Probenecid Inhibits Respiratory Syncytial Virus (RSV) Replication

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Abstract

RNA viruses like SARS-CoV-2, influenza virus, and respiratory syncytial virus (RSV) are dependent on host genes for replication. We investigated if probenecid prophylaxis or treatment inhibited RSV replication in three epithelial cell lines used in RSV studies, i.e., Vero E6 cells, HEp-2 cells, and in primary normal human bronchoepithelial (NHBE) cells, and in BALB/c mice. The studies showed that nanomolar concentrations of all probenecid regimens prevent RSV strain A and RSV B replication in vitro and RSV strain A in vivo, representing a potential prophylactic and chemotherapeutic for RSV.

Introduction

Respiratory syncytial virus (RSV) is the leading viral pathogen associated with lower respiratory tract disease in infants and young children worldwide also afflicting the elderly and immune-compromised\(^1,2\). Preventing RSV morbidity and mortality has been an effort of research and vaccine studies development for decades. RSV is responsible for >150,000 pediatric hospitalizations/year costing >$300 million in young children\(^3\). Therapeutic intervention is limited to inhaled ribavirin and palivizumab (Synagis), a humanized monoclonal antibody targeting the F protein. Ribavirin has shown mixed-to-poor results and palivizumab treatment is not fully effective\(^4,5\). Additionally, palivizumab is administered monthly to help protect high-risk infants from severe RSV disease throughout the RSV season, and although treatment reduces hospitalizations in treated infants by approximately 50%, its efficacy decreases as mutations in F protein are induced by treatment\(^6,7\). Unfortunately, there is no safe and effective RSV vaccine available despite years of effort, thus there is a need for effective RSV therapeutics.

As an alternative to developing and testing novel antiviral drugs, repurposed or repositioned drugs with known safety profiles is under investigation as this could reduce costs and the time needed for the development of new drugs. One example is the drug minocycline which is a tetracycline antibiotic with efficacy in bacterial infections as well as antiviral activity against influenza virus and other viruses including RSV in vitro\(^8\). There is room for improvement of the anti-RSV drug repertoire as FDA-approved drugs are not always advantageous owing to issues of tolerance and efficacy. The need for a greater understanding of virus-host interactions has resulted in the discovery and validation of several therapeutic drug targets for therapeutic intervention\(^9\). Antiviral drugs could target either viral proteins or cellular proteins. The antiviral drug approach typically leads to a narrow spectrum of antiviral activity with a likelihood of developing drug resistance. In contrast, a drug that targets a stage in the viral life cycle in a host cell could inhibit multiple viruses using that pathway.

By combining high-throughput screening (HTS) with RNA interference (RNAi), host gene silencing can lead to the rapid discovery of host genes and pathways for developing antiviral treatments\(^10–13\). We previously used RNAi to discover and validate drug targets as a means to filter and prioritize therapeutics\(^13–15\). Specifically, our laboratory used genome-wide RNAi screens to identify pro- and antiviral host genes that affect virus replication that resulted in repurposed drugs to inhibit influenza A virus
Using an RNAi screen to determine individual host genes from respiratory epithelial (A549) cells on influenza A virus replication (IAV), we discovered that organic anion transporter-3 (OAT3) is required for IAV replication. The OAT3 gene (SLC22A8) has 12 predicted transmembrane domains, is principally expressed in the kidney, and is important for urinary excretion of anionic metabolites. No gender-based differences in OAT3 expression have been reported in humans, and while single nucleotide polymorphisms (SNPs) have been identified in the solute carrier 22A6 (SLC22A6) gene, these are non-coding and do not translate into clinically significant interactions suggesting OAT3 is highly conserved. Although much focus has been on the kidney, OATs are localized to almost all epithelial barriers in the body. OAT3 is expressed in both human and mouse lung respiratory epithelial cells. We showed that transfection of A549 cells with a siRNA targeting the OAT3 gene completely silenced influenza virus replication while RNAi silencing of closely related transporters, i.e., OAT1, OAT2, OAT4, OAT7, and URAT1 did not affect IAV replication. Importantly, we showed that probenecid inhibits OAT3. Probenecid is a uricosuric agent and chemical inhibitor of OAT3, a well-described treatment for gout, and is a favorable candidate for antiviral drug repurposing because it is readily commercially available and has a benign clinical safety profile. Along with OAT3, probenecid is known to modify other ion channels and impact inflammatory responses. We showed probenecid prophylaxis or treatment reduced IAV replication in vitro and mice with a half-maximal inhibitory concentration (IC₅₀) for treatment of A549 cells infected with A/WSN/33 (H1N1) or A/New Caledonia/20/99 (H1N1) to be 5 x 10⁻⁴ and 8 x 10⁻⁵ µM, respectively.

