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Two novel fusion inhibitors of human respiratory syncytial virus

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ABSTRACT

To search for novel drugs against human respiratory syncytial virus (RSV), we have screened a diversity collection of 16,671 compounds for anti-RSV activity in cultures of HEp-2 cells. Two of the hit compounds, i.e., the N-(2-hydroxyethyl)-4-methoxy-N-methyl-3-(6-methyl[1,2,4]triazolo[3,4-a]phthalazin-3-yl)benzenesulfonamide (designated as P13) and the 1,4-bis(3-methyl-4-pyridinyl)-1,4-diazepane (designated as C15), reduced the virus infectivity with IC_{50} values of 0.11 and 0.13 μM respectively. The concentration of P13 and C15 that reduced the viability of HEp-2 cells by 50% was 310 and 75 μM respectively. Both P13 and C15 exhibited no direct virucidal activity or inhibitory effects on the virus attachment to cells. However, to inhibit formation of RSV-induced syncytial plaques P13 and C15 had to be present during the virus entry into the cells and the cell-to-cell transmission of the virus. The RSV multiplication in HEp-2 cells in the presence of P13 or C15 resulted in rapid selection of viral variants that were ~1000 times less sensitive to these drugs than original virus. Sequencing of resistant viruses revealed presence of amino acid substitutions in the F protein of RSV, i.e., the D489G for C15-selected, and the T400I and N197T (some clones) for the P13-selected virus variants. In conclusion, we have identified two novel fusion inhibitors of RSV, and the detailed understanding of their mode of antiviral activity including selection for the drug resistant viral variants may help to develop selective and efficient anti-RSV drugs.

1. Introduction

Human respiratory syncytial virus (RSV) is a highly contagious pathogen that targets superficial cells of respiratory epithelium frequently causing acute bronchiolitis and pneumonia in individuals with immature, inefficient, and/or impaired innate or adaptive immunity. These mainly include infants, elderly, and immunocompromised individuals of any age. Epidemiological data indicate that the prematurely born infants with underlying bronchopulmonary dysplasia, and infants suffering from congenital heart disease or neuromuscular impairment are at the greatest risk of developing severe RSV disease (reviewed in Welliver, 2003; Resch et al., 2009; Welliver et al., 2010). Among possible causes of increased disease severity in these patients are insufficient transfer of the RSV-neutralizing maternal antibodies and unusually narrow airways due to their incomplete development, inflammation or hyperreactivity, i.e., alterations that could facilitate the development of obstructive airway disease (Welliver, 2003). Although vaccines against a number of acute viral infections were developed, no vaccine is currently available for prevention of RSV-induced disease. The development of RSV vaccine, although troublesome (Murphy et al., 1986) is not unlikely since specific preparations of anti-RSV antibodies are prophylactically applied in groups of high-risk infants where they were reported to reduce incidence or severity of RSV disease (Groothuis et al., 1993; Johnson et al., 1997). Apart from aerosol formulation of ribavirin, no drug is currently approved for treatment of RSV infection. Ribavirin is known to affect infection of cultured cells by a number of different RNA viruses (reviewed in De Clercq, 2008), and in the case of RSV this activity was due to its anti-inosine monophosphate dehydrogenase activity that results in depletion of cellular pools of guanosine monophosphate (Leyssen et al., 2005). However, the effectiveness of ribavirin in treatment of RSV disease was reported to be suboptimal or uncertain (Collins et al., 2001).

RSV initiates infection of cultured cells by interaction between the G protein of the viral envelope and the cell surface glycosaminoglycan chains (Krusat and Streckert, 1997) especially these comprising iduronic acid such as heparan sulfate and chondroitin sulfate B (Hallak et al., 2000). Although it is uncertain whether glycosaminoglycans promote RSV infection in humans (Zhang et al., 2005; Monzon et al., 2006), sulfated polysaccharide mimetics of glycosaminoglycans are potent inhibitors of RSV infectivity (Vaheri, 1964; Witvrouw and De Clercq, 1997; Kimura et al., 2000). RSV invasion of cells can be efficiently blocked by targeting essential biological functions of the F protein of RSV envelope. During the biosynthesis of viral components, the precursor of F

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protein is cleaved at the furin cleavage sites yielding F1 and F2 subunits. The former, as a specialized fusion device, is composed of an amino-terminal fusion peptide followed by the heptad-repeat 1 (HR1), an intervening domain, and the HR2 located at the region of F1 proximal to the transmembrane domain (Lamb and Jardetzky, 2007). Insertion of the fusion peptide into cellular lipids followed by interaction of HR1 and HR2 to form a six-helix bundle complex is a driving force behind the RSV entry into the cells by fusion of the viral and cellular membranes (Reviewed in Harrison, 2008). A specific monoclonal anti-F antibody which neutralizes RSV infectivity is currently applied in prophylaxis of high-risk infants against this virus (Johnson et al., 1997). Furthermore, several low molecular mass compounds that interfere with the interaction between HR1 and HR2 repeats of the F protein and thereby with the RSV entry into the cells have been identified (Razinkov et al., 2001; Andries et al., 2003; Douglas et al., 2003; Ohki et al., 2003; Cianci et al., 2004; Luttick et al., 2007; Bonfanti et al., 2008). RSV comprises a negative strand genome and initiation of its transcription and replication requires presence in the virus particle of a polymerase complex including large polymerase, phosphoprotein, and M2-1 protein in addition to the nucleocapsid protein which encapsidates viral RNA to form a template for polymerase recognition and to protect it against degradation. The transcribed viral mRNAs are capped, methylated and polyadenylated indicating that the viral polymerase proteins possess methyl- and guanylyl-transferase activities, i.e., functional features thought to be promising targets for the development of antiviral drugs (Leyssen et al., 2008). Several candidate RSV drugs were found to target polymerase complex proteins. These include the quinazoline-diones (Mason et al., 2004) or the benzazepine thiophens (Sudo et al., 2005) for large viral polymerase, and the benzodiazepine derivatives for nucleocapsid protein (Carter et al., 2006; Chapman et al., 2007). Since the spread of RSV infection in human airway epithelium is believed to occur exclusively via the virus particles released from the apical membrane of ciliated cells (Zhang et al., 2002), any compound that prevent completion of budding or prevent virus egress from cells would limit progression of RSV disease.

