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Original Article

Characterization of an orally available respiratory syncytial virus L protein polymerase inhibitor DZ7487

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Abstract: Objectives: Respiratory Syncytial Virus (RSV) is a leading cause of death and hospitalization among infants and young children. People with an immunocompromised status are also at risk for severe RSV infection. There is no specific treatment for RSV infection available. Ribavirin, an antiviral drug approved for severe lung infection by RSV, has shown limited clinical efficacies with severe side effects. Additionally, given the genetic variability of RSV genomes and seasonal change of different strains, a broad-spectrum antiviral drug is highly desirable. The RNA-dependent RNA polymerase (RdRp) domain is relatively conserved and indispensable for the replication of the virus genome and therefore serves as a potential therapeutic target. Previous attempts to identify an RdRp inhibitor have not been successful due to lack of potency or high enough blood exposure. DZ7487 is a novel orally available small molecule inhibitor specifically designed to target the RSV RdRp. Here we present our data showing that DZ7487 can potently inhibited all clinical viral isolates tested, with large safety margin predicted for human. Methods: HEp-2 cells were infected by RSV A and B. Antiviral activities were assessed by *in vitro* cytopathic effect assay (CPE) and Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). DZ7487 antiviral effects in lower airway cells were evaluated in A549 and human small airway epithelial cells (SAEC) cells. DZ7487 induced RSV A2 escape mutations were selected through continuous culture with increasing DZ7487 concentrations in the culture medium. Resistant mutations were identified by next generation sequencing and confirmed by recombinant RSV CPE assays. RSV infection models in both BALB/c mice and cotton rats were used to evaluate DZ7487 *in vivo* antiviral effects. Results: DZ7487 potently inhibited viral replication of all clinical isolates of both RSVA and B subtypes. In lower airway cells, DZ7487 showed superior efficacy than the nucleoside analog ALS-8112. Acquired resistant mutation was predominantly restricted at the RdRp domain resulting asparagine to threonine mutation (N363T) of the L protein. This finding is consistent with DZ7487's presumed binding mode. DZ7487 was well tolerated in animal models. Unlike fusion inhibitors, which can only prevent viral infection, DZ7487 potently inhibited RSV replication before and after RSV *infection in vitro* and *in vivo*. Conclusions: DZ7487 demonstrated potent anti-RSV replication effect both in vitro and in vivo assays. It has the desired drug-like physical properties to be an effective orally available anti-RSV replication drug with broad spectrum.

Keywords: RSV, RdRp, DZ7487, L protein, oral, non-nucleoside

Introduction

Almost all children suffer from RSV infection before 2 years of age, and half experience reinfection [1]. Among infants and children, RSV accounted for 12-63% of all acute respiratory infections and led to 19-81% of all viral acute lower respiratory tract infection (ALRTI) associated hospitalizations [2]. RSV related ALTRI is the leading cause of death within the first year of life. Approximately half of children who experience severe RSV bronchiolitis will develop subsequent wheezing and asthma [3]. Furthermore, asthma severity in childhood influences clinical and lung function outcomes in adult life [4]. According to a study in 72 Gavi (the Global Alliance for Vaccines and Immunization) countries, the annual RSV-associated disease burden among children is estimated at an average of 20.8 million cases, 1.8 million hospital admissions, 40,000 deaths and $611 million (US) in discounted direct costs [5]. In addition to premature infants, RSV infection can occur at all ages. Elderly people with underlying car-

diopulmonary disease; immunocompromised adults, particularly hematopoietic stem cell transplant (HSCT) recipients, those undergoing intensive chemotherapy; and lung transplant patients are also at serious risk of severe RSV infection, which further exacerbates the disease burden.

Human RSV is a single stranded RNA virus and has only one serotype, which is classified into two subtypes, RSV A and B, based on the different reactions to antibodies against RSV long strain components [6]. The variety of RSV genotypes is determined mainly by the structural sequence of the envelope attachment protein (G) mucin-like domains [7, 8]. There are 13 A genotypes and 22 B genotypes that have been identified based on the second hypervariable region of the G gene [9, 10]. RSV-A includes GA1-GA7, SAA1, NA1-NA4, and ON1, and RSV-B includes GB1-GB4, BA1-BA6, BA7-BA10, SAB1-SAB3, SAB4, URU1-URU2, CB1, and BA-C [9]. In annual epidemics, these two subtypes can cocirculate [11]. Conflicting results have been reported regarding the severity of the subtypes due to different temporal and geographic factors. Some studies showed that the RSV A subtype predominates and corresponds to more severe disease in most years [12]. Others have shown that RSV B could lead to more severe infection [13] or that both subtypes could have equivalent severity [14]. The large variety of RSV genotypes also contributes to frequent reinfection throughout life [15]. Anti-RSV agents effective against most genotypes of RSV would be most helpful and convenient in the clinic, which has been a challenge for anti-RSV drug discovery efforts.

Vaccination is considered the most powerful, economical and effective method against infectious diseases. Severe RSV infection mostly occurs in infants less than 1 year old. Ideally, an active vaccine should be administered at birth or through maternal vaccination [16]. However, the immature immune system of infants and strict safety requirements impede infant vaccination [17, 18]. In addition, one study found that anti-RSV antibodies were poorly maintained in both adults and children, rarely last more than 6 months [19, 20], raising the question whether a RSV vaccine can provide longer term protection. Although there have been several clinical trials of vaccines, no vaccines have been approved yet [21]. The most promising vaccine candidate at the moment is Pfizer's RSVpreF (PF-06928316) which was granted for priority review by U.S. FDA for the prevention of RSV infection in older adults [22].