Viruses are dependent on co-opted host genes for replication. To determine if probenecid prophylaxis or treatment inhibited RSV replication, we tested nanomolar to micromolar concentrations of probenecid to prevent RSV strain A and strain B replication in epithelial cell lines and mice. Three cell lines were examined: Vero E6 cells, HEp-2 cells, and undifferentiated primary normal human bronchoepithelial (NHBE) cells, and male and female BALB/c mice were examined. The studies show that probenecid significantly reduces RSV replication in vitro and in vivo. These results are consistent with previous findings for influenza and SARS-CoV-2 suggesting that probenecid regimens are likely transferrable to other respiratory viruses which utilize solute carriers during replication, representing a potential host-directed pan-anti-viral.

**Results**

We used RNAi to discover host genes needed for IAV replication in A549 cells and showed that probenecid, an OAT3 (SLC22A8 gene) substrate and gene inhibitor, could reduce IAV replication in vitro and mice. The SLC family of solute carriers is expressed in both human and mouse tissues. Transfection of A549 cells with siRNA targeting the SLC22A8 gene completely silenced IAV replication. We showed that probenecid dramatically reduced IAV replication in vitro (IC₅₀ = 5 x 10⁻⁵ to 5 x 10⁻⁴ µM; p<0.05), and mice treated daily over 3 days with 25 mg/kg probenecid following lethal challenge (2 x 10³ PFU/mouse) with mouse-adapted IAV (A/WSN/33) were partially protected (60% survival; p<0.05). We
also showed that RNAi silencing of closely related transporters, i.e., OAT1, OAT2, OAT4, OAT7, and URAT1 did not affect IAV replication indicating a specific role of OAT3 to support IAV replication.

In this study, we determined if RSV replication in Vero E6 cells, HEp-2 cells, or NHBE cells infected with RSV A2, RSV B1, or Memphis-37 was affected by probenecid treatment. The different epithelial cell types were pretreated (prophylaxis) with differing probenecid concentrations (i.e., 100, 50, 25, 12, 6, 3, 1, 0.5, 0.2, 0.1, 0.05, 0.01, or 0 µM) and the effect of treatment on replication determined at 72h after infection by plaque assay. Probenecid prophylaxis resulted in a dose-dependent decrease in RSV A2 replication in all infected cells types with an IC$_{50}$/IC$_{90}$ = 0.07/0.63 uM in Vero E6 cells, 0.8/7.2 uM in HEp-2 cells, and 0.4/3.6 uM in NHBE cells (Figure 1). Cell viability was examined and as expected no cellular toxicity was evidently similar to earlier studies. Moreover, HEp-2 cells treated with IC$_{90}$ probenecid resulted in undetectable levels of OAT3 transcripts (Supplementary Table 1). Probencid treatment was very effective at inhibiting RSV A2 replication in all cells types (Figure 2). The IC$_{50}$/IC$_{90}$ = 0.1/2.7 uM in Vero E6 cells, 1.2/10.8 uM in HEp-2 cells, and 0.3/2.7 uM in NHBE cells. The results for probenecid prophylaxis showed the highest IC$_{50}$/IC$_{90}$ activity in Vero E6 cells and NHBE cells.

