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YM-53403, a unique anti-respiratory syncytial virus agent with a novel mechanism of action

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Abstract

We performed a large-scale random screening of an in-house chemical library based on the inhibition of respiratory syncytial virus (RSV)-induced cytopathic effect on HeLa (human cervical carcinoma) cells, and found a novel and specific anti-RSV agent, 6-{4-[(biphenyl-2-ylcarbonyl) amino]benzoyl}-*N*-cyclopropyl-5,6-dihydro-4*H*-thieno[3,2-*d*][1]benzazepine-2-carboxamide (YM-53403). YM-53403 potently inhibited the replication of RSV strains belonging to both A and B subgroups, but not influenza A virus, measles virus, or herpes simplex virus type 1. A plaque reduction assay was used to determine the 50% effective concentration (EC_{50}) value for YM-53403. The value, 0.20 μM, was about 100-fold more potent than ribavirin. The result of a time-dependent drug addition test showed that YM-53403 inhibited the life cycle of RSV at around 8 h post-infection, suggesting an inhibitory effect on early transcription and/or replication of the RSV genome. Consistent with this result, two YM-53403-resistant viruses have a single point mutation (Y1631H) in the L protein which is a RNA polymerase for both the transcription and replication of the RSV genome. YM-53403 is an attractive compound for the treatment of RSV infection because of its highly potent anti-RSV activity and the new mode of action, which differs from that of currently reported antiviral agents.

Keywords: Respiratory syncytial virus; YM-53403; Ribavirin; L protein

1. Introduction

Human respiratory syncytial virus (RSV), a member of the family Paramyxoviridae, is the most prevalent infectious agent of acute lower respiratory illness from infants to elderly people (Agius et al., 1990; Fleming and Cross, 1993; Falsey et al., 1995; Thompson et al., 2003). RSV is especially serious in premature infants and children with congenital heart disease or bronchopulmonary dysplasia (Hall and McCarthy, 2000). It is also a major cause of significant morbidity and mortality in certain immunosuppressed populations, including elderly people (Couch et al., 1997), as in the case with influenza viruses. Recent studies have begun to emphasize the importance of RSV infection in the development of childhood asthma and the triggering of serious acute respiratory conditions in adult patients with chronic pulmonary disease (Sigurs et al., 1995; Glezen et al., 2000).

Viable options for the prevention or treatment of RSV infections are limited. Up to now vaccines have not been successful, and none are licensed for use at this time. Prophylactic administration of palivizumab (Synagis™, MedImme, Inc.), a monoclonal antibody binding the fusion (F) protein, has been shown to significantly decrease the incidence of RSV-related hospitalizations among high-risk children (The IMpact-RSV Study Group, 1998). However, it must be administered for the period of the entire RSV season, about 5 months. Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is the only approved chemotherapy against RSV (Hruska et al., 1980; Hall et al., 1983, 1985; Ohmit et al., 1996), but, due to its controversial efficacy (Wyde, 1998) and the requirement of special equipment for administration, its utilization is reserved for only severely ill patients, including those with chronic lung disease, congenital heart disease, and

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immunodeficiency, as well as premature births. Thus, there is a clear need for new anti-RSV therapeutics with improved efficacy and ease-of-use.

In recent years, various groups have reported several structurally distinct small molecules. These include the disulfonated stilbenes CL387626 and RFI-641 (Wyde et al., 1998; Aulabaugh et al., 2000; Huntley et al., 2002), the benzimidazole derivatives R-170591 (Andries et al., 2003) and BMS-433771 (Cianci et al., 2004), and the triphenol-based molecule VP-14637 (Douglas et al., 2003). The mechanism of action studies indicated that all of these compounds interact specifically with the viral F protein and inhibit the process of viral entry.

We performed large-scale random screening of in-house chemical library based on the inhibition of RSV induced cytopathic effect on HeLa (human cervical carcinoma) cells and obtained a novel anti-RSV agent, YM-53403 which is 100-fold more potent than ribavirin. The time-dependent addition test on YM-53403 and the DNA sequence analysis of YM-53403-resistant mutants suggested that this compound inhibits RSV replication with a new mode of action which is different from previous anti-RSV agents. YM-53403 will therefore be a potential candidate for new anti-RSV therapy.