Palivizumab, which targets the envelope fusion protein (F), is an approved monoclonal antibody for the prevention of RSV infection in high-risk preterm infants [23]. However, the high cost and moderate efficacy limit Palivizumab's use [24, 25]. In 2022, AstraZeneca and Sanofi's monoclonal antibody Beyfortus (nirsevimab) was approved in the European Union for the prevention of lower respiratory tract disease caused by RSV infection in newborns and infants [26]. Passive antibody immunoprophylaxis has also been tested in infected patients with no apparent clinical benefits [27].

Small molecular drugs have also been pursued as anti-RSV therapies. Potential small molecules against RSV mainly include nucleoside analogs, fusion inhibitors, and polymerase inhibitors. Fusion inhibitors block the virus entry by interfering with viral fusion to the host cells. Once RSV entry step is completed, the fusion inhibitors will not be effective [28]. Both nucleoside analogs and polymerase inhibitors function through blocking viral replication and have the potential for post-infection treatment in the clinic [29]. Ribavirin, a synthetic nucleoside analog with broad virustatic activity, is the only antiviral agent licensed by the FDA for the treatment of severe RSV infections [30]. However, after encouraging data from industry-sponsored studies, a series of randomized trials were unable to demonstrate any improvement of bronchiolitis, leading to a rapid disappearance of ribavirin use for RSV infection [30]. Furthermore, nucleoside analogs have a potential genotoxicity risk, making nonnucleoside polymerase inhibitors more attractive for pediatric RSV treatment [31].

The RSV nucleocapsid includes four proteins: the nucleoprotein N, the phosphoprotein P, the transcription processivity factor M2-1, and the large polymerase subunit L [8]. The N, P, and L are required for RNA synthesis and thought to be potential targets for replication inhibition [32]. The N protein binds to the genome and antigenome to form a template with helical

nucleocapsids [8, 32, 33]. The P protein is the cofactor of RSV polymerase, recruiting multiple proteins to the polymerase complex such as L, RNA-free N (N0), N, and M2-1 to regulate RNA synthesis and transcription. The L protein is a viral RNA-dependent RNA polymerase that contains multiple enzyme activities required for RSV replication. To initiate the replication process, the L-P complex must replace N protein from the RNA template with the P protein [33-35]. For polymerase function, the L-P complex is the minimal component required to show RNA synthesis activity [32, 36, 37]. It is difficult to determine the P protein structure because the P protein is intrinsically disordered at the N- and C-terminal regions [38]. The L protein is a structurally conserved protein, particularly the RdRp domain [34]. Six conserved sequence motifs of RdRp constitute the catalytic center, the NTP/template/product entrance or exit tunnel, and are involved in the most critical step of RNA synthesis [39]. All of the above characteristics make the L protein, particularly RdRp, a very attractive target for broad spectrum anti-RSV drug development. No L protein inhibitor is approved yet, although several developing candidates are at early clinical studies.

Here, we describe a novel oral nonnucleoside RSV polymerase L protein inhibitor, DZ7487, which inhibits a broad panel of RSV A and RSV B clinical isolates and exerts postinfection therapeutic effects *in vitro* and *in vivo* by oral administration.

Materials and methods

Compounds

DZ7487 was synthesized by Dizal Pharmaceuticals. GS-5806 was purchased from MedChemExpress. ALS-8112 and PC786 were synthesized internally according to published structure.

Cells and viruses

HEp-2, A549 were purchased from the American Type Culture Collection (ATCC), SAEC was purchased from Lonza. HEp-2 was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Gibco; 10565-018) containing 10% Heat-Inactivated Fetal Bovine Serum (FBS). A549 was cultured in Ham's F-12 Nutrient Mix (Gibco; 11765054) containing 10% Heat-Inactivated FBS. SAEC was cultured in S-ALI™ Growth Basal Medium (Lonza; CC-3281). RSV A2, RSV B9320, RSV A Long were purchased from ATCC. RSV clinical isolation were gifted from AstraZeneca PLC. For RSV recombinant virus generation, RSV virus genome, L (with N363T mutation), N, P, M2-1 plasmid were transfected into BSR-T7 cell to package RSV. Seventy-two hours post transfection, supernatant was harvested and centrifuged to yield virus, then passaged in HEp-2 cells to establish virus stock, frozen at -80 degree for storage.

Cytopathic effect (CPE) assay

A total of 1500-3000 cells were seeded on a 384 well plate at day 0. Compounds were added into cells one hour ahead of RSV infection. RSV were added at MOI = 0.1, 1, and 7 for HEp-2, SAEC, and A549 cells, respectively. At day 6 when non-infected cells grow to 100% confluence and virus infected cells were completely dead, cell counting kit-8 (CCK-8) (Dojindo Laboratories; code CK04), were used to detect the cell viability by Optical density (OD) value which were measured by SpectraMax M5 microplate reader (Molecular Devices) machine. The cytopathic effect induced by virus was calculated by equation: inhibition % = (OD of the control - sample OD/OD of the control)/100%. The half maximal inhibitory concentration (IC50) and 90% inhibitory concentration (IC90) of CPE inhibition were calculated using four-parameter logistic equation.

Quantitative PCR

Ten thousand HEp-2 cells were seeded in 96-well plates, compounds were added one hour prior to infection. Two hours post infection, supernatants were removed and replaced with fresh medium containing compound and cultured for another 72 hr. Then, supernatant was harvested, and virus RNA was extracted and analyzed by RT-qPCR. Plaque-forming unit (PFU) equivalents were calculated based on the standard of RSV cDNA. Half maximal effective concentration (EC50) of supernatant viral load was calculated by PFU equivalents (PFUe). The sequences of the primers for RSV B 9320 N protein are: GCAGGTCTAGGCATAATGGG (forward) and TCTACATCATCTTCTTTGGGGTT (reverse); Primers for RSV A2 and A Long N protein are: AGATCAACTTCTGTCATCCAGCAA (forward)

and TTCTGCACATCATAATTAGGAGTATCAAT (reverse).

