EDP-938 is a novel non-fusion replication inhibitor of respiratory syncytial virus (RSV). It is highly active against all RSV-A and B laboratory strains and clinical isolates tested in vitro in various cell lines and assays, with half-maximal effective concentrations (EC_{50}s) of 21, 23 and 64 nM against Long (A), M37 (A) and VR-955 (B) strains, respectively, in the primary human bronchial epithelial cells (HBECs). EDP-938 inhibits RSV at a post-entry replication step of the viral life cycle as confirmed by time-of-addition study, and the activity appears to be mediated by viral nucleoprotein (N).

In vitro resistance studies suggest that EDP-938 presents a higher barrier to resistance compared to viral fusion or L protein inhibitors with no cross-resistance observed. Combinations of EDP-938 with other classes of RSV inhibitors lead to synergistic antiviral activity in vitro. Finally, EDP-938 has also been shown to be efficacious in vivo in a non-human primate model for RSV infection. EDP-938 is currently under evaluation in Phase 2 clinical studies.
**Introduction**

Respiratory syncytial virus (RSV) is a ubiquitous viral pathogen which can repeatedly re-infect a person throughout their lifetime. While most healthy children and young adults suffer mild symptoms with upper respiratory tract infections, progression to more serious lower respiratory tract infections do occur and RSV with significant mortality reported in at-risk groups such as infants, the immunocompromised, and the elderly [1–3]. RSV is the leading cause of respiratory induced hospitalizations, especially in children under 5, and is responsible for an estimated 3.4 million hospitalizations globally and 95,000–150,000 deaths per year [4,5]. In lower-middle income countries the majority of RSV-related fatalities occur in community settings, suggesting an underestimation of RSV’s true global impact [1,6].

Current prophylactic and therapeutic options for RSV are limited. Despite ongoing development efforts there is no approved vaccine or direct-acting antiviral against RSV. Treatment options include supportive care and the broad-spectrum antiviral ribavirin, whose usage is limited due to questionable efficacy and side effects [7–9]. For premature infants who are 6 months of age or younger at the start of the RSV season, the monthly injectable monoclonal antibody palivizumab is available, providing approximately 55% relative reduction in RSV-associated hospitalizations [10].

RSV is a non-segmented negative strand RNA virus of the family *Pneumoviridae*. Divided into subgroups A and B, its 15kb genome consists of 10 genes encoding 11 proteins. Transcription and replication involve the ribonucleoprotein complex which consists of the viral RNA genome or antigenome encapsidated by nucleocapsid N proteins. This structure provides protection from cellular degradation and acts as a guide for the RNA-dependant RNA polymerase L protein with assistance from P and M2-1 proteins [11].

A number of small molecule inhibitors directly targeting RSV have been identified. The majority of these compounds, known as fusion inhibitors, bind to the surface F protein, thus preventing viral entry into the cell. The first fusion inhibitor to show antiviral activity in a human RSV challenge study in healthy volunteers was presatovir (GS-5806) [12]. However, in subsequent phase 2 clinical trials presatovir failed to meet its virologic and clinical endpoints across a range of patient cohorts [13–15]. Additionally, resistance-associated mutations were selected quickly in patients receiving presatovir, suggesting a low barrier to resistance with this molecule [16,17]. Several other fusion inhibitors, JNJ-53718678 [18], ziresovir (AK0529) [19] and sisunatovir (RV521) [20], are currently under clinical development for RSV. Compounds targeting post-entry viral replication have also been described including the nucleoside inhibitor lumicitabine (ALS-8176) [21], non-nucleoside L polymerase inhibitors AZ-27 [22], PC786 [23] and JNJ-64417184 [24]. Among them, lumicitabine showed proof-of-concept efficacy in a human RSV challenge study [25] but its development was later terminated [26,27].

Another potential interesting target for RSV is the viral nucleoprotein (N), which is the most conserved viral protein and is essential for both viral replication and transcription [11]. A series of 1,4-benzodiazepine analogues were identified as specific inhibitors of RSV through cell-based antiviral screen of a diverse library of 20,000 compounds [28]. Later it was proposed that viral N protein is the target of the inhibitors mainly through *in vitro* resistance study [29]. The lead molecule, RSV-604, was advanced into human studies and showed some promising antiviral activities in a subset of stem cell transplant patients whose drug level was above its *in vitro* 90% maximal effective concentration (EC$_{90}$). However, the compound was later discontinued because of its suboptimal potency and the development challenge to achieve sufficient drug exposure [30].

Despite the challenges of development, there is a clear need for antiviral treatment options for RSV. EDP-938 was identified through a series of chemical optimizations based on...
1,4-benzodiazepine inhibitors of RSV [31]. Here we report the in vitro and in vivo antiviral activities of EDP-938. EDP-938 effectively blocks RSV replication by targeting a post-entry replication stage of the viral life cycle. In vitro resistance studies confirmed that it targets the viral N protein. EDP-938 is currently under evaluation in Phase 2 clinical studies.

**Results**

**In vitro activity of EDP-938 against RSV**

Based on reactivity of surface antigens to monoclonal antibodies and genetic variability, two subgroups of human RSV, A and B, have been delineated. Initially, the antiviral activity of EDP-938 was assessed in human epithelial cells (HEp-2) infected with RSV-A Long. As expected, visual inspection of the RSV-infected HEp-2 cell monolayers revealed an extensive cytopathic effect (CPE) in untreated cells. However, cells treated with EDP-938 exhibited a dose-dependent reduction in RSV-induced CPE. The cytoprotective effects of EDP-938 were independent of the RSV-A Long multiplicity of infection (MOI) (Fig 1A). To obtain a more quantitative measure of the antiviral activity of EDP-938, cellular ATP levels were determined. These data demonstrated that EDP-938 inhibited RSV induced CPE with a half-maximal effective concentration (EC\(_{50}\)) of 52 ± 12 nM (Fig 1B and Table 1). Using a reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay which monitors the reduction of viral RNA in the presence of compound, EDP-938 reduced viral load with an EC\(_{50}\) of 89 ± 16 nM. In line with previous publications [29], RSV-604 blocked RSV-A Long-induced CPE and inhibited viral replication with an EC\(_{50}\) of ~1.4 μM and ~2 μM, respectively (Fig 1C).