2. Materials and methods

2.1. Cells

HeLa (human cervical carcinoma) cells, MDCK (Madin-Darby canine kidney) cells, Vero (embryonic African green monkey kidney) cells, and MRC-5 (human embryonic lung fibroblast) cells were obtained from Rational Drug Design Laboratories (Fukushima, Japan). All cells except for the HeLa cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL of penicillin G, and 100 μg/mL of streptomycin. HeLa cells were maintained in the culture medium above with the addition of 1.6% glucose. The cells were subcultured every 3 to 4 days.

2.2. Viruses

Four strains of RSV (strains Long, A2, FM58-8, and SM61-48), influenza virus (A/Ishikawa/7/82/H3N2), measles virus (strain Sugiyama), and herpes simplex virus type 1 (HSV-1, strain KOS) were obtained from Rational Drug Design Laboratories (Fukushima, Japan). All RSV strains were propagated in HeLa cells using Eagle's MEM supplemented with 2% FBS, 1.6% glucose, 100 units/mL of penicillin G, and 100 μg/mL of streptomycin (maintenance medium; MM). Viral stocks were stored at −80 °C until used. HSV-1 was propagated in MRC-5 cells, influenza A virus in MDCK cells, and measles virus in Vero cells in MM without 1.6% glucose.

2.3. Compounds

YM-53403 (6-{4-[(biphenyl-2-ylcarbonyl)amino]benzoyl}*N*-cyclopropyl-5,6-dihydro-4*H*-thieno[3,2-*d*][1]benzazepine-2-carboxamide) and its derivatives used in this study were synthesized at Yamanouchi Pharmaceutical Co., Ltd. (Ibaraki, Japan). Ribavirin (1-(β-D-ribonuranosyl)-1,2,4-triazole-3-carboxamide) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.4. Antiviral assay

To evaluate anti-RSV activity, the plaque reduction method was carried out according to the modified protocol of Hosoya et al. (1992). Briefly, HeLa cells were seeded into a 24-well tissue culture plate at 200,000 cells/well and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 2 days. When the cells formed a monolayer, they were infected with RSV suspension containing 150 PFU in 0.2 mL, and incubated for 1 h at 35 °C. The cells were then washed three times with MM, and serial 5-fold dilutions of compounds in MM containing 0.8% methylcellulose were added. These were incubated for 3 days at 35 °C in a 5% CO_2 incubator. Finally, monolayer cells were fixed in 10% formalin in phosphate-buffered saline (PBS) and then stained with 0.02% crystal violet. The number of RSV plaques was counted under a microscope. Fifty percent effective concentrations (EC_{50}), the concentration of each test sample that was able to reduce RSV plaque formation by 50%, were calculated by logistic analysis. The plaque reduction method was also performed to evaluate the antiviral activity against measles virus (Kurokawa et al., 1993). Anti-influenza and anti-herpes viral assays were performed using the MTT method that has been reported previously (Sudo et al., 1994; Watanabe et al., 1994). The cytotoxicity of test compounds was evaluated in cultures of proliferating HeLa cells using the MTT method (Watanabe et al., 1994). The selective index (SI) represents the ratio of the 50% cytotoxic concentration (CC_{50}) to EC_{50}.

2.5. Virus yield assay

The RSV growth inhibition activity of YM-53403 was evaluated using virus yield assay. HeLa cell suspensions (3×10^4 cells/well) were added to each well in a 96-well flat-bottomed microtiter plate. When the cells formed a monolayer, RSV (MOI of 0.1) was inoculated onto the plates, which were incubated for 1 h at 35 °C and then washed three times with MM. YM-53403 or ribavirin was then added to each well, and the plates were incubated at 35 °C in a humidified atmosphere of 5% CO_2. The supernatants of the culture cells were collected every day from 1 to 6 days after infection. The virus infectivity of each culture was determined by plaque assay and expressed as PFU.

2.6. Time-dependent drug addition test for the anti-RSV activity of YM-53403

HeLa cell suspensions (6×10^4 cells/well) were added to each well in a 48-well flat-bottomed microtiter plate. When the cells formed a monolayer, RSV (MOI of 3) was inoculated and the plates were incubated for 60 min at 35 °C and then washed three times. At 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 h post-infection, 50 μM of YM-53403 or 100 μM of ribavirin was added to different wells. The supernatants from the respective wells were collected 28 h after infection. The virus titers of the respective supernatants were determined by plaque assay.