In this work we screened a collection of over 16,000 diverse compounds for anti-RSV activity. Two novel anti-RSV compounds were identified and appeared to target the fusion events mediated by the viral F protein.

2. Materials and methods

2.1. Cells and viruses

Human laryngeal epidermoid carcinoma (HEp-2) and baby hamster kidney (BHK-21) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% foetal calf serum (FCS), 60 μg/ml of penicillin, 100 μg/ml of streptomycin, and 8% tryptose–phosphate broth (for BHK-21). Unless otherwise stated, the cells were seeded the day prior to the experiments to be used at ~70% confluence. A laboratory strain A2 of RSV (Lewis et al., 1961) was purchased from ATCC (VR-1540). This preparation of A2 strain was reported to be free from adenovirus contamination (Cameron et al., 2003). The virus stock was prepared in HEp-2 cells (Hallak et al., 2000), and stored at −80 °C in the presence of 25% sucrose (Gupta et al., 1996).

2.2. Screening assay

The ChemBioNet library of 16,671 diverse compounds was obtained from the Leibniz Institute for Molecular Pharmacology (FMP, Berlin, Germany). The compounds were supplied at a concentration of 10 mM in 5 μl of DMSO in 384-well plate format. Control wells contained corresponding volume of the DMSO solvent. Prior to experiments, the compounds were diluted in 45 μl of de-ionized water and the plates were covered with the DMSO resistant aluminium tape (CLS-6569; Costar-Corning, NY, USA) and stored frozen at −20 °C.

Screening of the ChemBioNet library of compounds for anti-RSV activity was performed in the cluster 384-well cell culture plates (CLS-3701; Costar-Corning, NY, USA). The HEp-2 cells were seeded in these wells the day prior to the experiment to be used at ~70% confluence. The growth medium was removed from cells and 22 μl of DMEM supplemented with 2% heat-inactivated FCS and antibiotics (DMEM-FA) was added. Subsequently, the plates with library compounds were thawed for 20 min at 37 °C, centrifuged at 125 × *g* for 1 min, and 3 μl of each compound was transferred to HEp-2 cells using the 16-channel pipette (Finnpipette, Thermo Electron, Vantaa, Finland). After incubation for 10 min in the CO_2 incubator, ~350 plaque forming units (PFU) of RSV A2 strain in 25 μl of DMEM-FA was added. After shaking, the plates were centrifuged at 45 × *g* for 1 min, and left in the CO_2 incubator for 3–4 days. The cells were observed under the microscope for the protection against the virus induced cytopathic effect (CPE).

Larger quantities of hits C15, P13 and P13 analogs were purchased from the ChemDiv, Inc. (San Diego, CA, USA). ^{1}H NMR spectra (DMSO-d6) of C15 and P13 provided by the suppliers showed the correct structures for both compounds and purities of >95%. To further verify the identity and purity of the compounds after storage, both C15 and P13 were subjected to LCMS analysis using a Perkin Elmer Sciex API 150EX instrument, equipped with a Genesis C8 reversed phase column from Grace. LCMS (TSP) of C15 showed a peak at *m*/*z* 283.6, corresponding to M+1 in positive mode, indicating the correct structure. The LC trace (elution time 1.86 min) indicated a purity of >95%, and no substantial decomposition of the material over time. Likewise, LCMS (TSP) of P13 showed a peak at *m*/*z* 428.3, corresponding to M+1 in positive mode, also indicating the correct structure. The LC trace (elution time 4.05 min) indicated a purity of >95% for P13.

2.3. Virus radiolabeling and purification

Subconfluent monolayers of HEp-2 cells were infected with RSV A2 strain and incubated for 20 h at 37 °C. Then, the culture fluid was replaced with the methionine and cystine-free DMEM supplemented with 10% normal DMEM, 2% HI-FCS, antibiotics, and 50 μCi/ml of the expre^{35}S protein labelling mix (Perkin Elmer, Upplands Vasby, Sweden). After incubation for 24 h at 37 °C, the cells were scraped into the medium and centrifuged at 1000 × *g* for 10 min. Most of the supernatant fluid was removed and the cell pellet resuspended in the remaining medium. The cell suspension was then vortexed to release the virus (Hallak et al., 2000) and centrifuged at 4000 × *g* for 5 min. The virus-containing supernatant fluid was loaded on the top of the discontinuous gradient of 30, 40 and 50% sucrose and centrifuged at 25,000 rpm for 2 h at 4 °C using the SW 28.1 rotor. Finally, the fractions were collected from the bottom of the tube and their radioactivity and infectivity determined.