The activity of EDP-938 against both RSV-A and RSV-B was evaluated in vitro against multiple laboratory strains commonly used for antiviral screens in HEp-2 cells, human adenocarcinomic alveolar basal epithelial cells (A549), monkey kidney-derived cells (Vero), and Baby Hamster Kidney cells (BHK). Serial dilutions of the compound were added to cells infected with RSV-A at a MOI of 0.1 or RSV-B at an MOI of 0.5 and treated for 5 or 6 days, respectively. The EC\(_{50}\) values ranged from 28–72 nM for inhibition of CPE and from 54–110 nM for reduction in viral load as determined by RT-qPCR (Table 1) for the primate cell lines. However, a

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**Fig 1. Inhibition of RSV in vitro by EDP-938.** (A) Visualization of HEp-2 cell monolayers following RSV-A Long infection with or without treatment at indicated levels of EDP-938 with 5-day post infection (dpi) endpoint. (B) Percent cell viability measured by ATPlute and (C) percent viral RNA reduction measured by RT-qPCR in cells infected with RSV-A Long at MOI of 0.1. Data are mean ± standard error of the mean (SEM) from 89 and 77 individual experiments for EDP-938 and RSV-604, respectively, in the CPE assay and 8 separate assay runs for the RT-qPCR endpoint.

[https://doi.org/10.1371/journal.ppat.1009428.g001](https://doi.org/10.1371/journal.ppat.1009428.g001)
significant reduction of potency was observed in the BHK cell line, a finding previously observed for RSV-604 as well [32]. Together, these data show EDP-938 potently inhibits both RSV-A and B in various human and non-human primate cell lines using orthogonal assay read-outs.

RSV primarily replicates in the ciliated epithelial cells on the superficial layer of the airways. Primary human bronchial epithelial cells (HBECs) can be cultured and infected with RSV in vitro, which provides a more physiologically relevant system to evaluate potential antiviral drug candidates. The activity of EDP-938 was determined in HBECs infected with several RSV strains at a MOI of 0.1. RSV-A Long and M37 infected cells were treated for 6 days, whereas RSV-B VR-955 infected cells were treated for 7 days. As shown in Table 1, EDP-938 potently inhibited all 3 RSV strains tested in HBECs. EDP-938 was also interrogated using primary airway cells derived from cotton rats and neonatal lambs. Mirroring the results of the BHK cells, EDP-938 depicted reduced efficacy in these non-primate cell lines. EDP-938 was assayed for cytotoxicity in HEp-2, A549, Chinese hamster ovary (CHO), human embryonic kidney (HEK)-293 cells and the primary HBECs. There was no significant cytotoxicity with EDP-938 in HEp-2, A549, HEK-293 or HBEC at up to 50 μM, and the half-maximal cytotoxic concentration (CC₅₀) in CHO cells was ~27 μM (S1 Table). This measurement yields a selectivity index (antiviral EC₅₀/CC₅₀) of >2,380 in HBEC cells. In addition, EDP-938’s antiviral activity is highly specific to RSV, as it did not demonstrate significant activity against a broad panel of other viruses (S2 Table).

To demonstrate that the anti-RSV activity of EDP-938 is not affected by the genetic variability of the virus, USA-derived clinical isolates were obtained from Baylor University and Medical College of Wisconsin and tested in vitro for susceptibility to EDP-938. Serial dilutions of the compound were added to cells infected with RSV-A isolates at an MOI of 0.1 (except for isolate 629-9-2 at an MOI of 0.01) or RSV-B isolates at an MOI of 0.5 (expect for isolate 629-
24/2007 at an MOI of 0.0044) and were treated for 5 or 6 days, respectively. As shown in Fig 2A, EDP-938 was active against all the RSV-A and B clinical isolates tested with average EC₅₀ values of 76 nM and 121 nM, respectively. This is consistent with the fact that the viral N protein, which is the putative target of the antiviral effect of EDP-938, is the most conserved RSV protein [11].

To further expand testing against clinical isolates, the compound was assayed against a panel of 10 RSV-A and 10 RSV-B strains mainly isolated from children of age 2–200 weeks in the Netherlands. EDP-938 had average EC₅₀ values of 43 nM against RSV-A and 51 nM against RSV-B (Fig 2B) in the RSV Virospot reduction assay (ViroClinics).

The impact of protein-binding on the anti-RSV activity of EDP-938 was investigated in vitro in multiple cell types infected with various RSV strains. Unfortunately, human plasma or serum cannot be used directly in these cell-based studies because of the presence of RSV-neutralizing antibodies which interfere with the antiviral assay. Thus, purified human serum albumin or alpha-1-acid glycoprotein was added to the cell culture media at the physiologically relevant concentrations of 40 and 0.9 mg/mL, respectively. Albumin is the most abundant plasma protein whilst alpha-1-acid glycoprotein is known to be important in the binding and transport of many drugs. In this setting, a 2- to 5-fold shift in the potency of EDP-938 was observed in the presence of human serum albumin or alpha-1-acid glycoprotein across a range of RSV strains and cell types, suggesting that the effect of plasma protein binding may be minimal.