As RSV groups A and B co-circulate, and both groups may cause infection during a single season, it was important to determine the probenecid susceptibility to RSV A and RSV B particularly as it has been shown that the two groups have evolved separately for a considerable period. As for RSV A2, probenecid prophylaxis resulted in a dose-dependent decrease in RSV B1 replication in all infected cells types (Figure 3). There was no IC$_{90}$ for RSV B1 in the treated cell types because RSV B1 was not reduced 90% using the concentrations tested. Probencid prophylaxis resulted in an IC$_{50}$ = 0.85 uM in Vero E6 cells, 0.8 uM in HEp-2 cells, and 0.8 uM in NHBE cells (Figure 3, Table 1). Probencid treated Vero E6 cells infected with RSV B1 had an IC$_{50}$ = 2.0 uM, HEp-2 cells = 0.9 uM, and NHBE cells = 1.2 uM (Figure 4, Table 1). Similar to RSV A2 infected cells there was no cellular toxicity detected. The results showed that probencid prophylaxis or treatment was more effective for RSV A2 infected cell types compared to RSV B1.

Memphis-37 is an RSV A strain isolated from a pediatric case and used in studies in human adult subjects. Memphis-37 that is propagated in Vero E6 cells have been shown to develop a truncated G protein, thus the Memphis-37 strain used in these studies was propagated in HEp-2 cells. Probencid prophylaxis was effective at inhibiting Memphis-37 replication in all infected cells types (Figure 5). The IC$_{50}$/IC$_{90}$ = 0.03/0.27 uM in Vero E6 cells, 0.04/0.36 uM in HEp-2 cells, and 0.16/1.44 uM in NHBE cells (Table 1), and no effect on cell viability was detectable for any probenecid concentration. Treatment with probenecid inhibited Memphis-37 replication in all infected cells types as expected and was similar to RSV A2 and B1 studies (Figure 6). The IC$_{50}$/IC$_{90}$ = 0.4/3.6 uM in Vero E6 cells, 0.5/4.5 uM in HEp-2 cells, and 0.2/1.8 uM in NHBE cells (Table 1).

**Table 1. IC$_{50}$/IC$_{90}$ values.**
Table Legend. IC\textsubscript{50} and IC\textsubscript{90} values in NHBE cells, Vero E6 cells, and HEp-2 cells after treating with different probenecid concentrations and infecting with RSV A2, RSV B1, or Memphis-37. * = no IC\textsubscript{90} value for the RSV B1 virus as there was not a 90% reduction of virus titers with the concentrations of probenecid used.

Having shown probenecid to have potent activity on prophylactically or therapeutically treated cell types (Figures 1 - 6), we determined the effectiveness of prophylactic or therapeutic treatment in a BALB/c mouse model of RSV infection. Male or female 6-8-week-old BALB/c mice were intranasally (i.n.) infected with RSV strain A2. Mice were treated once with probenecid 24h before infection (prophylaxis) or 24h post-infection (treatment) dosed at 2 mg/kg or 200 mg/kg, or with PBS. As expected, there were no substantial clinical signs of disease determined by BAL cell infiltrates (Supplementary Table 2), and treatment did not affect antibody levels (Supplementary Figure 1). All probenecid regimens had significantly (p < 0.0001) reduced lung virus titer on days 3, 5, and 7 pi in female and male mice (Figures 7 and 8, respectively). As predicted from the in vitro results (Figures 1 and 2), there was a considerable reduction in the lung virus load in 2 mg/kg and 200 mg/kg probenecid-treated mice challenged with RSV A2. Maximum reductions of lung virus load occurred in mice pretreated with 200 mg/kg probenecid 24h before infection although substantial reductions in lung virus titer occurred following 2 mg/kg probenecid prophylaxis (Figures 7 and 8). Mice therapeutically treated once with 2 or 200 mg/kg probenecid 24h after RSV infection also had greatly reduced RSV A2 lung titers on days 3, 5, and 7 pi (Figures 7 and 8). Maximum reductions of lung virus load occurred in mice treated with 200 mg/kg probenecid, although significant (p < 0.0001) and substantial reductions in lung virus titers were observed in 2 mg/kg probenecid-treated mice. Moreover, RNA extracted from the lungs of mice treated with 200 mg/kg probenecid had markedly reduced OAT3 transcripts compared to PBS controls 2 dpi (Supplementary Table 1).