2.4. Viral plaque assays

HEp-2 cells growing in cluster 12-well plates were washed and 0.4 ml of DMEM-FA containing different test compounds at a final concentration range of 0.16–100 μM was added. Subsequently ~100 PFU of RSV A2 strain in 100 μl of DMEM-FA was added and the cells were incubated for 2–3 h at 37 °C in a humidified 5% CO_2 atmosphere. The medium was then collected and 1.5 ml of 1% methylcellulose solution in DMEM-FA supplemented with respective concentrations of test compounds was added. After 3 days of

incubation the cells were either stained with 0.25% crystal violet in water solution of 16% ethanol, 1% formaldehyde, and 1% acetic acid or immunostained using monoclonal antibody 131/G2 (Lifespan Biosciences, Seattle, WA, USA) specific for the G protein of RSV A2 strain.

2.5. Time-of-addition assay

The test compounds (20 μM) were incubated with HEp-2 cells for 2 h at 37 °C at different time points relative to the 2 h period of cell infection with ~100 PFU of RSV A2 strain. Cells in control wells received DMSO solvent at volumes corresponding to these present in the test compound. The cells were washed once following each period of incubation with the test compound or the virus, and then overlaid with 1% methylcellulose solution. In some experiments the methylcellulose overlay contained test compounds at 20 μM concentration. After 3 days of incubation at 37 °C, the cells were stained with crystal violet and the RSV plaques counted.

2.6. Virus inactivation assay

The test compounds (20 μM) and ~10^5 PFU of RSV A2 strain were mixed in a final volume of 500 μl of DMEM-FA and incubated for 15 min at 37 °C water bath. Following dilution of the mixture to the non-inhibitory concentration of a test compound, the residual viral infectivity was tested as described in Section 2.4.

2.7. Cytotoxicity assay

HEp-2 seeded in cluster 96-well plates were washed with DMEM-FA and 100 μl of the same medium containing test compounds at a concentration range 0.16–500 μM were added in duplicate. Specific concentrations of the test compound solvents (DMSO or water) were added to controls. The cells were incubated with test compound for 72 h at 37 °C in a humidified atmosphere containing 5% CO_2, and 20 μl of the CellTiter 96(R) Aqueous One Solution reagent (Promega, Madison, USA) was added to each well. After incubation of cells for 1–2 h at 37 °C, the absorbance at 490 nm against a background of 650 nm was recorded.

2.8. Preparation and analysis of the drug resistant RSV variants

Plaque purified variant of RSV A2 (~3×10^5 of input PFU) and the test compound at 10 μM concentration were co-incubated with HEp-2 cells for 4 days. The virus was harvested as described in Section 2.1, and then subjected to further nine consecutive passages in cells in the presence of 10 μM of respective compound. The viral variants that resisted the selective pressure from test compound were plaque purified from the limiting virus dilutions and then subjected to analysis of their nucleotide sequence as follows. RNA was extracted from RSV infected Hep-2 cells according to the manufacturer's protocol (QIAmp Viral RNA Purification Kit, Qiagen). The corresponding cDNA was prepared by RT-PCR using the First strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) and a forward primer matching the positions 87–105 of the nucleotide sequence of A2 strain of RSV (Collins, 1991; Connors et al., 1995). Amplification of the DNA fragments overlapping the viral genome was performed with forward primers matching the positions 87–105; 1432–1451; 2926–2947; 4149–4167; 7705–7726; 9620–9638; 11,446–11,464; and 13,138–13,157, and reverse primers 1576–1557, 3044–3025, 4586–4567, 5881–5862, 9761–9741, 11,860–11,842, 13,633–13,615, 15,118–15,101. The positions of sense primers for sequencing of the F gene were 5376–5396, 5787–5805, 6316–6335, 6760–6777 and 7287–7304, and of antisense primers were 5886–5868, 6366–6347, 6876–6858, 7335–7317 and 7842–7821.

2.9. Viral fusion assay

The assay was performed as described by Douglas et al. (2003). Briefly, BHK-21 cells in 96-well plates were infected with RSV A2 strain at a moi of 1 and incubated for 24 h at 37 °C. These cells were then transfected with a pT7EMCLuc plasmid (0.09 μg/well) expressing the firefly luciferase under control of the T7 promoter (Aoki et al., 1998) and incubated for 4 h at 37 °C. Another population of BHK-21 cells growing in 6-well plates was transfected with plasmid pCAGT7 (2 μg/well) that expresses T7 RNA polymerase (Okuma et al., 1999) and incubated for 18 h at 37 °C. Both plasmids were kindly provided by Prof. Y. Matsuura (Department of Molecular Virology, Osaka University, Japan). The cells transfected with pT7EMCLuc plasmid were washed and the test compounds at a final concentration range of 0.16–100 μM were added and incubated for 15 min at 37 °C. Meanwhile, the cells expressing T7 polymerase were dispersed with a trypsin-EDTA solution and added to cells transfected with pT7EMCLuc plasmid at a 1:1 ratio and incubated for 6 h at 37 °C. Finally, the cells were lysed using the passive buffer and the luciferase activity analysed with the luciferase assay system (Promega, Madison, WI, USA). Luminosity was measured for a period of 0.1 s in a LB940 Mithras (Berthold Technologies, Bad Wildbad, Germany) and expressed in relative light units.