2.7. Isolation and characterization of drug-resistant viruses

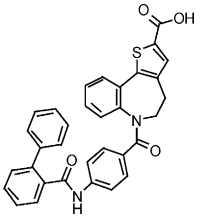
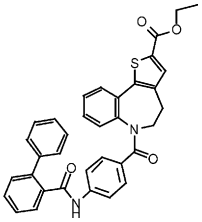
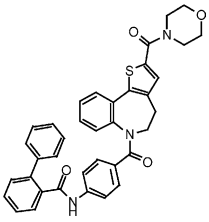
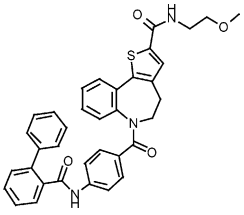
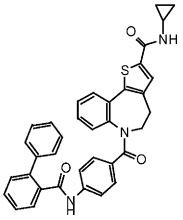
YM-53403-resistant viruses were isolated by serially culturing the RSV Long strain in HeLa cells 15 times in gradually increasing concentrations of the compound (up to 25 μM). The untreated control was cultured similary without YM-53403 (untreated WT), to rule out any genotypic drift. The susceptibility of the resultant viruses to YM-53403 was determined by the plaque reduction assay as described above. Sequence analysis of the full genome RNA of Long strain and the resistant strains were performed as outlined below. RNA was extracted from the supernatant of RSV-infected cells using ISOGEN (Nippon Gene Co., Ltd, Tokyo, Japan) and precipitated with isopropanol, according to the protocol provided by the manufacture. Reverse transcription (RT) of viral RNA to the specific cDNA of each gene was performed using a High Fidelity RNA PCR kit (Takara, Shiga, Japan) and proper primer sets. Amplified products were fractionated using 10% agarose gel electrophoresis, and then purified using a commercial kit (Microcon YM-100, Millipore, MA, USA). Purified gene products were directly sequenced on the ABI Prism 3700 genetic analyzer using an ABI BigDye® Terminator v3.1 Cycle Sequencing Kit (Applera Corporation, CT, USA). A single point mutation of the L protein was found using primers 5′-ATGTCTAAGGTATTTTTAGAACAA-3′ (nucleotides 13229–13252) and 5′-AATCCTTATATGTTTAGTTAATAG-3′ (nucleotides 7471–7490).

3. Results

3.1. Anti-RSV activity

To obtain a novel anti-RSV agent, we performed a random screening of an in-house chemical library based on the inhibition of RSV-induced cytopathic effect on HeLa cells. We found that compounds which have a 6-(4-aminobenzoyl)-5,6-dihydro-4*H*-thieno[3,2-*d*][1]benzazepine-2-carboxamide structure show antiviral activity against the RSV Long strain (Table 1). The most active compound, 6-{4-[(biphenyl-2-ylcarbonyl)amino]benzoyl}-*N*-cyclopropyl-5,6-dihydro-4*H*-thieno[3,2-*d*][1]benzazepine-2-carboxamide (YM-53403) dose-dependently inhibited RSV replication with an EC_{50} value of 0.2 μM by the plaque reduction method (Fig. 1). This was about 100-fold more potent than ribavirin (EC_{50} = 21.3 μM). Since YM-53403 and ribavirin exhibited cytotoxicity against proliferating HeLa cells with CC_{50}

Table 1
Anti-RSV activities and cytotoxicities of 6-{4-[(biphenyl-2-ylcarbonyl)amino]benzoyl}-5,6-dihydro-4*H*-thieno[3,2-*d*][1]benzazepine-2-carboxamide derivatives[a]

Compounds		EC_{50} (μM)[b]	CC_{50} (μM)[c]	*SI*
1		>25	N.T.[d]	–
2		>25	N.T.[d]	–
3		4.3 ± 1.1	>100	>23
4		0.98 ± 0.14	>100	>102
YM-53403		0.20 ± 0.05	82.3 ± 5.9	412
Ribavirin	–	21 ± 7	570 ± 30	27

[a] Data represent means ± standard deviations for three independent experiments.
[b] EC_{50} values were determined using the plaque reduction method described in Section 2.
[c] CC_{50} values were determined using the MTT assay.
[d] N.T., not tested.

