Small Molecules VP-14637 and JNJ-2408068 Inhibit Respiratory Syncytial Virus Fusion by Similar Mechanisms

Janet L. Douglas, Marites L. Panis, Edmund Ho, Kuei-Ying Lin, Steve H. Krawczyk, Deborah M. Grant, Ruby Cai, Swami Swaminathan, Xiaowu Chen, and Tomas Cihlar*

Gilead, 333 Lakeside Dr., Foster City, CA 94404

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Here we present data on the mechanism of action of VP-14637 and JNJ-2408068 (formerly R-170591), two small-molecule inhibitors of respiratory syncytial virus (RSV). Both inhibitors exhibited potent antiviral activity with 50% effective concentrations (EC_{50}s) of 1.4 and 2.1 nM, respectively. A similar inhibitory effect was observed in a RSV-mediated cell fusion assay (EC_{50} = 5.4 and 0.9 nM, respectively). Several drug-resistant RSV variants were selected in vitro in the presence of each compound. All selected viruses exhibited significant cross-resistance to both inhibitors and contained various single amino acid substitutions in two distinct regions of the viral F protein, the heptad repeat 2 (HR2; mutations D486N, E487D, and F488Y), and the intervening domain between HR1 and HR2 (mutation K399I and T400A). Studies using [3H]VP-14637 revealed a specific binding of the compound to RSV-infected cells that was efficiently inhibited by JNJ-2408068 (50% inhibitory concentration = 2.9 nM) but not by the HR2-derived peptide T-118. Further analysis using a transient T7 vaccinia expression system indicated that RSV F protein is sufficient for this interaction. F proteins containing either the VP-14637 or JNJ-2408068 resistance mutations exhibited greatly reduced binding of [3H]VP-14637. Molecular modeling analysis suggests that both molecules may bind into a small hydrophobic cavity in the inner core of F protein, interacting simultaneously with both the HR1 and HR2 domains. Altogether, these data indicate that VP-14637 and JNJ-2408068 interfere with RSV fusion through a mechanism involving a similar interaction with the F protein.

Respiratory syncytial virus (RSV) is a major cause of severe respiratory tract infections in pediatric, elderly, and immuno-compromised patients (7, 16, 19). Despite extensive research to develop a RSV vaccine, currently no vaccine has been approved. Prophylactic antibodies have been developed that effectively reduce the incidence and severity of RSV disease in the high-risk pediatric population (8, 9). However, the only antiviral treatment available for patients with RSV disease is ribavirin, a nucleoside analog with a suboptimal clinical efficacy (3). Several drug-resistant RSV strains have been identified as a result of either the high-risk pediatric population (8, 9). However, the only antiviral treatment available for patients with RSV disease is ribavirin, a nucleoside analog with a suboptimal clinical efficacy and safety profile (18). Recently, several promising small-molecule inhibitors with in vitro and in vivo anti-RSV activity have been identified. These include the disulfonated stilbenes CL387626 and RFI-641 (15, 22), the benzimidazole derivative JNJ-2408068 (formerly R-170591) (1, 21), the benzotriazole derivative BMS-433771 (4, 23), and the triphenol compound CL387626 and RFI-641 (15, 22), the benzimidazole derivative JNJ-2408068 (formerly R-170591) (1, 21), the benzotriazole derivative BMS-433771 (4, 23), and the triphenol compound VP-14637 (13) (D. C. Pevear et al., Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1854, 2000). Initial studies indicated that these inhibitors act early in the RSV replication cycle and mutations conferring resistance to these structurally diverse molecules map to various regions of the viral fusion (F) protein (1, 4, 14, 15).

The RSV F protein that mediates the fusion of viral envelope with host cell membrane consists of two disulfide-linked subunits, F1 and F2. The F1 subunit contains a hydrophobic fusion peptide at its N terminus, followed by two heptad repeats (HR1 and HR2) separated by almost 300 amino acids of intervening region (5). It is believed that a conformational change of the F protein homo-trimer leads to the formation of a stable HR1/HR2 six-helix bundle, which triggers the actual fusion of viral and cell membranes (11, 12, 24). Studying inhibitors of this process will not only increase our understanding of the fusion mechanism but may help to design more effective anti-RSV treatments.

We previously described the interaction of VP-14637 with the RSV F protein (6). In the present study, we focused on the potential functional similarities between VP-14637 and the structurally unrelated inhibitor JNJ-2408068 (Fig. 1).

MATERIALS AND METHODS

Cells and viruses. Hep-2 and BHK-21 cell lines were cultured in minimal essential medium plus 2 mM l-glutamine, 0.1 mM nonessential amino acids, and 10% fetal bovine serum. The BHK-21 cells were also supplemented with 10% tryptose phosphate broth. Primary chicken embryonic fibroblasts (CEF) were cultured in Dulbecco's minimal essential medium with 4.5 g/liter glucose, 4 mM glutamine, and 10% fetal bovine serum at 39°C. All cell lines were obtained from the American Type Culture Collection (Manassas, VA). The RSV strain A2 (American Type Culture Collection) and the attenuated vaccinia virus expressing T7 polymerase (MVA-T7), kindly provided by Bernard Moss (National Institutes of Health, Bethesda, MD), were grown and titered as previously described (6).

Plasmids. To generate pcDNA-F construct, the F gene from RSV A2 was obtained by reverse transcription-PCR amplification of RNA isolated from RSV-infected Hep-2 cells and cloned into pcDNA 3.1 expression vector (Invitrogen, Carlsbad, CA). To construct the F protein mutants, site-directed mutagenesis (QuickChange protocol from Stratagene) was performed on a fragment of F gene (nucleotides 916 to 1725), which had been subcloned into pBluescript II SK+ (Stratagene, La Jolla, CA). To verify the mutations, the inserts were sequenced using an ABI Prism BigDye terminator kit on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA). The fragments containing mu-
tations were cloned back into the pCDNA-F plasmid. Plasmids for the T7 expression system were described previously (6).