3. Results

3.1. Identification of anti-RSV hit compounds

The ChemBioNet diversity collection of 16,671 compounds, which was designed based on analysis of the World Drug Index for privileged substructures or scaffolds (Leibniz Institute for Molecular Pharmacology, FMP, Berlin, Germany), was screened at 60 μM for anti-RSV activity in HEp-2 cells growing in 384-well plate format. Based on microscopic observation, 221 compounds that at least partly protected cells from the RSV-induced syncytia were re-examined at concentrations 0.1, 1, and 10 μM in the 24-well plates. Thirteen hit compounds that at a concentration of 10 μM completely inhibited formation of viral syncytia while causing no visible cytotoxicity (cell lysis, altered cell shape) were selected for further studies. Two of these hit compounds, designated P13 and C15, were examined in more detail. The specificity of the screening procedure was supported by the observation that this assay frequently identified anti-RSV activity in several structurally similar compounds, i.e., in case of P13 two extra analogs and in case of C15 one additional analog were detected as anti-RSV hit compounds. Structurally, hit P13 is the N-(2-hydroxyethyl)-4-methoxy-N-methyl-3-(6-methyl[1,2,4]triazolo[3,4-a]phthalazin-3-yl)benzenesulfonamide (Fig. 1A) while hit C15 is the 1,4-bis(3-methyl-4-pyridinyl)-1,4-diazepane (Fig. 1B). The concentrations of P13 and C15 that reduced the number of RSV plaques in HEp-2 cells by 50% (IC_{50}) were 0.11 and 0.13 μM respectively (Fig. 1). The concentrations of P13 and C15 that reduced the viability of HEp-2 by 50% (CC_{50}) were 310 and 75 μM respectively (Fig. 1). Note that some cytotoxicity of P13 observed at 500 μM might be due to DMSO solvent (Fig. 1A). Hence, the selective index (CC_{50}/IC_{50}) values were 2818 for P13 and 577 for C15. Note that even at the relatively high concentrations both compounds did not completely block the development of RSV plaques (Fig. 1). These escape plaques were of smaller size and of non-syncytial phenotype as compared to plaques formed in the absence of inhibitor. The specificity of these plaques was confirmed by immunostaining with monoclonal antibody against the G protein of RSV A2 strain (data not shown).

To elucidate significance of particular structural features of P13, we assayed antiviral potency of 15 commercially avail-

Table 1
Anti-RSV activity of analogs of the test compound P13.

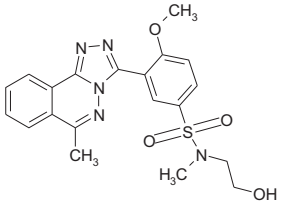
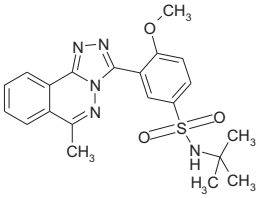
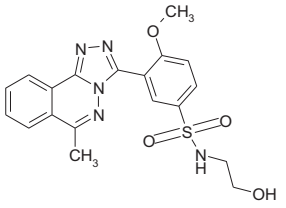
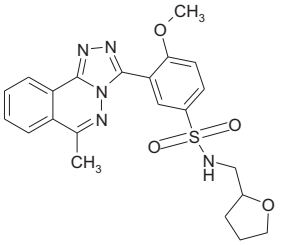
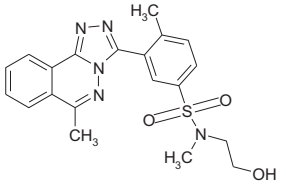
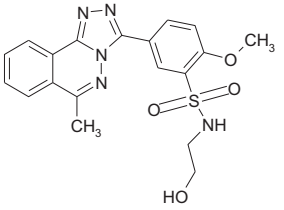
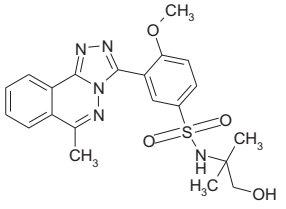
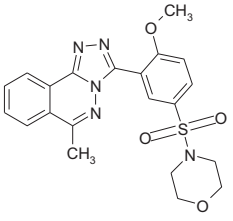
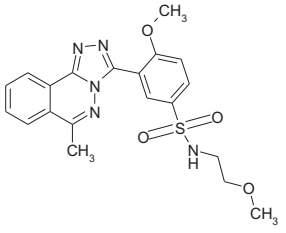
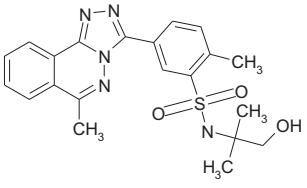
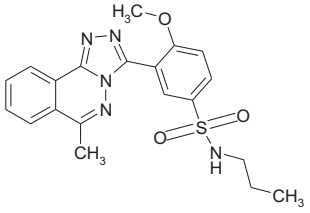
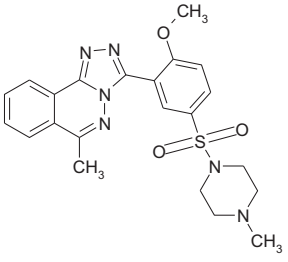
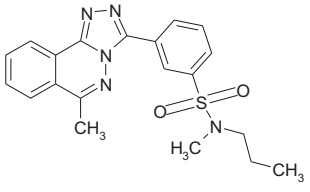
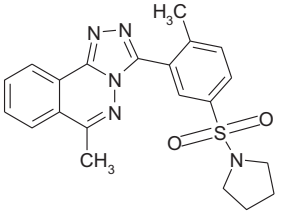
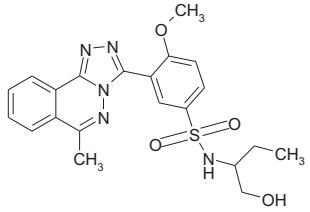
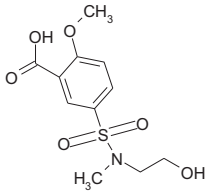
Analog	Structure	P13/analog (IC_{50} ratio)	Analog	Structure	P13/analog (IC_{50} ratio)
P13		1	8		0.38
1		0.5	9		2.61
2		0.17	10		>0.006
3		2.4	11		0.8
4		2.4	12		>0.006
5		0.13	13		1.5
6		>0.006	14		>0.006