**In vitro resistance study**

To understand the mechanism of action of the inhibitor and evaluate the potential impact of resistant variants, efforts were undertaken to generate and characterize drug resistant virus in vitro. RSV-A Long virus was passaged in the presence of increasing concentrations of EDP-938. The generation of EDP-938-resistant virus required a low starting concentration of drug relative to its in vitro EC₅₀ (1x EC₅₀). The concentration of EDP-938 was gradually increased (up to 64x EC₅₀ in one resistance mutant) over 15 passages. Initiating viral passage at higher concentrations of EDP-938 or attempting to increase the concentration faster or higher resulted in clearance of the virus rather than selection of resistance. In contrast, the selection of resistance mutants using the fusion inhibitor GS-5806 [12] or L polymerase inhibitor AZ-27 [33] began at 10x their respective EC₅₀ values and was quickly increased to 16,384x EC₅₀ and 1,024x EC₅₀, respectively (S1 Fig).

Five populations of RSV-A Long and two populations of RSV-B VR-955 drug-resistant virus were generated. The RSV-A viruses were plaque purified to derive multiple resistance clones (#1–7). The RSV-B-resistant viruses became non-cytopathic and, therefore, plaque purification could not be performed. As a result, viruses were tracked as populations using
RT-qPCR. Plaque purified RSV-A viruses and RSV-B resistant populations were assayed for susceptibility to EDP-938 as well as RSV-604 and GS-5806 as controls. As shown in Table 2, two of the resistance clones conferred a 60-fold increase of EC$_{50}$ against EDP-938. They also showed cross-resistance against RSV-604 but remained fully sensitive to GS-5806 as indicated by the lack of shift in compound potency. For each EDP-938 resistant strain, the complete genome was sequenced to identify mutations. All of the EDP-938 resistant viruses contain mutations in the RSV N protein (Table 2), which is consistent with previous resistance studies of structurally related compounds such as RSV-604 [29]. Interestingly, three of the resistant viruses also have mutations in the G glycoprotein, the function of which is unclear since G is responsible for viral attachment but not replication. The N protein is the most conserved protein of the virus while the G protein is the least [11]. It is possible that some of the G mutations selected represent compensatory mutations or random drift rather than conferring resistance. While GS-5806 and AZ-27 lost all ability to inhibit their resistant viruses (Table 2), these strains showed no signs of cross resistance when treated with EDP-938 or compounds targeting other mechanisms of action from those used to induce resistance.

To confirm the findings from the EDP-938 in vitro resistance studies, recombinant RSV viruses containing site-specific mutations in N were generated and tested against EDP-938, RSV-604, and GS-5806 in HEP2 cells. The key mutations identified in RSV N as shown in Table 2 were incorporated into recombinant viruses using site-directed mutagenesis in combination with an RSV reverse genetics-based approach. As shown in Table 3, mutations at N residues M109 and I129 caused the largest EDP-938 EC$_{50}$ fold shifts relative to recombinant wild-type RSV. EDP-938 had an EC$_{50}$ of ~1.2 μM against the M109K mutant, which corresponded to a 26.9-fold increase over its EC$_{50}$ against the recombinant RSV wild-type. The EC$_{50}$ against the I129M mutant was 170 nM, which corresponded to a 3.7-fold increase over wild-type. Recombinant viruses containing multiple mutations with N were also generated and tested. EDP-938 had an EC$_{50}$ of ~2 μM against the recombinant virus harboring the Q102L/M109T/I129M triple mutants, which corresponded to a 42.4-fold increase relative to wild-type recombinant RSV and was comparable to the original resistance clone #1 selected with EDP-938 (Table 2). Moreover, recombinant RSV viruses containing the previously reported N mutations (I129L, L139I, and I129L/L139I) that confer resistance to RSV-604 [29] were also generated and evaluated. As shown in Table 3, the modifications at I129 and L139 produced RSV-

### Table 2. Characterization of EDP-938 resistant viruses.

| Virus            | Mutations in RSV N protein | Mutations in RSV G protein | EC$_{50}$ Fold Change vs. WT |
|------------------|---------------------------|---------------------------|------------------------------|
|                  |                           |                           | EDP-938 | RSV-604 | GS-5806 |
| RSV-A Long       |                           |                           | 1       | 1       | 1       |
| Wild-Type (WT)   |                           |                           | 1       | 1       | 1       |
| Plaque purified EDP-938 resistant clones | #1 Q102L, M109T, I129M | K205G, K213G, T219A | 60 | 4.6 | 0.7 |
|                  | #2 T29S, S134T             |                           | 3.3 | 3.1 | 0.9 |
|                  | #3 S134T                   |                           | 2.6 | 2   | 0.8 |
|                  | #4 V90A, S134T             |                           | 3.8 | 3.2 | 1.5 |
|                  | #5 M109I                   | R8H                       | 3.1 | 0.7 | 1.5 |
|                  | #6 M109K                   |                           | 67 | 3.3 | <0.23 |
|                  | #7 K136R                   |                           | 2.7 | 2.2 | 1.2 |
| RSV-B VR-955     | Wild-Type                  |                           | 1       | 1       | 1       |
| Population 1     | M109K, L139Q*             |                           | 42 | NA | 0.2 |
| Population 2     | M109T                     |                           | 6.6 | NA | 0.2 |

* Observed as a dual WT/Mutant population

https://doi.org/10.1371/journal.ppat.1009428.t002
EDP-938 shifts that were comparable to the previously published observations for RSV-604-resistant viruses [29]. Taken together, these findings support the observations from the in vitro EDP-938 resistance studies.

It is important to note that all the EDP-938-resistant strains appeared to replicate at a much slower rate than the wild-type virus and showed less CPE in infected cells. The growth kinetics of two mutant viruses that conferred the highest levels of resistance against EDP-938 were assessed in a viral fitness assay compared to the wild-type virus. HEp-2 cells were infected with the mutant and wild-type virus at the same MOI of 0.01 and monitored over time. As shown in Fig 3 and S3 Table, the amount of replication-competent virus generated was ~100–400 times lower than wild-type, suggesting reduced viral fitness.