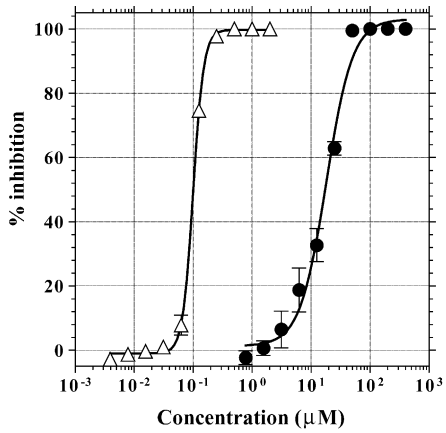


Fig. 1. Dose-response curve of the anti-RSV activity of YM-53403. Anti-RSV activities were measured by the plaque reduction method using RSV Long strain and HeLa cells. Virus input was 150 PFU per well. YM-53403 (△); ribavirin (●).

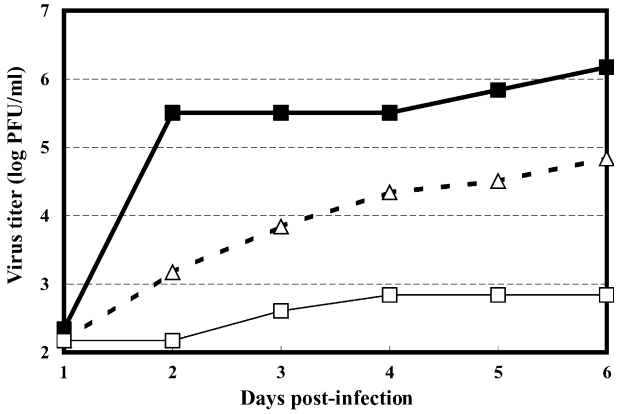


Fig. 2. Effect of YM-53403 on extracellular RSV yield during multiple rounds of replication. Control (untreated) (■); 2.5 μM YM-53403 (□); 50 μM Ribavirin (△). HeLa cells were infected with RSV (MOI = 0.1). The virus titer of each culture was determined by the plaque assay.

values of 82.3 μM and 570 μM, respectively, YM-53403 had a superior selective index (SI = CC_{50}/EC_{50}), 412, compared to ribavirin (SI = 27).

The potency of the derivatives vary depending on the substituents at the 2-position on the thiophene ring (Table 1). The compounds with carboxylic acid or an ethoxycarbonyl group at the 2-position on the thiophene ring (compounds 1 or 2) exhibited no anti-RSV activity. The substituents containing carboxamide are crucial for antiviral activity against RSV. Although the morpholine carboxamide derivative (compound 3) was somewhat less active, *N*-(2-methoxyethyl) carboxamide moiety (compound 4) led to the increased anti-RSV activity with an EC_{50} value of 0.98 μM. Since CC_{50} values of both compounds 3 and 4 were more than 100 μM, compound 4 had a superior SI (>102), which is equal to YM-53403. In addition, cytotoxicity assays using HeLa monolayer cells were also performed, and YM-53403, compounds 3 and 4 did not exhibit cycotoxicity at 100 μM (data not shown).

3.2. Virus yield assay

In the virus yield assay, HeLa cells were infected with RSV at a MOI of 0.1, and the inhibitory effects of YM-53403 and ribavirin on extracellular virus yield in multiple rounds of replication were determined after different incubation times (1, 2, 3, 4, 5, and 6 days post-infection). A large amount of infectious virus production (3.2×10^5 PFU/mL) was seen in untreated supernatants from day 2 post-infection. YM-53403 and ribavirin both demonstrated rapid and significant antiviral activities from day 2 to day 6. Over 5 days, 2.5 μM YM-53403 showed 2.7 $\log_{10}$PFU to 3.3 $\log_{10}$PFU decrease in RSV titer, while 50 μM ribavirin resulted in 1.2 $\log_{10}$PFU to 2.3 $\log_{10}$PFU decrease (Fig. 2).

3.3. Antiviral spectrum

The antiviral activity of YM-53403 against several RSV strains, including subgroups A and B, were evaluated using the plaque reduction assay. As shown in Table 2, YM-53403 inhibited the replication of all tested RSV strains at EC_{50} values ranging from 0.20 to 0.38 μM. The antiviral activities of YM-53403 against tested RSV strains were 76–105 times more potent than those of ribavirin. On the other hand, YM-53403 did not inhibit the replication of measles virus, influenza A virus, or HSV-1. From these results, the antiviral activity of YM-53403 seem to be specific to RSV.