Table 1 (*Continued*)

Analog	Structure	P13/analog (IC_{50} ratio)	Analog	Structure	P13/analog (IC_{50} ratio)
7		0.71	15		>0.006

able analogs of this compound (Table 1). Replacement of the methyl-triazolophthalazin with carboxyl group (analog **15**) ablated anti-RSV activity indicating that the former structure is essential for antiviral activity. Likewise, attachment of methoxyl group to benzenesulfonamide is required for anti-RSV activity as removal of this group (**6**), replacement with methyl group (**2**) or changing its attachment site (**10**) has reduced or abolished antiviral potency. However, removal of N-methyl and simultaneous substitution of hydroxyl-dimethylethyl (**3**), methoxyethyl (**4**), tetrahydrofuranylmethyl (**9**) but not tert-butyl group (**8**) for N-hydroxyethyl group (**P13**) potentiated antiviral activity by ~2.5 fold. Given the fact that P13 did not completely inhibit development of RSV plaques even at a relatively high concentration, an extent of inhibition of viral plaque formation by all analogs at a concentration of 20 μM was tested. Analog **13** exhibited near-complete reduction of the RSV plaque-forming activity at this concentration (data not shown).

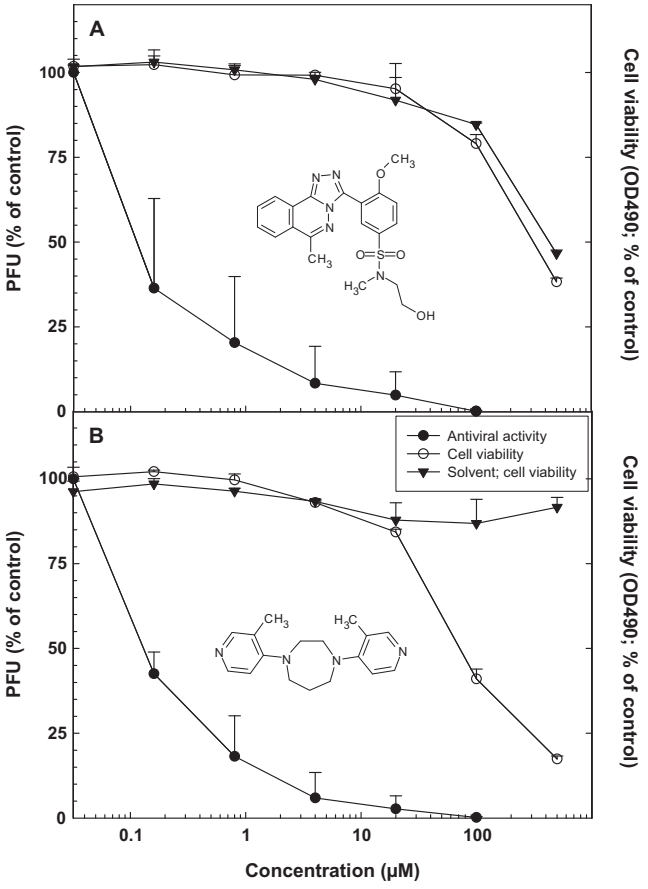


Fig. 1. Anti-RSV activity and cytotoxicity of compounds P13 and C15. In infectivity assay, P13 (A) or C15 (B) were added to HEp-2 cells at a concentration range of 0.16–100 μM, followed by the addition of ~100 plaque-forming units (PFU) of RSV A2 strain and incubation with cells for 2–3 h at 37 °C in a humidified 5% CO_2 atmosphere. The medium was then replaced with the methylcellulose solution supplemented with identical concentrations of test compounds and the cells incubated until the development of viral plaques. Two separate experiments were performed in duplicate and the results are presented as a mean percentage of a number of viral plaques developed in the presence of test compound relative to the number of viral plaques developed in the presence of specific concentrations of the P13 or C15 solvents. In the cytotoxicity assay, HEp-2 cells were incubated for 72 h at 37 °C in a humidified 5% CO_2 atmosphere with specific concentrations of P13 (A) or C15 (B). Subsequently, the MTS tetrazolium reagent was added and the dye absorbance measured at 490 nm. The results are expressed as a percentage of absorbance value detected with the test compounds relative to that developed in cells treated with P13 or C15 solvents. Each data point is a mean ± SD of duplicate determinations from two separate experiments.