**Mechanism of action**

In order to help determine which step of the viral life cycle EDP-938 blocks multiple in vitro time-of-addition studies were performed. In the first study, EDP-938 was administered to cells infected with RSV-A Long at a MOI of 0.1 at 2 hours pre-infection (-2) or 2, 6, or 24 hours
post-infection (hpi), and viral CPE evaluated 5 days later. As shown in Fig 4A, EDP-938 maintained about 80% of its cytoprotective activity for the cell population from RSV-induced CPE when given up to 24 hpi. The EC\textsubscript{50} values of EDP-938 for cytoprotection were 49 nM, 55 nM, 76 nM, and 66 nM following compound addition at -2 h, 2 h, 6 h, and 24 h, respectively. A second time-of-addition study explored the ability of EDP-938 to protect not just a population experiencing a spreading infection, but individual cells already infected with RSV. Using a MOI of 3, which infects approximately 95% of all cells, virus was cold-adsorbed to the cells for 1 hour to synchronize infection before transfer to 37°C to induce fusion (marking the 0 hpi time point). Compound was added at times indicated and a 24 hpi RT-qPCR endpoint was employed to constrain viral replication cycle to a single complete cycle. As shown in Fig 4B, cells were either treated with DMSO, EDP-938 or the fusion inhibitor GS-5806 at either 2x or 20x their respective EC\textsubscript{50}. Compared to DMSO treatment, EDP-938 inhibited RNA production at all time points up to 8 hpi infection, however, compound efficacy began to diminishing at 6 hpi and beyond. In contrast, the antiviral efficacy of GS-5806 was reduced when added at 0 hpi and completely lost when added at 8 hpi (Fig 4B). These results suggest that EDP-938 works at a post-entry, replication step of the viral life cycle.

EDP-938 inhibits an early stage of RSV replication following fusion. Following entry into a cell, nucleocapsids are transported from the plasma membrane into the cytoplasm, where viral transcription/replication “factories” are established. These “factories” manifest initially as small granules containing N, P, L, M2-1 and viral RNAs, and increase in size over time to form inclusion bodies [34,35]. Studies with RSV have shown that these inclusions can be formed with just the N and P proteins, and are dynamic liquid organelles which can readily dissociate and re-associate [36,37]. Given that resistance data suggest that N protein is the target of EDP-938, we investigated if the compound disrupted formation of the “factories”. RSV synchronously-infected HEp-2 cells were analyzed by immunofluorescence microscopy using an N-protein specific antibody. At 8 hpi, in the absence of compound, small RSV inclusions were detected in almost all cells (Fig 5A). In the presence of EDP-938, the majority of the cells did not present inclusions, however, in a few cells, inclusions were visible. These data suggest that EDP-938 did not inhibit liquid organelle formation per se (as they were present in some cells) but inhibited a prior step such that in most cells there was insufficient RSV protein for inclusion body formation.

To examine this more closely, we analyzed the effect of EDP-938 on accumulation of RSV RNAs. Again, HEp-2 cells synchronously-infected with RSV at 4°C were treated with either DMSO or EDP-938 upon transfer to 37°C (0 hpi). RNA isolated from DMSO treated cells showed that positive sense N RNA (likely representing N mRNA) increased significantly
between 2 and 6 hpi (Fig 5C). However, in cells treated with EDP-938 transcription was inhibited with no significant increase above the background at any of the times post infection. These data indicate that EDP-938 inhibits RSV primary transcription and/or processes prior to onset of primary transcription.

**In vitro combination study**

The combination effect of EDP-938 with other RSV inhibitors of different mechanisms/targets was investigated *in vitro*. RSV-A Long infected HEp-2 cells were treated with various concentrations of EDP-938 either alone or in combination with the fusion inhibitor GS-5806, non-nucleoside I polymerase inhibitor AZ-27, nucleoside inhibitor ALS-8112 [38], ribavirin, or palivizumab. The combination of two compounds always led to a greater inhibition than a single agent alone.

To determine whether the effect of the combination was synergistic, additive or antagonistic, the data were analyzed by the Loewe additivity model using Calcusyn software [39], which gave a Combination Index (CI) value at various effective concentrations of the compounds. A CI value of 0.9–1.1 indicates additivity, <0.9 suggests synergy, and >1.1 suggests antagonism. The combinations of EDP-938 with other RSV inhibitors showed moderate synergy. As an experimental control, adding EDP-938 to itself was additive. For details see S4 Table.

**In vivo efficacy in African green monkey model**

African Green monkeys (AGMs) are permissive for human RSV infection and support a relatively higher level of viral replication compared to cotton rats and BALB/c mice. It has been previously validated as a non-human primate model for assessing *in vivo* efficacy of potential RSV vaccines and antiviral drugs [38]. To evaluate the activity of EDP-938 in the AGM model, 4 animals each were treated with 100 mg/kg BID with EDP-938, RSV-604, or vehicle control via oral gavage starting 24 hours prior to RSV challenge and continuing for an additional 5 days. Samples were collected through bronchoalveolar lavage (BAL) and nasopharyngeal (NP) swab at various time points to determine viral load. As shown in Fig 6A, there was robust viral replication in the RSV infected AGMs of the vehicle control group with peak viral titer of ~10^6 copies/mL on Day 5 post infection. RSV-604 treatment did not result in significant RSV viral load reduction compared to vehicle control. In contrast, EDP-938 completely suppressed viral replication in the animals. Viral load in the NP swab samples was also determined. As shown in Fig 6B, EDP-938 treatment led to a 4-log_{10} viral load reduction to below the limit of detection (100 copies/mL) in both BAL on Day 5 and NP swab on Day 7 when the viral titer reached peak in the vehicle control group.