F protein ELISA assay

HEp-2 cells were seeded into a 96 well plate (at 1 × 10^4/well) one day before infection. Compound was added to cells 1 h before inoculation and 6, 24, 48 h after inoculation with RSV A Long. Three days post infection, supernatant was removed, and cells were fixed with acetone, incubated with anti-RSV F fusion protein antibody (Sigma; MAB858-1) overnight, washed twice, and then incubated with secondary antibody (Sigma; AP124P) for 2 hrs. After washing and addition of the substrate, OD value was determined in a SpectraMax M5 microplate reader (Molecular Devices). The IC90 of virus inhibition was calculated using four-parameter logistic equation.

Cell cytotoxicity

Cells were seeded into a 384 well plate. Compounds were added into cells at the specified concentrations. Six days later, cell viability was measured using CCK-8 assay. The 50% cytotoxicity concentration (CC50) of compound were calculated using four-parameter logistic equation.

Escape mutation induction

Escape mutations were selected by dose escalation treatment of DZ7487. HEp-2 cells were seeded in 6-well plates; the second day 100% cell confluence were required. Two folds of EC50 compound concentration was used to treat RSV 1 hour prior to RSV addition at MOI 0.1. Between day 4 to day 7, cells cytopathic effect was observed. If more than 70% of cells were cytopathic, the virus was transferred to a new HEp-2 cell plate and compound concentration was double; if less than 70% of cells were cytopathic, passaged the virus to a new HEp-2 cell plate, and kept with the same compound dose for treatment. After the 20th passage, we harvested the supernatant and cells for virus RNA extraction and perform reverse transcription for next generation sequencing analysis.

Pharmacokinetics

Animals were administrated with different doses and plasma was collected at different time point post dosing. Plasma drug concentration were analyzed by LC-MS/MS. Protein binding of compound was determined by equilibrium dialysis.

RSV infection model

All animal studies were conducted according to the guidelines approved by Institutional Animal Care and Use Committees of Dizal Pharmaceuticals or Children's Hospital of Chongqing Medical University. BALB/c mice (female, 6- to 7-week-old, 18 to 20 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd. Cotton rats (female, 6-week-old, 80 g) were provided by Children's Hospital of Chongqing Medical University. BALB/c mice and cotton rats were intranasally infected with 1 × 10^6 PFU or 1.5 × 10^6 PFU of RSV A2 strain virus. Compounds were dosed orally every 12 hours. For BALB/c mice, the first dose was initiated as indicated, and animals were sacrificed at day 4 or day 7; for cotton rats, the first dose was initiated 2 hours before RSV inoculation and animals were sacrificed at day 3. Lung tissues were harvested for viral load detection and histopathology analysis. Virus load in all animal lung homogenates was determined by quantitative PCR.

Histopathology

Lung tissues were fixed in 10% neutral-buffered formalin solution. Paraffin sections (3-4 μm in thickness) were prepared and stained with Hematoxylin and Eosin (H&E) prior to the observation by light microscopy.

Statistical analysis

Results are represented as means ± standard errors of the means. EC50, IC50, IC90, and CC50 were calculated using four-parameter logistic equation in GraphPad Prism software. The selectivity index was calculated as the ratio of the CC50 and IC50. Multiple comparison was performed by analysis of variance (ANOVA) using the GraphPad Prism Software. Statistical significance was defined as a P value of 0.05 or lower.

Results

DZ7487 effectively blocks RSV replication

To identify the potential lead compounds capable of inhibiting RSV viral replication, we