3.4. Time-dependent drug addition test for anti-RSV activity of YM-53403

To investigate the mechanism for the anti-RSV activity of YM-53403, we performed a time-dependent drug addition test. At different time points, 50 μM YM-53403 and 100 μM ribavirin were added to HeLa cells infected with a high RSV MOI (MOI = 3). Development of syncytia reached more than 90% at 28 h after virus exposure in untreated control wells. At this time point, supernatant fluids were collected, and the extracellular virus yields were determined by plaque-titration. As shown in Fig. 3, ribavirin, which is known to inhibit RNA synthesis during the virus replica-

Table 2
Specificity of antiviral activities of YM-53403[a]

Virus	Strain (type)	EC50 (μM)		Ratio
		YM-53403	Ribavirin	
RSV	Long (A)	0.20 ± 0.05	21 ± 7	105
RSV	A2 (A)	0.28 ± 0.06	23 ± 7	82
RSV	FM58-8 (A)[b]	0.38 ± 0.07	29 ± 3	76
RSV	SM61-48 (B)[b]	0.29 ± 0.01	27 ± 7	93
Influenza	A/Ishikawa/7/82 (H3N2)	>100	45 ± 0	–
Measles	Sugiyama	>100	87 ± 4	–
HSV-1	KOS	>100	>400	–

[a] Data represent means ± standard deviations for three independent experiments.
[b] Clinical isolates of RSV.

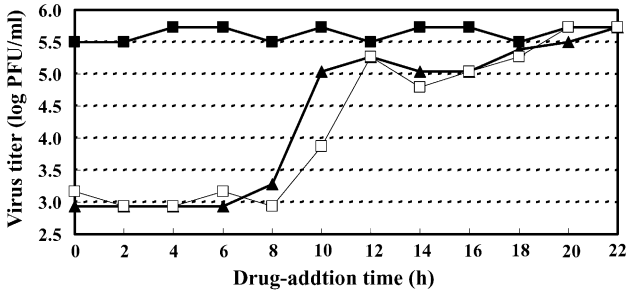


Fig. 3. Time-dependent drug addition test of YM-53403. HeLa cells were infected with RSV (MOI = 3). YM-53403 (50 μM) and ribavirin (100 μM) were added at the indicated times, and after 28 h incubation, the virus titer of culture medium was determined by the plaque assay. (■) virus control, (□) YM-53403, (▲) ribavirin.

tion cycle (Gilbert and Knight, 1986), had inhibited RSV growth until 8 h post-infection. YM-53403 had also inhibited RSV replication until 8 h post-infection, suggesting that YM-53403 is active at the same stage of the viral life cycle as ribavirin.

3.5. Isolation and characterization of YM-53403-resistant RSV viruses

In order to verify the specific target of YM-53403, we isolated and characterized YM-53403-resistant variants. The EC_{50} values of YM-53403 to the mutant viruses were >25 μM (Table 3), that is more than 125 times weaker than the EC_{50} value to the wild-type Long strain (EC_{50} = 0.20 μM). Conversely, the antiviral effects of ribavirin against the mutant strains were similar to that of the wild type. Next the cDNA of a full viral RNA genome was sequenced and the amino acids of all proteins were compared between untreated WT and resistant strains (YM-53403-R 01 and 02). Although YM-53403-R 01 and 02 were derived from two independent selection experiments, they contained the same single point mutation (Y1631H) in the L protein. Other gene segments of YM-53403-resistant viruses, NS1, NS2, N, P, M, SH, G, F, and M2, had no mutations at both the cDNA and amino acid levels (data not shown). These results suggest that YM-53403-R 01 and 02 acquired their drug-resistance based on the mutation within the L protein.

Table 3
Characterization of RSV variants resistant to YM-53403

Virus	EC50 (μM)[a]		L genotype
	YM-53403	Ribavirin	
RSV Long	0.20 ± 0.05	21.3 ± 6.5	Wild type
Untreated WT[b]	0.33 ± 0.07	32.7 ± 6.0	Wild type
YM-53403-R01	>25	32.3 ± 4.9	Y1631H
YM-53403-R02	>25	24.4 ± 0.2	Y1631H

[a] EC50 values were determined in the plaque reduction method described in Section 2. Data represent means ± standard deviations for three independent experiments.