As previously reported\textsuperscript{23}, a population pharmacokinetics (pop-PK) model was developed to characterize probenecid PK using a one-compartment structure with saturable elimination and first-order absorption. Simulations using the final pop-PK model to generate probenecid exposure profiles comparing 600 mg twice daily, 900 mg twice daily, or 1800 mg once daily administration was completed and free drug concentrations were calculated (Table 2).
Table 2
Probenecid steady-state concentration and free drug concentrations after different probenecid doses.

| Dose (mg) | Frequency | Steady state concentration (mg/ml) | Steady-state concentration (mM) with 95% protein binding |
|-----------|-----------|-----------------------------------|--------------------------------------------------------|
| 600       | BID       | 30.1                              | 5.27                                                   |
| 900       | BID       | 92.5                              | 16.2                                                   |
| 1800      | QD        | 64.9                              | 11.4                                                   |

The doses examined are predicted to provide plasma concentrations exceeding the protein binding adjusted IC$_{50}$/IC$_{90}$ values for all RSV strains under all study conditions. The projected doses are below the maximum allowable FDA-approved dose and have been generally safe and well-tolerated with no significant side effects.

Discussion

There are currently only two FDA-approved drugs for RSV: palivizumab, a monoclonal antibody for the prevention of RSV in high-risk children, and ribavirin, approved for the treatment of severe RSV disease. Both of these drugs have questionable effectiveness$^{30}$. Despite the availability of these approved drugs, RSV remains a worldwide health concern due to the lack of a safe and effective vaccine, and substantial morbidity and some mortality across a spectrum of ages, i.e., the young and old. Several promising antiviral candidates with different mechanisms of action$^{30,31}$ are helping to advance development$^{32,33}$, however, new options are being considered as RSV disease intervention still is needed. Drug repurposing (or repositioning) is a strategy for identifying new uses for approved or investigational drugs beyond their original scope to facilitate antiviral development. There are two spaces in the drug repurposing and development area, i.e., direct-acting antivirals and host-targeted antivirals. The majority of FDA-approved antivirals are direct-acting typically rely on structural similarity or identical enzymatic activity, e.g., RNA-dependent RNA polymerase (e.g., Remdesivir) and proteases (e.g., Lopinavir).

Previously, siRNA screens were performed using siRNA libraries and A549 cells infected with WSN/33 IAV$^{12,13,34}$. The screens and validation confirmed that the OAT3 gene was needed for IAV replication as siRNA silencing completely blocked IAV replication. It is likely that OATs transport viral components needed for IAV assembly and replication, a process also used for SARS-CoV-2 replication$^{24}$, and likely RSV replication. Initially, we showed that probenecid treatment inhibited IAV replication by reducing OAT3$^{13}$. Probenecid is a chemical inhibitor of OAT transport, particularly OAT1 and OAT3$^{19,35,36}$. Host gene pathway analysis suggested that respiratory virus replication intersects OAT activity and that OATs may be needed for transport of viral constituents needed for viral replication using a similar process of OAT-mediated vectorial transport as for sodium and chloride ions across the airway lumen$^{37,38}$. In HEp-2
cells and BALB/c mice treated with probenecid, OAT3 expression was reduced (Supplementary Table 1), suggesting a role for OATs (including OAT3) in RSV replication. Probenecid has also been shown to modify pannexin 1 channels important role in intercellular communication but may also be relevant to disease.  