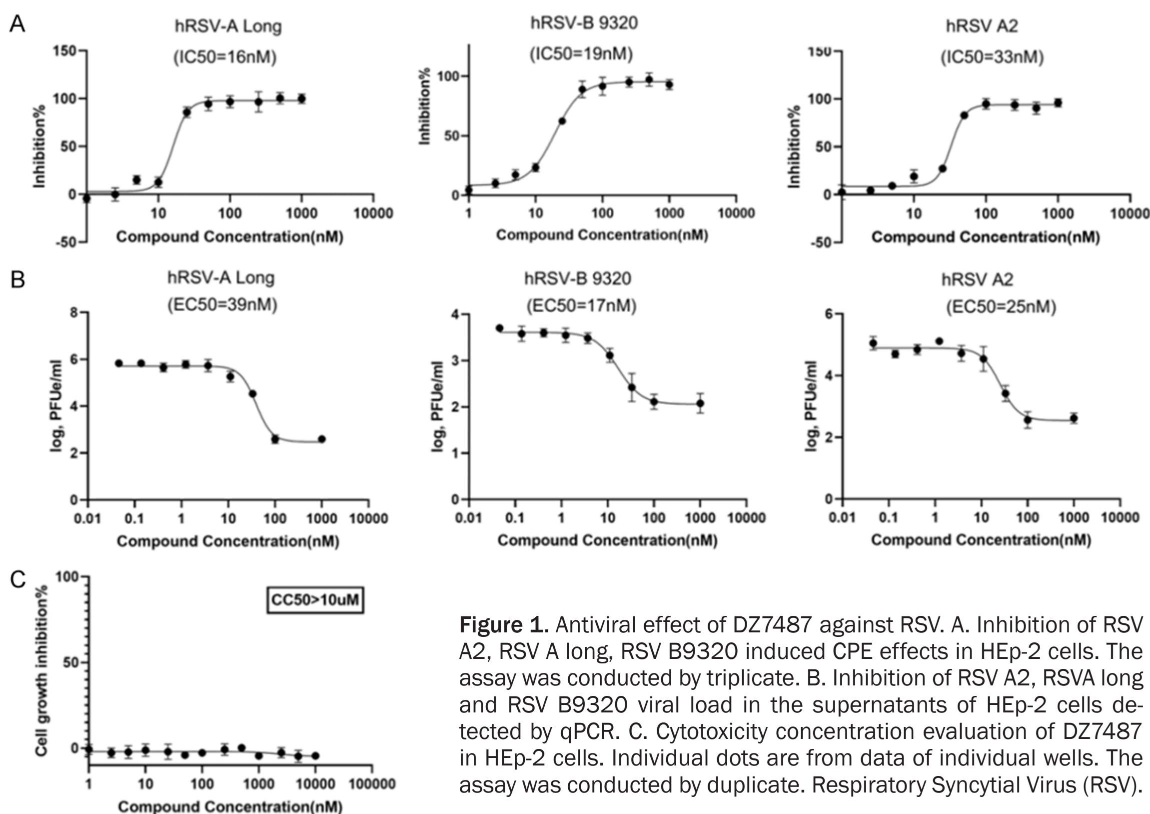

Figure 1. Antiviral effect of DZ7487 against RSV. A. Inhibition of RSV A2, RSV A long, RSV B9320 induced CPE effects in HEp-2 cells. The assay was conducted by triplicate. B. Inhibition of RSV A2, RSVA long and RSV B9320 viral load in the supernatants of HEp-2 cells detected by qPCR. C. Cytotoxicity concentration evaluation of DZ7487 in HEp-2 cells. Individual dots are from data of individual wells. The assay was conducted by duplicate. Respiratory Syncytial Virus (RSV).

Table 1. Antiviral CPE effects of DZ7487 against RSVA and B clinical isolates in HEp-2 cells

RSV A strains	laboratory	Clinical isolates of RSV A											
	A long	A22	A27	A29	A37	A39	A41	A44	A46	A55	A56	A121	Average ± SD
IC50 (nM)	16	22	30	26	21	31	25	24	26	27	24	29	26±3
RSV B strains	laboratory	Clinical isolates of RSV B											
	B9320	B28	B36	B62	B5	B9	B27						Average ± SD
IC50 (nM)	19	27	19	15	10	14	29						19±8

Respiratory Syncytial Virus (RSV), cytopathic effect assay (CPE).

designed and screened our small molecular libraries using *in vitro* CPE assays in the human laryngeal epithelial cell line HEp-2 infected by laboratory strains of RSV strain A Long and RSV strain B 9320. After rounds of lead optimization, DZ7487 was selected as one of the lead candidates. DZ7487 demonstrated potent viral replication activities in CPE assays with IC50 values of 16 nM, 19 nM and 33 nM against RSV A Long, RSV B 9320 and RSV A2, respectively (**Figure 1A**). The supernatant of the infected cells was collected, and RSV N gene expression was quantified using RT-qPCR as a measure of viral replication. **Figure 1B** shows the EC50 for DZ7487 anti-replication activities, which were consistent with CPE assay results and confirmed DZ7487 as a potent inhibitor of RSV replication. The cytotoxicity assay of DZ7487 revealed that DZ7487 showed no apparent cytotoxic effect at concentrations up to 10 μM (**Figure 1C**), with a selectivity index (SI) of >300 between DZ7487 antiviral effects and cell cytotoxicity.

DZ7487 blocks RSV viral replication in multiple clinical isolates

To evaluate DZ7487's potential as a broad-spectrum RSV replication inhibitor, we tested DZ7487 in multiple RSV clinical isolates (**Table 1**). A total of 11 strains of A subtype and 6 B subtype clinical isolates were tested. DZ7487

Table 2. Antiviral CPE effect of DZ7487 and ALS-8112 in different airway cell line

Cell type	IC50 (nM)	
	DZ7487	ALS-8112
A549	10	1884
SAEC	37	1205

Cytopathic effect assay (CPE), small airway epithelial cells (SAEC).

potently inhibited all these clinical isolates with almost equal potencies, between 10 and 31 nM.

DZ7487 protects respiratory tract cells from RSV infection induced cell death

Effect of DZ7487 blocks RSV replication and thus protects respiratory tract cells from RSV infection-induced lysis were evaluated. As cell sensitivities to RSV infection differ, pilot experiments were performed to optimize the multiplicity of infection (MOI). DZ7487 effectively protect SAEC and A549 cells from RSV infection-induced cell death, with IC50 of 37 and 10 nM, respectively (**Table 2** and **Figure 2A**, **2B**). The cytotoxicity of DZ7487, measured as CC50, for SAEC and A549 is 12.235 and 44.456 μM, respectively (**Figure 2C**, **2D**). The calculated selectivity index (SI) for SAEC is 331-fold and 4446 for A549 (**Table 3**). In the same assay, the nucleoside analog ALS-8112 showed much weaker antiviral activity (IC50 = 1205, 1884 nM in SAEC and A549, respectively) (**Figure 2A**, **2B** and **Table 2**).

Unlike anti-fusion inhibitors, DZ7487 blocks viral replication and protects host cells after viral infection

RSV virus initiates its infection into host cells through its fusion protein F which guides virion fusion to host cell membrane. Blocking F protein and stopping virion fusion to target cell membrane have been attempted as a therapeutic approach to prevent viral infection. GS-5806 is one of the better studied fusion inhibitors. GS-5806 showed dose-dependently prevent virion production (**Figure 3A**). It can completely prevent virion production at low single digit nanomolar concentration if the compound is added 1 hour before virus is added to the cell culture. The maximal inhibition decreased 30% if GS-5806 was added 6 hours after the virus. The inhibition effect diminished when GS-5806 was added 24 hours post infection. In contrast, DZ7487 can maintain 100% inhibition 24 hours after infection (**Figure 3B**). Our data replicated earlier reports on fusion inhibitors: fusion inhibitors inhibit RSV at an early stage of viral infection and are inactive when added after RSV infection occurs [28]. **Table 4** showed the calculated antiviral IC90 shift pre- and post-viral infection. GS-5806 could not achieve more than 70% maximal inhibition post infection while DZ7487 showed only a 2.4-, and 2.5-fold IC90 increase at 6 and 24 h post-inoculation, respectively. Clearly, being able to block virus replication further after infection is of clinical significance. Together, these data highlight DZ7487's potential as an effective therapy in clinic.

