 717311, 14 p. (2021)

   Q1 IF_{2020}:7.561

7. Physico-Chemical, *In vitro* and Ex Vivo Characterization of Meloxicam Potassium-Cyclodextrin Nanospheres

   Patrícia Varga , Rita Ambrus , Piroska Szabó-Révész , **Dávid Kókai** , Katalin Burián , Zsolt Bella , Ferenc Fenyvesi , Csilla Bartos
   PHARMACEUTICS 13: 11 Paper: 1883, 14 p. (2021)
   https://doi.org/10.3390/pharmaceutics13111883

Q1 **IF_{2020}:6.321**

Cumulative impact factor:36.54

Abstracts related to the subject of the thesis:

1. Katalin, Burián; Bettina, Magyari; Tímea, Mosolygó; Valéria, Endrész; Dezső, Virók; **Dávid, Kókai**
   Effects of mucolitics in *in vitro* and *in vivo Chlamydophila pneumoniae* infection
   Acta Microbiologica Et Immunologica Hungarica 64: Suppl.1. pp. 115-116., 2 p. (2017)

2. Katalin, Burian; **David, Kokai**; Tímea, Mosolygó; Dezso, Virok; Valeria, Endresz
   N-acetyl-cysteine increases the replication of *Chlamydia pneumoniae* and prolongs the clearance of the pathogen from mice
   In: 28th European Congress of Clinical Microbiology and Infectious Diseases (28th ECCMID)(2018)

3. **David Kokai**; Dora Paroczai; Dezso Virok; Valeria Endresz; Katalin Burian
   Antimicrobial Effect Of The Commonly Used Mucolytic Agent, Ambroxol
   In: A Magyar Mikrobiológiai Társaság 2018. évi Nagygyűlése és a XIII. Fermentációs Kollokvium :(2018) 70 p. p. 33

4. **Dávid, Kókai**; Dóra, Paróczai; Dezső, Virók; Valéria, Endrész; Katalin, Burián
   Antimicrobial effect of the commonly used mucolytic agent, ambroxol
   Acta Microbiologica Et Immunologica Hungarica 66: Suppl. 1 pp. 52-52, 1 p. (2019)

5. **Kókai, Dávid**; Paróczai, Dóra; Burián, Katalin
   Egy mukolitikum *in vitro* és *in vivo* anti-chlamydiális hatással
   Medicina Thoracalis (BUDAPEST) 73: 1 pp. 15-16, 2 p. (2020)

6. **David, Kokai**; Dora, Paroczai; Dezso, Virok; Valeria, Endresz; Katalin, Burian
   Ambroxol Possesses An Antichlamydial Effect *In vitro* And *In vivo* (2020)
   18th German Chlamydia Workshop 2020 2020-02-05 Lübeck, Germany

Abstracts not related to the subject of the thesis:

1. **Dávid, Kókai**; Dóra, Paróczai; Dezső, Virók; Valéria, Endrész; Dezső, Csupor; Katalin, Burián
   Growth modulating effect of Hedera helix extract on bacteria
   Acta Microbiologica Et Immunologica Hungarica 66: Suppl. 1pp. 156-157. 2 p.(2019)

2. **Kókai, Dávid**; Paróczai, Dóra; Virók, Dezső Péter; Endrész, Valéria; Somogyvári, Ferenc; Bozó, Renáta; Burián, Katalin
   Viscum Album Tumorellenes Hatásának Vizsgálata (2020)
   A Magyar Mikrobiológiai Társaság 2020. évi Nagygyűlése és a XIV. Fermentációs Kollokvium 2020-10-14 Kecskemét, Magyarország

3. **Dávid, Kókai**; Dóra, Paróczai; Dezső, Péter Virók; Valéria, Endrész; Ferenc, Somogyvári; Renáta, Bozó; Katalin, Burián
   Antitumor Effect of Viscum Album
   Acta Microbiologica Et Immunologica Hungarica 68: Supplement 1 p. 24 (2021)

4. **Dávid, Kókai**; Dóra, Paróczai; Zain, Baaity; Dezső, P. Virók; Valéria, Endrész; Rita, Ambrus; Renáta, Bozó; Katalin, Burián
   Investigation of antiviral properties of hyaluronic acid (2021)
   23rd Annual Conference of the European Society for Clinical Virology