![Fig 5. EDP-938 prevents primary transcription of RSV mRNAs.](https://doi.org/10.1371/journal.ppat.1009428.g005)
Discussion

RSV is a common respiratory virus that is responsible for almost 60,000 hospitalizations among children <5 years of age in the United States [40]. In the current study, we describe the identification and characterization of novel clinical candidate, EDP-938, for the treatment of RSV. EDP-938 has nanomolar activities in vitro against a broad range of RSV-A and -B laboratory strains and clinical isolates. Time-of-addition studies indicate a post viral entry mechanism-of-action as EDP-938 maintains cytoprotection and RNA inhibition beyond initial infection. This contrasts with other known small molecule fusion inhibitors such as GS-5806 which are only effective prior to viral infection [41]. EDP-938’s profile more closely resembles other known replication inhibitors such as the N-protein inhibitor RSV-604, or AZ-27, ALS-8112, and PC786, each of which target the viral L protein, responsible for acting as the RNA-dependent-RNA-polymerase, capping, and methyltransferase enzyme.

The emergence of resistant viral strains on exposure to antiviral agents has the potential to impact the long-term utility of a therapeutic agent. As a result, we extensively characterized the resistance profile generated by EDP-938. Compared to resistance selection for the fusion inhibitor GS-5806 or the non-nucleoside L inhibitor AZ-27, the selection of RSV strains resistant to EDP-938 was difficult. Although were ultimately successful in the generation of resistant mutations, the level of EDP-938-resistance observed was inversely correlated with viral fitness levels. For example, the N\textsuperscript{M109K} mutation yielded a 67-fold EC\textsubscript{50} shift, but also resulted in a loss of CPE and a 100-fold reduction in infectious virion production. This resistance profile may bode well for the clinical use of EDP-938 as viral resistance appears hard to develop, and when it does such mutations carry with them significant reductions in viral fitness.

Upon full genome sequencing, 9 resistant variants were identified with mutations in the N and G proteins. While every resistant viral clone or population had at least 1 N mutation, only a third had G mutations. The N\textsuperscript{M109K} mutation granted the greatest resistance to EDP-938. Interestingly, the M109 amino acid displayed several variants, emerging multiple times across both RSV-A and -B strains. To the best of our knowledge, none of these mutations has previously been reported. Further analysis revealed that most of the EDP-938 mutations generally cluster within one ~24 angstrom solvent exposed section of the N protein, a region believed to interact with the RSV P protein (Fig 7) [42,43]. However, it seems unlikely that EDP-938 inhibits N-P interactions because its efficacy was diminished when added after 6 hpi (Fig 5), a relatively early time in the RSV replication cycle, and had no effect on either mRNA transcription or genome replication in a minigenome system. Of note, the structurally-related small-molecule inhibitor RSV-604 also generated resistant mutations within this same region of the
N protein (residues 105–139) and, compared to the prototype RSV L inhibitor AZ-27, had minimal effect in a replicon assay [32]. The N protein is the most conserved of the RSV proteins, while G is the least conserved. For these reasons, we conjecture that EDP-938 is either directly targeting the N protein or is mediating its antiviral activity through the N protein via another yet unknown actor, possibly a cellular protein, given that its activity is host dependent. Further, we conjecture that EDP-938 is either inhibiting primary (but not secondary) transcription, or another very early step in the viral replication cycle. The observed G protein mutations may be acting as compensatory mutations, or perhaps are the result of random genetic drift across the ~15 passages of the virus in cell culture.

While monotherapy treatment of viral infections such as acyclovir for herpesviruses or oseltamivir for influenza exist, combining treatments targeted to distinct aspects of viral replication have often proven superior to monotherapy options. The combination potential of EDP-938 was evaluated with the either ALS-8112, AZ-27, GS-5806, ribavirin, or palivizumab. EDP-938 displayed additivity with itself and ribavirin, while presenting moderate levels of synergy with the other antiviral agents tested. Chronic viral infections may benefit more from combination therapy than acute viral infections for several reasons. With many acute infections, the host's immune system can clear the viral pathogen if afforded the time to do so, as opposed to chronic infections where the time to mount an effective immune response is not typically a critical factor. Chronic conditions often go untreated for longer periods as compared to acute ailments, enabling chronic infections more time to diversify their genetic pool through selection and genetic drift. Additionally, chronic conditions often necessitate prolonged treatment courses as compared to acute infections, allowing the chronic viral pathogen more time to develop resistance mutations, something which combination therapy could mitigate against. While RSV is generally considered an acute infection, it has yet to be determined whether monotherapy or combination treatment of RSV will be required to effectively repress viral replication in a clinical setting.

The African green monkey model was selected for EDP-938 in vivo studies for its higher level of viral replication versus cotton rats and BALB/c mice. RSV-604, a compound whose
mechanism of action likely matches that of EDP-938 has previously been shown to have an antiviral effect in a phase 2 study of RSV-infected patients following stem cell transplantation. In this previous trial, patients who achieved RSV-604 levels in excess of the EC$_{90}$ experienced a 2.31 log reduction in viral load [14]. In the African Green monkey study presented here, EDP-938 displayed an ~4-log$_{10}$ reduction in peak viral load, while RSV-604 was unable to reduce viral load in a statistically significant manner. Additionally, following the cessation of treatment on day 4-post infection, no viral rebound was observed in the BAL fluid above the limit of detection. The lack of antiviral activity observed with RSV-604 could be a function of relatively poor potency compared to EDP-938 (Fig 1) and/or insufficient tissue exposure. While in this study the animals were not sacrificed and thus the drug level in the lung could not be determined, plasma samples were taken at 12h after the last dose (C$_{\text{trough}}$) to confirm drug exposure. EDP-938 had a mean plasma C$_{\text{trough}}$ of 1479 nM, which was ~15x of its EC$_{90}$. In contrast, RSV-604, given at the same dose, had a mean plasma C$_{\text{trough}}$ of 637 nM, which was well below its EC$_{90}$. This was likely the reason why EDP-938 worked in the model while RSV-604 did not. It is reasonable to project that given the same dose or exposure EDP-938 should perform better than RSV-604 in RSV infected patients as well.