3.2. Mode of anti-RSV activity of P13 and C15

To investigate whether P13 or C15 exhibit a direct virucidal activity, these compounds were incubated with RSV particles and then the mixtures tested for residual virus infectivity at the non-inhibitory compound concentrations. The infectious titres of RSV incubated with P13 or C15 or in the absence of these compounds were similar (Table 2) indicating that P13 and C15 did not posses a direct virus-inactivating activity.

To investigate whether anti-RSV activities of P13 and C15 were due to inhibition of virus adsorption to cells, purified ^{35}S-methionine/cysteine labelled RSV particles were incubated with respective test compound (20 μM) during the 90 min period of virus attachment to HEp-2 cells at 4 °C. In comparison with the RSV binding in the absence of inhibitor there were 87.18% and 102.02% of viral cpm attached in the presence of P13 and C15 respectively (mean of triplicate determinations; data not shown). These results indicate that P13 and C15 exhibited little or no inhibitory effect on the attachment of RSV particles to cells. This suggests that these compounds did not interfere with the step of RSV binding to the cell surface glycosaminoglycan receptors.

To identify a step of the infectious RSV cell cycle affected by P13 and C15, these compounds were added to cells at different time points relative to the virus infection. When the incubation of P13 with cells was limited to a 2 h period occurring prior to the virus addition to cells, no inhibitory effect was observed (Fig. 2A). Under the same experimental conditions, C15 reduced the virus infectivity by ~50%. This observation together with the lack of C15 effect on virus attachment to cells suggest that this compound may to some extent bind to cellular components other than glycosaminoglycans and interfere with the post-attachment step(s) of RSV infection of cells. P13 and C15 also reduced RSV infectivity when added 2 h

Table 2
Lack of virucidal activity associated with the test compounds P13 or C15.

Compound	Residual RSV infectivity (PFU/ml)	
	Experiment 1	Experiment 2
P13	1.32×10^5	3.16×10^5
C15	1.3×10^5	3.27×10^5
Control (no compound)	1.36×10^5	3.36×10^5

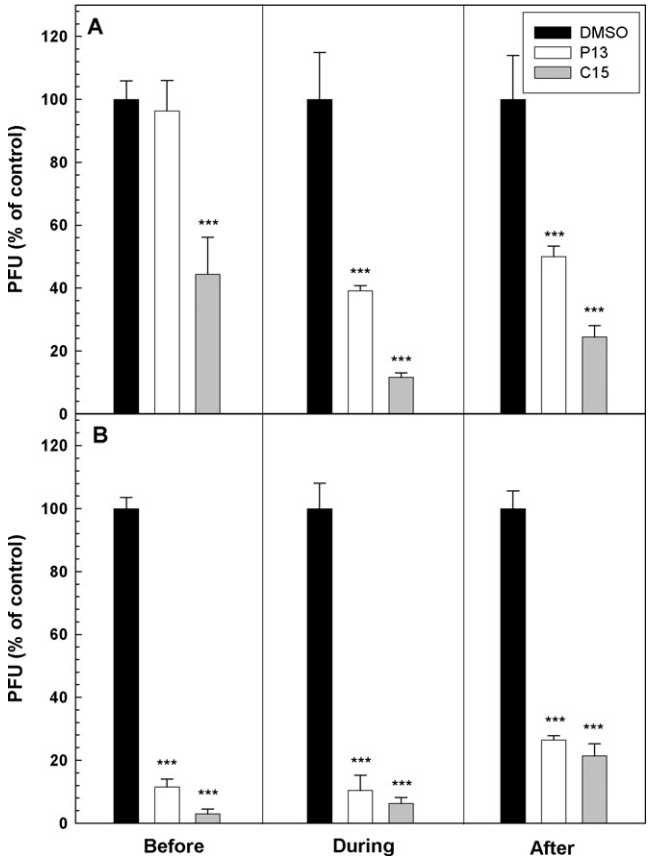


Fig. 2. Anti-RSV activity of P13 and C15 added at different times relative to virus infection of cells. The test compounds (20 μM) were incubated with HEp-2 cells for 2 h at 37 °C which occurred either before, during, or after a 2 h period of infection of cells with ~100 plaque forming units (PFU) of RSV A2. The cells were washed once following each period of incubation with the test compound or the virus, and then overlaid with 1% methylcellulose solution containing no test compounds (A) or supplemented with P13 or C15 at 20 μM (B). Two separate experiments were performed in duplicate and the results are presented as a mean percentage of a number of viral plaques developed in the presence of test compound relative to the mock-treated controls. Statistically significant differences as related to DMSO controls at P values of <0.005 (***).