In this study, probenecid pretreatment of Vero E6 cells, HEp-2 cells, or NHBE cells was very effective at preventing RSV replication. The IC$_{50}$ and IC$_{90}$ of probenecid prophylaxis against RSV A2 was IC$_{50}$/IC$_{90}$ = 0.07/0.63 uM in Vero E6 cells, 0.8/7.2 uM in HEp-2 cells, and 0.4/3.6 uM in NHBE cells. Similarly, the IC$_{50}$ of probenecid treatment of RSV B1 infected Vero E6 cells was IC$_{50}$ = 0.85 uM, 0.8 uM for HEp-2 cells, and 0.8 uM for NHBE cells. Importantly, comparable IC$_{50}$/IC$_{90}$ results following probenecid prophylaxis or treatment of Memphis-37 infected cells were evident. These results along with the previous IAV and SARS-CoV-2 results show that nanomolar concentrations of probenecid reduce virus replication. Importantly, probenecid administered prophylactically before RSV infection resulted in reduced lung virus titers in vivo. Likewise, probenecid given therapeutically at 24h post-RSV infection also resulted in reduced lung virus titers demonstrating the versatility of probenecid as a chemotherapeutic. Importantly, human plasma concentrations for probenecid are projected to exceed the protein binding adjusted IC$_{50}$/IC$_{90}$ value over the dosing interval providing adequate coverage against the tested strains. Future studies should evaluate the in vivo efficacy of probenecid against other RSV strains and determine optimal treatment regimens and dosing.

Materials And Methods

Cells and Cell Culture Vero E6 cells (ATCC; CRL-1586) and human epithelial (HEp-2) cells (ATCC; CCL-23) were propagated in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Hyclone) at 37°C with 5% CO$_2$. Vero E6 cells and HEp-2 cells were maintained in log-phase in T75 cm$^2$ culture flasks (ThermoFisher) and HEp-2 was used for virus propagation. HEp-2 and Vero E6 cells depend largely on RSV G protein binding to cell surface glycosaminoglycans (GAGs). GAG-dependent infection is reduced by a single passage of RSV in Vero E6 cells. Normal human bronchial epithelial (NHBE) cells (Lonza) from a healthy male donor were expanded, cryopreserved, and maintained in bronchial epithelial cell growth medium (BEGM; Lonza) through 15 population doublings and were used undifferentiated.

Viruses RSV A2 (ATCC VR-1540), RSV B1 (ATCC VR-1580), or Memphis-37 (a clinical strain of human RSV strain A obtained from Meridian Life Science) were propagated and quantified on HEp-2 cells and Vero E6 cells then stored at -80°C as described previously. HEp-2 cells and Vero E6 cells were maintained in Dulbecco's modified essential medium (DMEM) supplemented with glutamine and 5% fetal bovine serum (5% DMEM; Gibco). Virus titers were determined using a methylcellulose plaque assay as described.

In vitro probenecid inhibition assays A working stock of probenecid (Sigma) was dissolved in DMSO (Sigma) and dilutions of the working stock were resuspended in PBS (Gibco). Cellular toxicity was
determined using a ToxiLight Bioassay (Lonza). Vero E6 cells, HEp-2 cells, or Undifferentiated NHBE cells were plated overnight at $10^4$ cells/well in 96-well flat-bottom plates (Costar). Cells were either pretreated for 24h prior to infection (prophylactically) or therapeutically at 24h post-infection with probenecid at different concentrations, i.e. 100, 50, 25, 12, 6, 3, 1, 0.5, 0.2, 0.1, 0.05, 0.01, or 0 µM. Subsequently, the media and probenecid were removed and the cells were infected with RSV A2, RSV B1, or Memphis-37 at an MOI = 0.1. At 72h post-infection the plates containing the cells were frozen at -80°C the freeze-thawed 3X and the cell-free supernatants were used for log_{10} dilutions in RSV plaque assays.

In vivo inhibition studies BALB/c male and female mice (6-8 weeks old) were obtained from Charles River and rested a week before use. All experiments and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia. All experiments were performed with five mice per group and repeated twice independently. To evaluate lung virus titers, probenecid was administered intraperitoneally (i.p.) at doses and time points pre- or post-RSV infection as indicated in the Results. Briefly, 2 mg/kg or 200 mg/kg of probenecid in PBS were i.p. delivered to the mice. On days 3, 5, and 7 bronchoalveolar lavage (BAL) samples were collected from individual mice and analyzed. BAL cell yield was determined by counting the total cell number, and cell viability was determined by Trypan blue (Sigma) exclusion. Smears for cell differentiation were prepared by cytocentrifugation (Shandon), and cell differentiation was performed by microscopy on cytospun slides after staining with hematoxylin and eosin staining where at least 100 cells were counted for macrophages, polymorphonuclear (PMN) cells, lymphocytes, and eosinophils. At each time point, sera were collected, and the lungs were isolated to determine virus titers by PFU/ml analyses. For virus titration analyses, lung homogenates were serially diluted, and the titer was determined on Vero E6 cells.