In summary, EDP-938 is a potent nanomolar inhibitor of all assayed RSV strains in vitro. An in vitro selectivity index of over 2,380 suggests an excellent safety window. It displays a high barrier to resistance as compared to compounds targeting fusion or polymerase proteins. Mutation mapping suggests a mechanism of action mediated by the N protein. Resistance mutations which do arise appear to come at a severe fitness cost to the virus, thus minimizing concerns of the widespread emergence of drug resistant strains. EDP-938 displays additive to synergistic effects when given in combination with other anti-RSV compounds and treatments which suggests a potential for combination therapy options. Clinical studies evaluating the effects of EDP-938 in patients with RSV infection are currently ongoing.

**Materials and methods**

**Ethics statement**

The African Green Monkeys (Chlorocebus), used in this study were housed at BIOQUAL, Inc., in accordance with the recommendations of the Association for Assessment and Accreditation of Laboratory Animal Care International Standards and with the recommendations in the Guide for the Care and Use of Laboratory Animals—National Institutes of Health. The Institutional Animal Use and Care Committee of BIOQUAL approved this experiment (protocol no. 16–105). When immobilization was necessary, the animals were sedated by intramuscularly injection with 10 mg/kg of Ketamine HCl. All efforts were made to minimize suffering. Details of animal welfare and steps taken to ameliorate suffering were in accordance with the recommendations of the Weatherall report, "The use of non-human primates in research.” Animals were housed in an air-conditioned facility with an ambient temperature of 21–25°C, a relative humidity of 40%-60% and a 12 h light/dark cycle. Animals were socially housed when possible or individually housed if no compatible pairing could be found. Animals also received appropriate environmental enrichment. The animals were housed in suspended stainless steel wire-bottomed cages during the quarantine period and in HEPA filtered microisolator caging during the RSV challenge phase, to minimize virus transmission. The animals were provided with a commercial primate diet and fresh fruit twice daily, with water freely available at all times. RSV infected AGMs were humanely euthanized in accordance with the American Veterinary Medical Association Guidelines on Euthanasia, 2020.
**In vitro compound screen against RSV laboratory strains**

HEp-2, Vero, and BHK were seeded at 8,000 cells per well (cpw) in growth medium (Dulbecco’s Modified Eagle Medium (DMEM) with 1% GlutaMAX, 1% Penicillin/Streptomycin (P/S), 1% Non-Essential Amino Acids (NEAA), 10% Fetal Bovine Serum (FBS)) or (Minimum Essential Medium (MEM) with 10% FBS, 1% NEAA, 1% P/S) or (Eagle’s Minimum Essential Medium with L-Glutamine, with 10% FBS, 1% P/S) respectively. A549 seeded at 3,000 cpw in F-12K Kaighn’s Modification nutrient mixture with 10% FBS, 1% NEAA, 1% P/S. Cells seeded at time of infection in 96-well plates, final well volume 100μL. Compounds suspended in DMSO, serially diluted and added to wells at 0.5% final DMSO concentration. Viral stocks stabilized with 25% sucrose, previously titered using plaque assay, were added at desired MOI. Sucrose added to cells-only control wells to match percent added with virus. Unless otherwise noted, RSV-A strains were tested at 0.1 MOI and incubated for 5 days in a 37˚C, 5% CO₂ humidified incubator, and RSV-B at 0.5 MOI for 6 days. Cytopathic effect (CPE) determined using ATPlite (Perkin Elmer) measured with Envision luminometer. For RT-qPCR, RNA was extracted from whole well using Ambion RNeasy-96 Automated Kit, and RT-qPCR run using Applied Biosystems TaqMan RNA-to-CT 1-Step Kit. Primer/probes set for N gene (RSV-A: for: CATCCAGCAAATACACACATC, rev: TTCTGCACATCATAATTGGA-GTATCAA, probe: FAM-CGGAGCACAGGA-GAT-NFQ-MGB; RSV-B: for: CTGTCACTCCAGCAAATACACT-ATTCA, rev: GCACATCATAATTGGGAGTGTCA, probe: FAM-CGTAGTACAGGAGATAA-NFQ-MGB) run with 1kb N gene standard of known concentration. All EC₅₀s were calculated using the XLFit four-parameter logistic model 200 in Microsoft Excel.

**In vitro compound screen against RSV clinical isolate strains**

RSV clinical isolates were obtained from Baylor University and Medical College of Wisconsin. MOI of 0.1 used for all clinical isolates except RSV-A 629-9-2 (0.01) and RSV-B 629-24/2007 (0.0044) due to low titer. Virospot reduction assay was run by ViroClinics (Rotterdam, the Netherlands) against 20 additional RSV clinical isolates [44,45].

**Activity of EDP-938 in Primary Human Bronchial Epithelial Cells (HBECs), Primary Cotton Rat Lung Cells, and Primary Neonatal Lamb Bronchial Epithelial Cells**

Primary undifferentiated HBECs obtained from ATCC or PromoCell. Maintained in ATCC Airway Epithelial Cell Basal Medium with HLL Supplement, L-glutamine, Extract P, Airway Epithelial Cell Supplement or PromoCell Airway Epithelial Cell Growth Medium with Supplement Mix and 1% P/S respectively. Collagen-coated plates seeded with 8,000 cpw and infected at 0.1 MOI. Incubated 6 or 7 days with RSV-A and -B strains respectively. Primary cotton rat lung cells were harvested and maintained in Minimum Essential Medium with 2mM L-Glutamine, Earle’s Balanced Salts, 30 μg/mL Gentamicin, 2% P/S, 2% Amphotericin B. Assay was run using Minimum Essential Medium with 2mM L-Glutamine, Earle’s Balanced Salts, 10% FBS, 1% P/S, 1% NEAA, 10 ng/mL hEGF, 1x Lactalbumin Hydrolysate. Collagen-coated plates seeded with 8,000 cpw and infected at 0.1 MOI. Incubated 5 days with RSV-A strain. Primary neonatal lamb bronchial epithelial cells were harvested and maintained in Bronchial Epithelial Cell (BEC) Growth Medium. Seeded at 10,000 cpw in BEC medium, then washed twice with SF-DMEM the next day. Infected at 0.1 MOI, incubated 6 days with RSV-A strain.
Cytotoxicity
HEp-2, A549, and CHO cells were seeded at 3,000 cpw in their respective growth medium. CHO growth medium used was RPMI medium 1640 with 10% FBS, 1% P/S, 1% GlutaMAX. HEK-293 cells seeded at 10,000 cpw in DMEM growth medium. Undifferentiated HBECs were seeded in collagen-coated 96-well plates at 7,500 cpw in appropriate growth medium. Compound added and incubated for 3 days (4 days for HBECs) as described above. Plates read with ATPlile and CC\textsubscript{50} calculated as EC\textsubscript{50}s as described above. SI = CC\textsubscript{50}/EC\textsubscript{50} within a cell line.