Mutations at the RdRp domain of the L protein abolish DZ7487's anti-replication effect

To evaluate whether DZ7487 directly targets L protein RdRp domain of RSV, we selected DZ7487 resistant RSV A2 virus by passaging virus in the presence of increasing concentrations of DZ7487 in HEp-2 cell. After 20 passages, the treatment concentration was increased to 16 times higher than the initial IC50 for wild type RSV A2 (**Figure 4A**). The 20th passage virus was collected and analyzed by next-generation sequencing. Among the individual viral genomes sequenced, 87% of them have a point mutation at position 1088 of the RSV L gene, which causes an asparagine to threonine change at amino acid 363 (N363T) in the RdRp domain of the L protein (**Figure 4B**, **4C**). Other mutations have also been detected but occur with much lower frequencies.

To confirm the causality of N363T mutation and resistance to DZ7487, we generated recombinant RSV with the N363T mutation and evaluated its sensitivity to DZ7487. Compared to wildtype, N363T mutation caused about a 9-fold shift of DZ7487 IC50, from 52 nM to 447 nM (**Figure 4D** and **Table 5**). Mutations that disrupt drug binding are often selected as the most efficient escape mechanism. The fact that N363T mutation on L protein is the dominant resistant mutation further supports that the L protein RdRp domain is the target of DZ7487. It is worthwhile to note that N363 is well conserved in all isolates of both RSV A and B subtypes, which could explain why DZ7487 is active against all isolates tested.

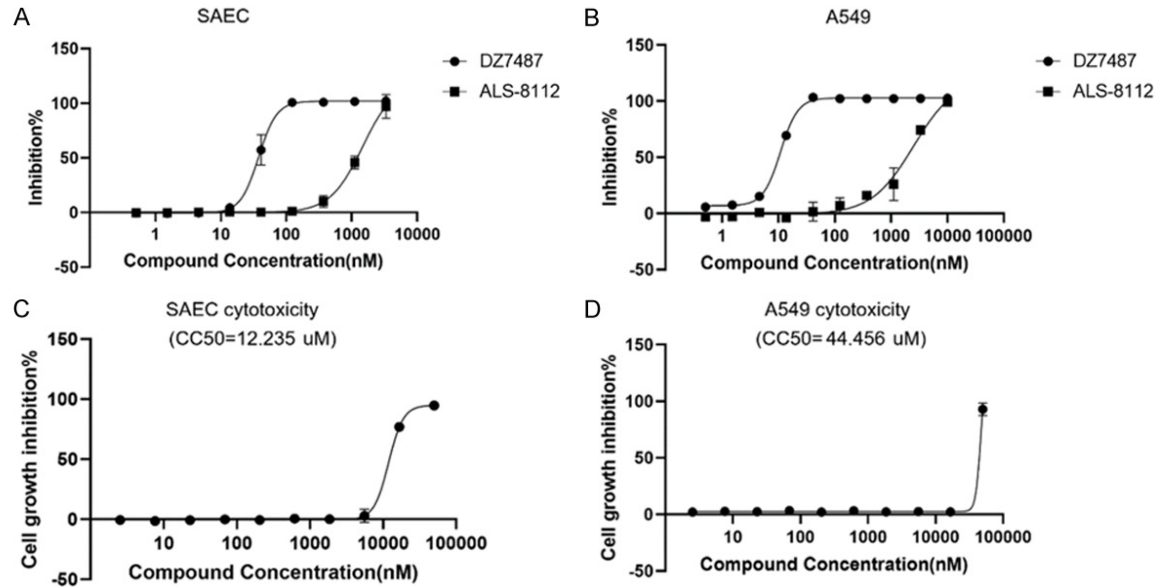

Figure 2. Antiviral CPE effect of DZ7487 against RSV in different airway cell lines. (A, B) Inhibition of RSVA long induced CPE effects of DZ7487 and ALS-8112 in SAEC (A) and A549 cells (B). (C, D) Cell cytotoxicity of DZ7487 in SAEC and A549 cells, respectively. Cells were infected with 100 tissue culture infectious dose (TCID50) of RSVA long 1 hr post compound treatment, and cell viability were determined by CCK-8 assay at day 5 post inoculation. Respiratory Syncytial Virus (RSV), cytopathic effect assay (CPE), small airway epithelial cells (SAEC).

Table 3. Selectivity index (SI) of DZ7487 in different airway cell line

Cell type	DZ7487		
	IC50 (nM)	CC50 (nM)	SI
A549	10	44456	4446
SAEC	37	12235	331

Small Airway Epithelial Cells (SAEC).

DZ7487 is potent against other RSV mutants

We next evaluated whether DZ7487 could overcome resistance mutations reported against other RSV inhibitors and avoid cross resistance. As shown in **Table 6** and **Figure 5**, DZ7487 fully maintained its potency to inhibit RSV replication with diverse mutations, including L138F and F140L/N517I in the F protein induced by GS-5806, Y1631H in the L protein induced by PC786, and QUAD mutations (M628L, A789V, L795I, and I796V in the L protein) induced by ALS-8112.

DZ7484 showed strong anti-RSV effect in animal models

After confirming the antiviral effect of DZ7487 *in vitro*, we further evaluated DZ7484 anti-viral activities in animal models. As shown in **Figure 6A**, DZ7487 showed linear dose-exposure relationship from 10 mg/kg to 75 mg/kg. Twice daily dosing of 10 mg/kg DZ7487 is required to achieve steady state drug concentration above IC90 continuously in mice. DZ7487 exposure in the lung was much higher than that in the blood (**Figure 6A**), suggesting drug accumulation in lung tissues. Animals could well tolerate up to 70 mg/kg twice daily dosing (**Figure 6B**) without evidence of toxicity and body weight decrease.