The BAL cell pattern reflects the inflammatory cell profile in the lung. Neither prophylactic nor therapeutic probenecid treatment with 2 mg/kg or 200 mg/kg probenecid had substantial effects on the differential cell counts or BAL leukocyte subpopulations at days 3, 5, or 7 pi (Supplementary Table 2). Further, no substantial differences in BAL cells were evident by smears despite the reduced RSV lung titers in the probenecid-treated mice highlighting the anti-RSV effects of the drug.

Lung virus titers Lung viral titers from RSV-infected mice were determined as previously described. Briefly, lungs were homogenized in 1 ml of sterile Dulbecco PBS per lung, and 10-fold serial dilutions in serum-free DMEM (Gibco) were added to confluent Vero cell monolayers in 24-well plates. After adsorption for 2h at 37°C, cell monolayers were overlaid with 2% methylcellulose, incubated at 37°C for 6 days, and then enumerated by immunostaining with anti-F protein monoclonal antibody, 131-2A.

RSV-specific ELISA Antibodies against RSV prevent disease by various mechanisms including virus neutralization, antibody-dependent cellular cytotoxicity (ADCC), and complement-mediated neutralization. To determine if probenecid treatment affected the anti-RSV antibody response, the sera from four female mice per group collected at days 7 pi were diluted (1:40) and assayed by ELISA using a modified protocol as described. The ELISA detects both neutralizing and non-neutralizing antibodies and the use of RSV
A2 lysate antigen provides a way to detect antibodies against multiple RSV proteins. As expected, there were very low IgG, IgG1, and IgG2a levels as these mice received a primary infection and sera were collected 7d post-challenge (Supplementary Figure 1).

OAT3 Expression SLC22A8 (OAT3) transcripts were evaluated by qPCR as previously described\textsuperscript{34,45,46}. For \textit{in vitro} studies, HEp-2 cells were plated in 96-well tissue culture plates (Corning) and treated with the IC\textsubscript{90} of probenecid [7.2µM] or DMSO only control for 24 h. RNA was isolated by RNAzol RT (Molecular Research Center) and digested with DNase1, and total RNA was quantified by Nanodrop (ThermoFisher). cDNA first-strand synthesis was performed using LunaScript (New England Biolabs) as described by the manufacturer. cDNA was used as a template for qPCR in Luna Universal qPCR master mix (New England Biolabs). For \textit{in vivo} studies, BALB/c lung RNA was extracted by RNAdvance Tissue (Beckman Coulter) at indicated time points and processed as described above. Primer pairs used are:

| Gene                  | Primer Left (5'-3')         | Primer Right (5'-3')         |
|-----------------------|-----------------------------|-----------------------------|
| SLC22A8 (huOAT3)      | TGCAATGAATGCGAATGAGG        | CGGTTCGCATAACACATA          |
| ACTB(huB-actin)       | CATGTACGTTGCTATCCAGGC       | CTCTTAAATGTCACGCACGAT       |
| SLC22A8 (msOAT3)      | CATACTCACTCCTGCACTCATC      | CCAGGGAATCTCAAAGGAAAA       |
| ACTB(msB-actin)       | CAGCCTTCCTTTGGGTATG         | GGCATAGAGGTCTTTACGGATG      |

Gene expression was determined, and raw Ct values or fold change (reciprocal of 2\(\Delta\Delta\text{Ct}\)) are presented normalized to a housekeeping gene. Data represent mean Ct values ± 95% confidence interval, or SEM, respectively, of three independent repeats.