Viral specificity
EDP-938 were tested against human metapneumovirus and parainfluenza virus 3 using PIV-3\textsubscript{JS}\_GFP and hMPV-Can97-83\_GFP (ViraTree) in LLCMK2 cells (ATCC, cat# CCL-7) at Katholieke Universiteit Leuven. The activities against human rhinovirus 14 (1059 strain), MERS coronavirus (EMC strain), influenza A (H1N1) and B (Brisbane/60/2008) viruses were determined in Hela-Ohio, Vero 76, and MDCK cells at NIAID/Utah State University. Another human coronavirus, HCoV-229E, was assayed in MRC-5 cells at Imquest Biosciences, Inc. (Frederick, Maryland). The activity against Measles virus Edmonston strain was evaluated in MRC-5 cells at Southern Research Institute (Frederick, Maryland). Hepatitis B virus (HBV) and hepatitis C virus (HCV) activities were evaluated at Enanta Pharmaceuticals Inc. using HepAD38 cells and a subgenomic genotype 1b HCV replicon kindly provided by Dr. Christoph Seeger (Fox Chase Cancer Center) and Dr. Ralf Bartenschlager (Heidelberg University), respectively.

Time-of-addition
As above, but 8,000 HEp-2 cpw seeded 1-day pre-infection. 2h pre-infection compound or media added. Plates incubated 2h. Viral infection at 0.1 MOI, incubated 1h. Viral inoculum removed, cells washed, and media replaced with compound for -2h sample. Compound added at 2h, 6h, and 24h post-infection to appropriate plates. 5dpi cell viability calculated as described above.

For single round infection synchronized infection 25,000 HEp-2 cpw seeded 1-day pre-infection. Plates brought to 4˚C, viral infection at 3 MOI calculated for 30,000 cpw incubated at 4˚C 1hr. Compound added at 0h plate and plates moved to 37˚C. Compound added at 2h, 4h, 6h, and 8h post-incubation (when plates moved to 37˚C) to appropriate plates. 24 hpi viral copies measured with RT-qPCR as described above.

Synchronized infection and analysis of primary viral transcription
To synchronize infection, HEp-2 cells seeded with or without coverslips, were chilled on ice for ~ 5 minutes, washed twice with ice-cold serum free Opti-MEM and infected with a chilled RSV A2 inoculum at an MOI of 3 in Opti-MEM containing 2% FBS. The virus was allowed to adsorb for 2 h at +4˚C (without disturbance), after which at 0 hpi the inoculum was supplemented with 37˚C pre-warmed media (Opti-MEM containing 2% FBS) containing DMSO or 1000 nM EDP-938 (diluted in DMSO, such that the concentration of DMSO was equivalent in each well) and incubated at 37˚C. For immunofluorescence microscopy analyses, at 8 hpi cells seeded on coverslips were fixed with 5% formaldehyde, 2% sucrose in phosphate buffered saline for 30 min, permeabilized with 0.5% lgepal, 10% sucrose in phosphate buffered saline for 20 min, and incubated with an antibody toward RSV N protein (Serotec). Following washing in phosphate buffered saline, cells were incubated with isotype specific secondary antibody, labelled with Alexafluor 588 and DAPI. Cells were analyzed by fluorescence microscopy. For RT-qPCR analyses, cells were harvested at the indicated times and RNA extracted using Trizol
(ThermoFisher) according to manufacturer’s instructions, except that following isopropanol precipitation it was resuspended in 2 M NTE (2 M NaCl, 40 mM Tris [pH 7.4], 1 mM EDTA), extracted with phenol and chloroform, and precipitated with ethanol. RNA pellets were resuspended in 50 μl of RNase free water. 500 ng of RNA was annealed simultaneously to a GAPDH primer and N specific primer to detect positive sense RNA and reverse transcribed using superscript IV (ThermoFisher) according to manufacturer’s instructions. 2 μl of cDNA was then subjected to qPCR. Amplicons were detected using iTaq Universal Sybr Green Supermix (Bio-Rad), and quantified using the ΔΔCt method relative to the mean expression of housekeeping gene GAPDH. Primer sets for N gene (for- ATGGGAGAGGTAGCTCCAGA, rev- AGCTCTCCTAATCACGGCTG) and GAPDH (for- GTCGGAGTCAACGGA TT, rev- AAGCTTCCCGTTCTCAG). The data presented represent one of two independent experiments, performed with slightly different timepoints. In each experiment, the reverse transcription step was performed twice and each set of cDNA was analyzed in duplicate by qPCR.