We then established an RSV A2 infection model in BALB/c mice. Animals were administered different doses of DZ7487 to identify the most efficacious dose (**Figure 7A**). DZ7487 potently inhibited *in vivo* RSV A2 replication from 10 mg/kg to 70 mg/kg in a dose dependent manner (**Figure 7B**). Since 70 mg/kg is well tolerated (**Figure 6B**), a dose regime of 70 mg/kg twice daily was deployed for further efficacy studies. To test whether DZ7487 could provide protection after infection, we tested DZ7487 activity 1-2 hour before viral inoculation, 24 hours, and 48 hours after RSV inoculation (**Figure 7C**). Lung tissues were harvested on Day 4 and Day 7 after inoculation and viral load in lung tissues were measured. DZ7487 exhibited similar efficacies (**Figure 7D**) up to 2 days after viral infection.

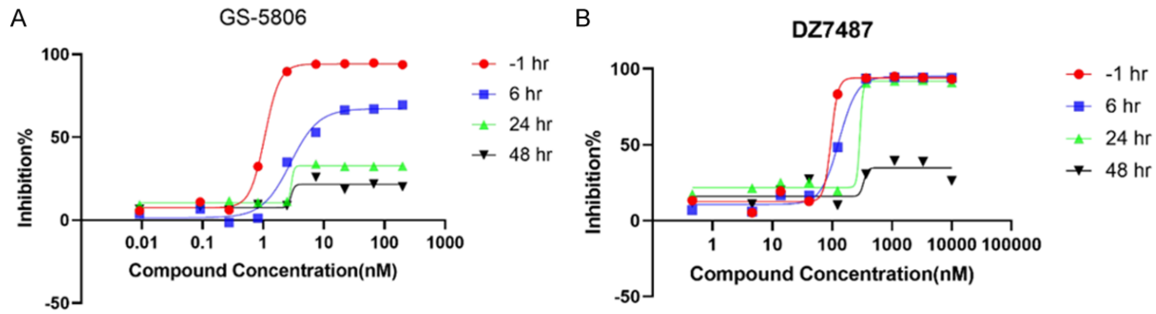

Figure 3. Time-of-addition assay of DZ7487 compared with a known fusion inhibitor in HEp-2 cells. (A) Inhibition of virus replication by fusion inhibitor GS-5806 detected by F protein ELISA assay. DZ7487 was added 1 hour ahead of RSV A Long inoculation or 6, 24 and 48 hours post inoculation, respectively. ELISA was conducted at day 3 post inoculation. (B) Inhibition of virus replication by DZ7487 added at different time point as in (A).

Table 4. IC90 fold change for different addition time of DZ7487 and fusion inhibitor

Compound	IC90 fold change*		
	6 hours	24 hours	48 hours
DZ7487	2.4	2.5	>70
GS-5806 (fusion protein inhibitor)	>80	>80	>80

*IC90 fold change was calculated as (IC90 of indicated time of addition post inoculation)/(IC90 of 1 hour ahead of inoculation).

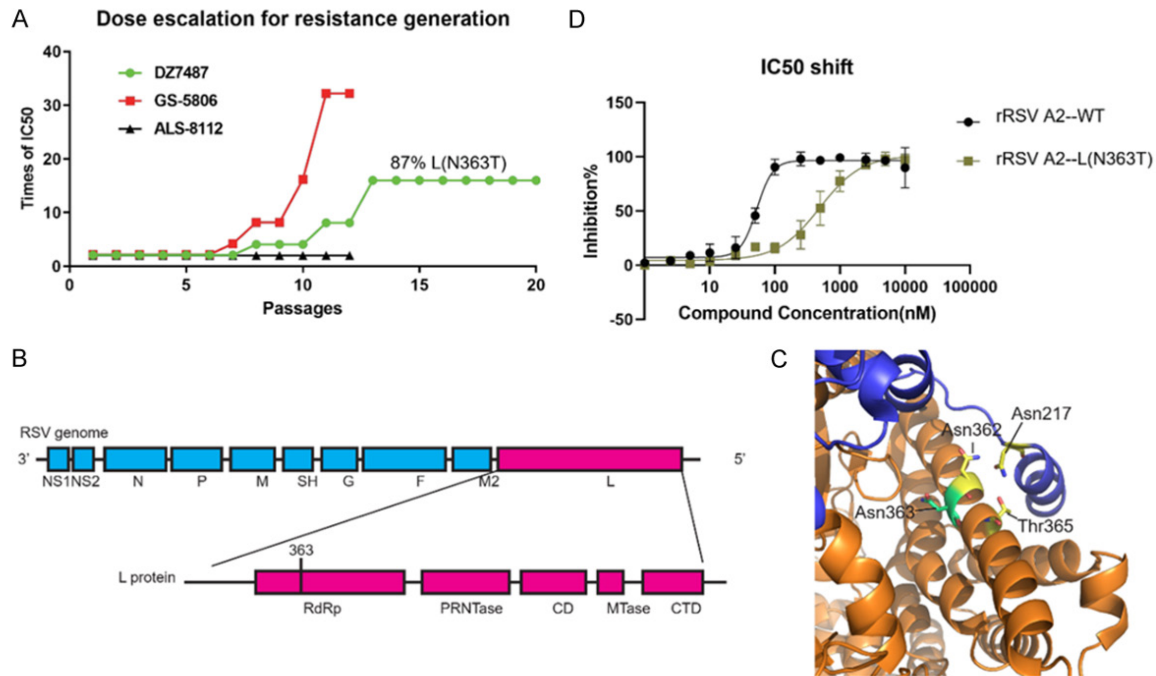

Figure 4. Susceptibility of DZ7487 resistant virus L (N363T) to RSV inhibitors. A. Resistance virus induction in HEp-2 cells against DZ7487, GS-5806 and ALS-8112. B. The linear map shows the position of mutation site Asn 363 in L protein. C. Structure of L protein and P protein interaction and Asn 363 position. (PDB: 6PZK). D. IC50 of DZ7487 was evaluated by inhibition of cytopathic effect in wild-type and N363T mutation recombinant virus strain, respectively. The experiment was done by triplicate.