**Statistical analysis.**

Statistical analyses were done using the Student’s t-test or one-way analysis of variance (ANOVA), as indicated. Results were calculated as means ± standard errors. Values of p<0.05 were considered significant.

**Declarations**

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**Author contributions**

J.M., H.B., D.M., F.D.S., and R.T. contributed to the design, data analysis, and the writing of the manuscript; J.M., H.B., L.J., T.C., and Z.B.R. contributed experiment support; all authors reviewed and contributed to
the preparation of the final manuscript.

**Competing interests**

D.M., H.B., and R.T. have ownership in TrippBio. There is no conflict of interest for any authors.

**Other**

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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**Figures**
**Figure 1**

Cell lines were prophylactically treated with probenecid 24h before RSV A2 infection. Probenecid prophylaxis significantly (**** p < 0.0001) inhibited the virus replication in Vero E6 cells, HEp-2 cells, and NHBE cells compared to control (DMSO only). Viral titers were determined by plaque assay. The IC$_{50}$ and IC$_{90}$ values are shown in Table 1.

**Figure 2**

Cell lines were treated with probenecid 24h after RSV A2 infection. Treatment significantly (**** p < 0.0001) inhibited the virus replication in Vero E6 cells, HEp-2 cells, and NHBE cells compared to control (DMSO only). Viral titers were determined by plaque assay. The IC$_{50}$ and IC$_{90}$ values are shown in Table 1.

**Figure 3**
Cell lines were prophylactically treated with probenecid 24h before RSV B1 infection. Probenecid prophylaxis significantly (**** p<0.0001) inhibited the virus replication in Vero E6 cells, HEp-2 cells, and NHBE cells compared to control (DMSO only). Viral titers were determined by plaque assay. The IC\textsubscript{50} values are shown in Table 1, IC\textsubscript{90} values are not available as the virus was not reduced by 90%.

![RSV B1- probenecid treatment](image)

**Figure 4**

Cell lines were treated with probenecid 24h after RSV B1 infection. Treatment significantly (**** p<0.0001) inhibited the virus replication in Vero E6 cells, HEp-2 cells, and NHBE cells compared to control (DMSO only). Viral titers were determined by plaque assay. The IC\textsubscript{50} values are shown in Table 1, IC\textsubscript{90} values are not available, as the virus was not reduced by 90%.
**Figure 5**

Cell lines were prophylactically treated with probenecid 24h before RSV Memphis-37 infection. Probenecid prophylaxis significantly (**** p < 0.0001) inhibited the virus replication in Vero E6 cells, HEp-2 cells, and NHBE cells compared to control (DMSO only). Viral titers were determined by plaque assay. The IC$_{50}$ and IC$_{90}$ values are shown in Table 1.

**Figure 6**

Cell lines were treated with probenecid 24h after RSV Memphis-37 infection. Treatment significantly (**** p < 0.0001) inhibited the virus replication in Vero E6 cells, HEp-2 cells, and NHBE cells compared to control (DMSO only). Viral titers were determined by plaque assay. The IC$_{50}$ and IC$_{90}$ values are shown in Table 1.
Figure 7
Lung virus titers from female BALB/c mice. The mice received 2 mg/kg or 200 mg/kg probenecid 24h before infection (prophylaxis) or 24h pi (treatment). The mice were i.n. infected with $1 \times 10^6$ PFU of RSV A2. At days 3, 5, and 7 pi, the lungs were harvested, and virus titers were determined by plaque assay. There is a significant ($**** p < 0.0001$) reduction in lung viral titers with all probenecid treatments compared to control (PBS).

Figure 8
Lung virus titers from male BALB/c mice. The mice received 2 mg/kg or 200 mg/kg probenecid 24h before infection (prophylaxis) or 24h pi (treatment). The mice were i.n. infected with $1 \times 10^6$ PFU of RSV A2. At days 3, 5, and 7 pi, the lungs were harvested, and virus titers were determined by plaque assay.
There is a significant (**** p < 0.0001) reduction in lung viral titers with all probenecid treatments compared to control (PBS).

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