after infection of cells in agreement with previous report (Roymans et al., 2010) that this class of compounds exhibits inhibitory effects when added up to 3 h post-infection. There was greater inhibition of RSV infectivity when P13 or C15 were present during the 2 h period of virus infection than before this period ($P < 0.005$ for P13 and $P < 0.01$ for C15; t-test). Likewise, there was somewhat better inhibition of RSV when P13 or C15 were present during rather than after infection of cells although these differences were not statistically significant. These results together with the inability of P13 and C15 to block the virus attachment to cells suggest that these compounds target, at least in part, the virus entry into the cells. Addition of the test compounds to cells at either −2 h, 0 h or +2 h relative to the infection with RSV, followed by the continuous presence of compounds until the development of viral plaques, gave the greater reduction of RSV infectivity when the compounds were added either prior to ($P < 0.005$ for P13; $P < 0.01$ for C15) or at the beginning of virus infection of cells ($P < 0.005$ for P13; $P < 0.05$ for C15) than after infection of cells (Fig. 2B). In contrast, addition of test compounds 16 h after the virus infection of cells, i.e., at the beginning of formation RSV-induced syncytial plaques, did not substantially reduced the number of viral plaques, however these plaques exhibited smaller size and a non-syncytial phenotype (data not shown).

Table 3
Mutations in the F protein of the RSV variants resistant to fusion inhibitors.

Amino acid change	F1 subunit region	Inhibitor	Reference
F140I	Fusion peptide	BMS-433771	Cianci et al. (2004)
V144A	Fusion peptide	BMS-433771	Cianci et al. (2004)
N197T	Heptad repeat 1	P13	This report
D392G	Cysteine-rich domain	BMS-433771	Cianci et al. (2004)
K394R	Cysteine-rich domain	BMS-433771	Cianci et al. (2004)
		JNJ-2408068	Roymans et al. (2010)
S398L	Cysteine-rich domain	JNJ-2408068	Andries et al. (2003)
T400I	Cysteine-rich domain	P13	This report
T400A	Cysteine-rich domain	VP-14637	Douglas et al. (2003)
D486N	Heptad repeat 2	JNJ-2408068	Andries et al. (2003)
F488Y	Heptad repeat 2	VP-14637	Douglas et al. (2003)
D489Y	Heptad repeat 2	BMS-433771	Cianci et al. (2004)
D489G	Heptad repeat 2	C15	This report

To more precisely define the site of anti-RSV activity of P13 and C15, the virus was subjected to 10 consecutive passages in HEp-2 cells in the presence of these compounds (10 μM). The first syncytial plaques that developed in the presence of test compounds were already seen in passage 2 and their number rapidly increased in subsequent passages. To verify the extent resistance of the drug-passaged virus both this virus as well as an original input virus were tested for their sensitivity to P13 or C15 in the dose-dependent viral plaque assay as described in Section 2.4. The virus variants that escaped selective pressure from test compounds appeared to be >1000 and 1000 times less sensitive to P13 and C15, respectively. Several viral plaques that developed at the limiting dilutions of the resistant viruses were collected. Except for the first 90 nucleotides comprising the non-coding fragment of the NS1 gene, an entire genome of resistant and original viruses was sequenced. Nucleotide sequence analysis of four plaque variants resistant to P13 revealed presence of the c6859t nucleotide change in the genome fragment coding for the F protein. This mutation was predicted to results in the T400I amino acid substitution in the cysteine-rich region of F1 subunit (Table 3). Furthermore, two of these plaque purified variants contained an additional nucleotide substitution (a6250c)

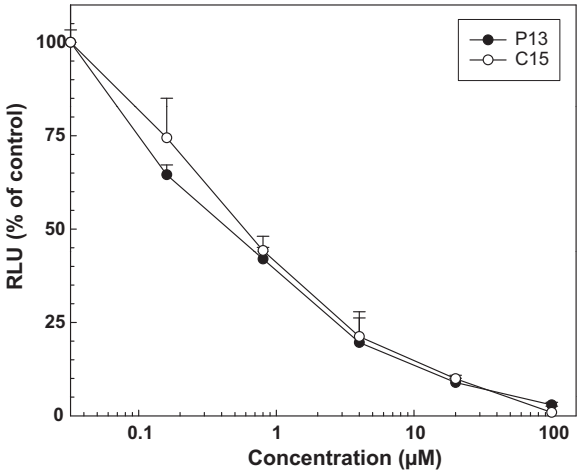


Fig. 3. Compounds P13 and C15 target the RSV induced cell-fusing activity. BHK-21 cells that were infected with RSV and transfected with pT7EMCLuc plasmid were mixed with cells expressing T7 RNA polymerase and co-incubated in the presence of specific concentrations of test compounds. The luciferase activity, induced by the RSV mediated fusion of cells, is expressed as a percentage of a number of relative light units (RLU) detected in the presence of test compound relative to the mock-treated controls. The mean number of RLU in the mock treated control cells were 52,925 and 82,070 in assays with P13 and C15 respectively. The mean number of background RLU in co-cultivated non-infected cells that were transfected with respective plasmids was 1340. Two separate experiments were carried out in duplicate for each compound.

resulting in the N197T amino acid change in the HR1 repeat of the F1 subunit. Moreover, viral variants resistant to P13 also exhibited the a4133c nucleotide substitution located in the segment of the M gene that do not code for M protein. Although unlikely, this segment of the M gene could be alternatively translated, however, an identity and significance of this putative novel RSV protein is not known (Satake and Venkatesan, 1984). All three plaque variants resistant to C15 carried a single nucleotide mutation (a7126g) resulting in the D489G amino acid substitution in the HR2 repeat of the F1 protein (Table 3). These results indicate that both P13 and C15 are inhibitors of the RSV-induced fusion.