Because airway symptoms are limited in BALB/c mice with RSV infection, we further established a cotton rat RSV infection model and obtained similar results. RSV infection

Table 5. IC50 and IC90 comparison between RSV A2 WT and RSV A2 LN363T mutation virus in HEp-2 cells

Compound	IC50 (nM)		IC50 fold change (L(N363T)/WT)	IC90 (nM)		IC90 fold change (L(N363T)/WT)
	RSV A2 WT	RSV A2 LN363T		RSV A2 WT	RSV A2 LN363T	
DZ7487	52	447	9	108	2178	20

Respiratory Syncytial Virus (RSV).

Table 6. IC50 fold change between wild type and mutant RSV A2 in HEp-2 cells

RSV mutants	IC50 fold change*			
	DZ7487	ALS-8112	PC786	GS-5806
WT	1	1	1	1
N363T	9	1	1	1
L138F	1	0	1	>9000
F140L/N517I	1	1	1	>9000
QUAD	1	5	1	1
Y1631H	0	1	95	1

*IC50 fold change was calculated as (IC50 of RSV mutants)/(IC50 of WT RSV A2). Respiratory Syncytial Virus (RSV).

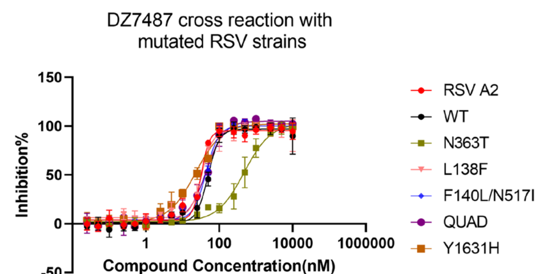

Figure 5. Effect of DZ7487 against RSV mutants resistant to reported anti-RSV compounds. Indicated mutant RSV were inoculated into HEp-2 cell 1 h post DZ7487 treatment (MOI = 0.5-1). Antiviral effects were evaluated through inhibition of RSV induced CPE. Serial diluted DZ7487 were dosed triplicated respectively and cell viability were reflected through CCK-8 assay at day 5 or day 6 post inoculation. Respiratory Syncytial Virus (RSV).

caused severe tracheal necrosis and lung tissue inflammation. DZ7487 treatment significantly decreased the lung RSV loads. No apparent tracheal necrosis and lung tissue inflammation were observed in treated animals (**Figure 7E**, **7F**), which suggests that DZ7487 could benefit RSV related ALRTI patients in the clinic.

Discussion

Among the small molecule anti-RSV drugs, stopping virus replication became the focus of therapeutic strategy due to its post entry antiviral activity. Although several promising reagents have been initiated in clinical trials, they have had many problems to resolve. In the replication process, the L protein is the most important component, containing the polymerase domain for virus replication. Several L protein inhibitors have been reported. Among them, PC786 showed good preclinical efficacy and antiviral effects against RSV in a healthy volunteer challenge study [40]. PC786 is a nebulized drug, that is minimally water soluble, highly plasma protein bound and has a low systemic concentration [40]. Clinical studies showed that serum RSV loads have a strong correlation with mechanical ventilation and death compared to bronchoalveolar lavage viral loads [41, 42], suggesting systemic exposure is needed for effective RSV treatment.

We demonstrated DZ7487 as an orally available potent RSV replication inhibitor with good pharmacokinetic properties. Due to its size and structural complexity, it is not yet possible to directly measure DZ7487 binding to L protein RdRp domain. The fact that N363T mutation located on the RdRp domain is by far the dominant resistance mutation suggests that DZ7487 direct interaction with this residue is critical for DZ7487 to disrupt RSV genome replication. This is in contrast with some previously claimed L protein inhibitors where resistant mutations are located outside of RdRp functional domain and capping domain [43, 44]. Given the importance of the RdRp domain for viral replication, it is one of the most conserved domains across different strains, especially the asparagine residue at the 363 position. We searched publicly available RSV sequences deposits and found no N363T mutation reported (NCBI blast with the N363T sequence), suggesting the critical importance of asparagine at this position. This may explain DZ7487's almost equal potencies against all clinical isolates from both A and B subtypes as they all have this conserved N at the 363 position. PC786, on the other hand, show 400-fold difference in

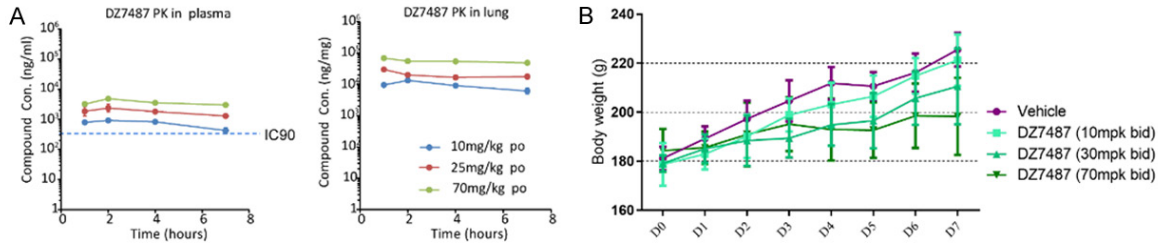

Figure 6. DZ7487 pharmacokinetics and tolerability in rat. A. Blood and lung pharmacokinetics of DZ7487 administrated by oral dose at 10, 25, 70 mg/kg respectively in healthy BALB/c mice. B. Body weight change of Wistar Han rats after 7 days of DZ7487 bid dose po.