**In vitro resistance study**

**RSV drug resistance selection.** Both T25 flasks and 24-well plates were utilized to generate drug resistant mutants (DR/DRMs). Cells (HEp-2 for RSV-A Long, and A549 for RSV-B VR-955) were seeded well below confluency. Viral infection initiated at 0.05–0.1 MOI. EDP-938 initially administered at 1X, 4X, 10X, 8X, and 50X EC\textsubscript{50} anywhere from 3–10 times each. GS-5806 and AZ-27 initiated at 10X and 50X EC\textsubscript{50} 1 time each. Passed 1 DMSO-only culture to track DMSO / random genetic drift. All samples had 0.1% final DMSO concentration. Collected upon first of ~70% CPE or 7dpi for RSV-A. RSV-B lost CPE effect after several passages and was monitored by RT-qPCR. Virus was collected by scraping cells into media, adding 25% sucrose, using LN2 for 2x snap freezes of pellet mixing in supernantant between each freeze. Passaging was done with 10–100μl of viral stock. Stocks were passed at 0.5X, 1X, and 2X EDP-938 from the previous passage each round. Loss of virus resulted in backtracking or passage of a lower concentration stock. Successful growth resulted in discard of active lower concentration stocks. Virus stored long term at -80°C. Each RSV-A DR population was plaque purified using 0.3% agarose overlay in growth media (3 plaques picked each) and amplified by passage with the top respective concentration of EDP-938.

**Sanger sequencing.** RNA extracted as above. RT-PCR used SuperScript One Step Long Kit with primers covering the entire RSV genome in sections. Gel purified product sent to Quintara Biosciences for Sanger sequencing. Chromatograms from DMSO/WT results compared to DRMs in house.

**Characterization of RSV drug-resistant strains.** RSV-A DR strains titered by plaque assay as previously described [46]. CPE and RT-qPCR compound testing performed as described above at a MOI of 0.1. DMSO passed virus used as WT control. EDP-938, RSV-604, and GS-5806 tested against each strain. Fold shift = DRM EC\textsubscript{50} / WT EC\textsubscript{50}.

Viral fitness performed as CPE assay above with compound omitted, 8,000 HEp-2 cpw, 0.01 MOI, and 6dpi endpoint. Inoculum used for “day 0” pfu value. CPE quantified as above on days 0–6. Pfu samples collected days 1–6 by adding equal volume media +50% sucrose, scraping cell debris and collecting entire well as above. RSV-B DRMs infected using RT-qPCR-determined genomic equivalence with RT-qPCR readout as above vs. DMSO passed WT strain.

**RSV reverse genetics system**

Preparation and recovery of recombinant WT and mutant RSV was performed using the bacterial artificial chromosome (BAC)-based system described in Stobart et al [47]. Mutagenesis
performed on a BAC containing the antigenome of RSV A2-line19F (pSynk-AZ) using an Agi-
lent site-directed mutagenesis kit following manufacturer’s instructions. Upon mutation con-
firmation, WT or modified versions of pSynk-AZ along with the four helper plasmids and a
plasmid encoding T7 polymerase were transfected into Vero cells. Cells incubated at 37˚C.
Replaced culture media every 2–3 days until detection of mKATE2 fluorescent foci indicative
of active recombinant RSV propagation. Cells expanded and incubated at 37˚C until mKATE2
fluorescence detection in >75% of a T25 flask. Working viral stocks were expanded in HEp2
cells. Titration performed by a modified plaque assay in HEp2 cells, assessing the number of
fluorescent foci 7 dpi. Mutations confirmed by Sanger sequencing as described previously.

**In vitro combination study**

As described above with 8,000 HEp-2 cpw, 0.1 MOI RSV-A Long, 5-day endpoint, and CPE
readout. Compounds added in serial 1.3-fold 6-point dilutions across both X- and Y-axes of
plate. Plates run in quadruplicate with one uninfected plate for cytotoxicity. Combination
indexes calculated using Loewe additivity model using CalcuSyn software from Biosoft.

**African green monkey model**

A study of EDP-938 and RSV-604 in the African Green monkey (AGM) animal model was
performed by BIOQUAL, Inc (Rockville, Maryland). Methods were similar to those run in a
study by Ispas et al. [48]. Six (6) male and 6 female AGMs (Chlorocebus) of 4–5 kg were pur-
chased from PrimGen (Hines, Illinois). Each animal was challenged with 2 x 10^5 PFU of RSV
A2 viral inoculum (Virapur, San Diego, CA). Four animals (2 per sex) in each group were
treated with EDP-938, RSV-604, or vehicle control (0.5% w/v methylcellulose). The animals
were dosed via oral gavage twice daily (every 12 hours), starting 24 hours prior to RSV chal-
lenge, and then continuing for an additional 5 days (6 total days of dose administration).
Bronchoalveolar lavage (BAL) samples and nasopharyngeal (NP) swabs were collected for viral
load determination.

**Supporting information**

S1 Table. *In vitro* cytotoxicity of EDP-938.
(TIF)

S2 Table. *In vitro* activity of EDP-938 against other viruses.
(TIF)

S3 Table. Fitness of resistance mutant vs. wild-type (WT) virus.
(TIF)

S4 Table. Analysis of the effect of combination of EDP-938 with other RSV inhibitors.
(TIF)

S1 Fig. Passage history of *in vitro* selection of RSV resistant strains. Viral passaging in the
presence of increasing concentrations of compound. In each Panel, grayscale curves represent
stocks unable to survive with black markers denoting the terminal passage. Resistant strains
are indicated by colored lines. (A) RSV-A Long passaged in the presence of EDP-938. 7
attempts to grow virus at 4x and 8x the EDP-938 EC_{50} value resulted in immediate loss of
virus. EDP-938 increases were attempted every passage; all failures but terminal omitted from
graph. (B) RSV-B VR-955 passaged. 20 separate attempts resulted in total loss of virus. Some
curves slightly offset to display better. (C) GS-5806- and (D) AZ-27-induced viral resistance.
Compound increases were not attempted every passage.

**Acknowledgments**

We would like to thank Lijuan Jiang and Kellye Daniels (Enanta Pharmaceuticals, Inc.) for their support of the project, Pedro Piedra (Baylor University) and Kelly J. Henrickson (Medical College Wisconsin) for providing the RSV clinical isolates in the US, Viroclinics Bioscience BV for testing clinical isolates in the Netherlands, Martin Moore (Emory University) for providing the RSV reverse genetics system, and Hanne Andersen Elyard (Bioqual, Inc) for conducting the African green monkey study.

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