[b] Viruses cultured in the absence of test compound.

4. Discussion

The nucleoside analogue ribavirin is the only clinically-available drug for anti-RSV therapy. Although ribavirin inhibits IMP dehydrogenase in host cells, the mechanism of its antiviral activity against RSV is not clear (Gilbert and Knight, 1986). Moreover, it is used only as an inhalant for the treatment of RSV infections. The in vitro anti-RSV activity of ribavirin is not strong and its clinical efficacy is controversial (Wyde, 1998). A random screening of an in-house compound library resulted in the discovery of a novel class of RSV inhibitor, YM-53403, which exhibited prominent anti-RSV activity against both subgroups A and B. YM-53403 EC_{50} values against all evaluated RSV strains were 76–105-fold more potent than those of ribavirin. In addition, the selective index of YM-53403 (SI = 412) was superior to that of ribavirin (SI = 27). It has been reported that ribavirin inhibits both DNA and RNA viruses (Markland et al., 2000), and in our study, ribavirin inhibited the replication of some RNA viruses, influenza A virus, measles virus, and RSV with EC_{50} values of 45, 87, and 21 μM, respectively, but not the DNA virus, HSV-1. In contrast, YM-53403 did not inhibit the replication of any viruses except for RSV. These results indicate that YM-53403 is a potent and specific anti-RSV agent whose mode of action is different from that of ribavirin.

Although there has been a sustained effort to find new and better agents that can effectively prevent or control RSV infections, all of the new anti-RSV compounds have a similar mechanism-of-action that inhibit the earliest steps of viral replication (i.e. virus attachment or penetration). For example, NMSO3 inhibits the interaction of RSV G protein with its receptor (Kimura et al., 2000, 2004). VP-14637 (Douglas et al., 2003), R-170591 (Andries et al., 2003), BMS-433771 (Cianci et al., 2004), and RFI-641 (Razinkov et al., 2001; Huntley et al., 2002) which is a successor of CL387626 (Wyde et al., 1998) interfere at the virus entry stage, inhibiting the function of RSV F protein, which in turn play a role in lipid membrane fusion. The time-dependent drug addition test suggested that the mode of action for YM-53403 is different from those of RSV entry inhibitors, and that it will interfere with the viral primary transcription and/or replication steps of virus genome RNA.

The sequence analysis of YM-53403-resistant viruses revealed that their L gene contained one sequence alteration, Y1631H, which suggests that YM-53403 interacts with the L protein. The RSV L protein is an essential component of the viral RNA polymerase, along with the phosphoprotein (P) and nucleocapsid (N) proteins (Grosfeld et al., 1995; Yu et al., 1995). Interaction of the RSV L protein with the N and P proteins promotes the formation of a transcriptase complex that is essential for viral RNA transcription and replication (Garcia-Barreno et al., 1996). In addition to the N, P, and L proteins, several viral proteins are required for RSV RNA synthesis. The antitermination function of M2-1 is essential for processive RNA synthesis and suppression of transcription termination in the intergenic regions (Collins

et al., 1995; Hardy and Wertz, 2000). M2-2 has been postulated to have a role in regulating the switch between the viral RNA transcription and replication processes (Bermingham and Collins, 1999). We also sequenced the P, N, and M2 gene segments of the YM-53403-resistant virus, but the other gene segments showed no sequence changes (data not shown). There are some reports that amino acid changes in the RSV L protein affect the viral RNA transcription and/or replication steps. Juhasz et al. (1999) reported that the live-attenuated RSV vaccine candidate *cpt*530/1009 (Phe-521 to Leu and Met-1169 to Val, respectively, according to the amino acid sequence of the L protein) was partially restricted for RNA replication, mRNA synthesis, and virus growth. Grosfeld et al. (1995), using the minireplicon system, also showed that RNA synthesis was completely ablated by the substitution of Asn for Asp at position 989 in the RSV L protein. YM-53403 is the first small molecule reported that can efficiently inhibit RSV replication, presumably by interfering with the RSV polymerase activity of the L protein. YM-53403 is an attractive and novel anti-RSV agent because of its potent anti-RSV activity and good selectivity index as well as the new mechanism of action, which differs from that of currently reported antiviral agents.

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