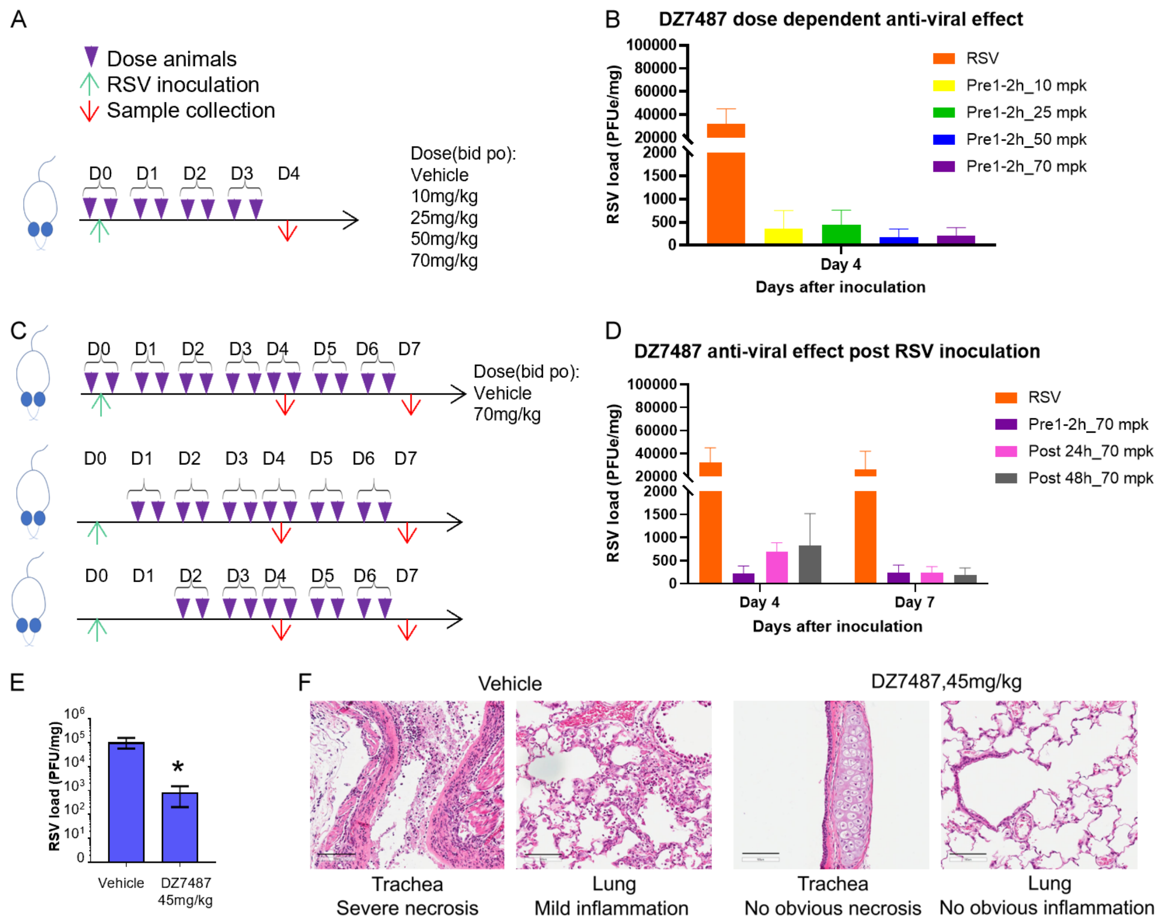

Figure 7. Effect of oral DZ7487 against RSV A2 in BALB/c mice and cotton rats. (A) Experimental design for DZ7487 efficacy evaluations in BALB/c mice. (B) Dose dependent effect of DZ7487 against RSV infection in BALB/c mice. (C) Experimental design for time-of-addition effect of DZ7487 in BALB/c mice. (D) Effect of DZ7487 treated pre- or post-RSV inoculation in BALB/c mice. (E) Lung tissue viral load (convert to PFU) in cotton rats infected with RSV A2 and treated with 45 mg/kg bid po DZ7487 for 3 days. (F) Pathological evaluation of RSV A2 infected cotton rats in (E). H&E staining at 20 × magnification. Scale bar 100 μm. Respiratory Syncytial Virus (RSV).

potency between A and B strains [44]. Since both A and B subtypes can circulate during the same season, a drug with broad antiviral activity against both is clearly advantageous [11].

Similar to previous reports, our data showed that fusion inhibitors can be useful if it is given before virus infection. Once RSV enters the cell, the fusion inhibitors are no longer effective. Although theoretically fusion inhibitors could still provide protection against new round of infection, the rapid virion production after infection make it a lot more challenging [45]. Our data showed that 6 hours after initial infec-

tion only about 70% of maximal inhibition could be achieved and no effect at all if drugs are given 24 hours after infection (**Figure 3A**). As a genome replication blocker, DZ7487 showed no significant difference in blocking viral load product both *in vitro* and *in vivo*, given before or after viral infection.

Clinically RSV infection symptoms usually show up around 2 days after exposure to the virus. By the time the symptoms are obvious, it is too late to treat the patients with a fusion inhibitor in [46]. In a 6-year retrospective study in elderly patients with a median age of 61 years, the median time between symptom onset and RSV molecular testing was 4.9 days for all patients and 7.8 days for patients who died [47]. Therefore, it is important for high-risk patients to obtain effective antiviral treatment after symptom onset and diagnosis.

DZ7487 has the desired profile to meet this unmet medical need. It is a potent, broad spectrum viral replication blocker, with good animal safety margin. It showed dose-dependent PK profile with nice drug-like properties. In addition to suppress viral load in lung after infection, it demonstrated significant pathological improvement as well. It is currently under pre-IND evaluation before human clinical studies.

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Disclosure of conflict of interest

DZ7487 is an investigational drug of Dizal Pharmaceuticals.

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