To further substantiate this finding, the effect of P13 and C13 on the RSV-induced cell-to-cell fusion was tested using a luciferase reporter activation assay. To this end, the RSV infected BHK-21 cells were transfected with plasmid pT7EMCLuc expressing luciferase under control of the T7 promoter and then mixed with target cells transfected with plasmid PCAGT7 to express T7 RNA polymerase. The RSV-induced cell-to-cell fusion, measured by quantification of the induced luciferase activity (Fig. 3), was inhibited by P13 and C15 with IC_{50} values of 0.45 and 0.58 μM respectively. Note that P13 or C15 affected the RSV cell-fusing (Fig. 3) and the RSV plaque-forming (Fig. 1) activities with similar IC_{50} values that differed by ~4–5-fold. This indicates that both these compounds are inhibitors of the RSV induced fusion.

4. Discussion

We have identified two novel inhibitors of RSV infectivity in cultured cells, i.e., the benzenesulfonamide-based P13 and the diazepane-based C15 compound. These inhibitors appeared to target the viral F protein and interfere with both the virus entry into the cells and the virus-induced fusion of cells. To date, several different inhibitors of RSV fusion have been identified. These include the dendrimer-like disulfonated stilbene RFI-641 (Razinkov et al., 2001), the substituted benzimidazole JNJ-2408068 (Andries et al., 2003) and its morpholinopropylaminobenzimidazole derivative TMC353121 (Bonfanti et al., 2008), the triphenol-based molecule VP-14637 (Douglas et al., 2003), the diacetyltartaric acid esters of mono and diglycerides DATEM (Ohki et al., 2003), the benzotriazole derivative BMS-433771 (Cianci et al., 2004), and the imidazoisoindole derivative BTA9881 (Luttick et al., 2007). Although structurally different, it is likely that these compounds exhibit similar mode of antiviral activity (Douglas et al., 2005; Roymans et al., 2010). The fusion inhibitors of RSV including our P13 and C15 did not block the virus attachment to cells (Douglas et al., 2003; Cianci et al., 2004; this report), an activity mediated by the viral G protein. Instead, the results of the time-of-addition experiments invariably demonstrate the greatest activity of these inhibitors at the stage of RSV entry into the cells and the cell-to-cell spread activity of the virus (Andries et al., 2003; Douglas et al., 2003; Cianci et al., 2004; this report). These results are in line with observation that this group of compounds efficiently inhibit the RSV induced cell–cell fusing activity (Douglas et al., 2003; this report). Furthermore, analysis of RSV variants resistant to the different fusion inhibitors revealed that of all of them carried resistance mutations in the F1 subunit of F protein that promotes RSV entry into the cells by fusion of viral and cellular membranes. The F1 subunit is composed of an amino-terminal fusion peptide followed by HR1, an intervening globular domain, and the HR2 repeat located near the transmembrane domain. Mutations conferring resistance to fusion inhibitors occurred most frequently in the HR2 domain (Andries et al., 2003; Douglas et al., 2003; Cianci et al., 2004; this report) but also in the cysteine-rich region of an intervening domain (Andries et al., 2003; Douglas et al., 2003; Cianci et al., 2004; this report), and fusion peptide (Cianci et al., 2004). Some RSV variants generated with our fusion inhibitor P13 also contained the D197T resistance mutation in the HR1 repeat. The F protein is present in the virus particle in the pre-fusion conformation which was not targeted by the fusion inhibitors as these compounds did not cause a direct inactivation of infectivity of RSV particles (Andries et al., 2003; this report). However following binding of RSV virions to susceptible host cells, the trimeric F protein adopts an extended conformation, a core of which comprises a coiled-coil structure of three HR1 domains with the outermost exposed fusion peptide to interact with lipids of cell plasma membrane (reviewed in Harrison, 2008). It was postulated that the fusion inhibitors target the three identical shallow pockets made by adjacent HR1 domains of this intermediate (Douglas et al., 2005). Following insertion of fusion peptide into cellular lipids, this intermediate collapses to form a six-helix bundle complex of trimeric HR1 and HR2 repeats, the aim being to appose viral and cellular lipid membranes, then merge them and produce a fusion pore for the passage of viral nuleocapsid into cytoplasm. Hence, inhibitors of RSV fusion may either impair the binding of HR1 to HR2 repeats or stabilise the HR1/HR2 complex in a distorted conformation (Douglas et al., 2005; Roymans et al., 2010), an event that could discontinue the RSV entry into the cell by fusion of viral and cellular lipids.

Peptides derived from HR repeats can inhibit viral fusion via mechanism partly similar to that of fusion inhibitors. Attachment of cholesterol to these peptides dramatically potentiated their anti-paramyxovirus activity very likely due to the high affinity of these modified peptides for lipid rafts of cell plasma membrane (Porotto et al., 2010), i.e. the preferred sites of the RSV-cell fusion events. It is not known whether the low molecular weight fusion inhibitors would benefit of such modification.

In conclusion, we report on identification of two novel fusion inhibitors of RSV. We believe that the detailed understanding of their mode of anti-RSV activity including selection for the drug resistant virus variants may help to develop an effective anti-RSV compound.

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