Compounds. VP-14637 {5,5'-bi-1-((4-amino-1H-tetrazolyl)limino)methyl]-2,4'-methylenebis[3H]VP-14637 (specific activity, 4 Ci/mmol) was prepared by Moravek Biochemicals (Brea, CA). JNJ-2408068 {2-[[2-[[1-(2-aminoethyl)-4-piperidinyl]amino]-4-methyl-1H-benzimidazol-1-yl]-6-methyl-3-pyridinol]} (Fig. 1) was synthesized in nine steps from the commercially available 2-nitro-3-methyl benzoic acid as described previously (Janssens et al., patent no. WO 01/00612, and WO 01/00615). Ribavirin was obtained from Sigma. The HR2-derived peptide T-118 (10) was synthesized by Genemed Synthesis (South San Francisco, CA).

Antiviral activity. Hep-2 cells were seeded into 96-well plates at 1,000 cells per well. After 24 h, the cells were infected with RSV at a multiplicity of infection (MOI) of 0.1 and incubated at 37°C for 2 h. The inoculum was replaced with fresh media containing serial dilutions of the tested inhibitors. Following a 4-day incubation, the media was removed and the cells were stained with crystal violet (0.1% in 20% methanol). The RSV-induced cytopathic effect was determined spectrophotometrically by reading absorbance at 630 nm, and the concentration of inhibitor that reduced the cytopathic effect by 50% relative to untreated control (EC50) was calculated by nonlinear regression using Prism software (GraphPad, San Diego, CA).

Viral fusion assay. The fusion assay was described previously (6). Briefly, BHK-21 cells were infected with RSV for 24 h at an MOI of 0.5 and then transfected with a T7-driven luciferase plasmid (T7-Luc) for 5 to 7 h. During the infection, another population of BHK-21 cells was transfected with a plasmid expressing T7 polymerase. After transfection, the virus-infected cells were washed and fresh medium containing the tested inhibitor was added to the cells. The second population of cells transfected with the T7 polymerase plasmid was trypsinized and overlaid onto the virus-infected, T7-Luc-transfected cells in a 1:1 ratio. After 6 to 7 h, the mixed population of cells were lysed and analyzed for luciferase activity using the luciferase assay system (Promega, Madison, WI). Luciferase activity was measured as relative light units in a TopCount NXT (Packard Bioscience).

Selection and characterization of drug-resistant viruses. Viruses resistant to VP-14637 or JNJ-2408068 were selected by passaging RSV in Hep-2 cells in the presence of increasing concentrations of either inhibitor. The starting drug concentration for VP-14637 was 5 nM. For JNJ-2408068, two concentration ranges were used for selection. The low range was 10 to 100 nM and the high range was 25 to 2,000 nM. Control viruses were grown in parallel to the same passage without drug. Susceptibility of the resultant viruses to VP-14637 and JNJ-2408068 were included as controls. Both VP-14637 and JNJ-2408068 were approximately 1,000- and 10,000-fold more potent than T-118 and ribavirin, respectively, at inhibiting RSV replication in the antiviral assay. Similarly for the fusion assay, both inhibitors had EC50 values in the low nanomolar range compared to 3.5 µM for T-118. These data confirm that the antiviral effects of both VP-14637 and JNJ-2408068 are mediated by their ability to inhibit the RSV fusion process.

To further characterize the interactions of these compounds, a VP-14637 binding assay with RSV-infected cells was per-
TABLE 1. Effect of RSV inhibitors on virus replication, virus-induced cell fusion, and [3H]VP-14637 binding to virus-infected cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>RSV replication EC50 (nM)</th>
<th>RSV fusion EC50 (nM)</th>
<th>[3H]VP-14637 binding a</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP-14637</td>
<td>1.4 ± 0.7 d</td>
<td>5.4 ± 2.7 d</td>
<td>9.3 ± 2.9</td>
</tr>
<tr>
<td>JNJ-2408068</td>
<td>2.1 ± 1.1</td>
<td>0.9 ± 0.6</td>
<td>2.9 ± 1.8</td>
</tr>
<tr>
<td>T-118</td>
<td>1,500 ± 560d</td>
<td>3,500 ± 1,900d</td>
<td>&gt;20,000</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>33,000 ± 11,500</td>
<td>NA b</td>
<td>NA</td>
</tr>
</tbody>
</table>

a Inhibition of RSV replication was determined in Hep-2 cells infected with RSV A2. Cells were incubated in the presence of various inhibitor concentrations for 4 days, and the cytopathic effect was quantified spectrophotometrically following the staining of cells with crystal violet. The results represent the mean ± standard deviations from two to six independent experiments.

b The RSV-induced cell fusion was determined in virus-infected BHK-21 cells (MOI = 0.6) overlaid with uninfected BHK-21 cells for 6 h. The fusion process was quantified based on the luciferase reporter enzyme activity.

c Binding was determined in RSV-infected Hep-2 cells. Forty-eight hours after the infection, cells were incubated with 10 nM [3H]VP-14637 for 90 min in the absence or presence of various concentrations of tested compounds and the cell-bound radioactivity was determined.

d Data from reference 6.

TABLE 2. Characterization of RSV variants selected in the presence of VP-14637 or JNJ-2408068

<table>
<thead>
<tr>
<th>Virus a</th>
<th>Concentration range (nM)</th>
<th>Fold resistance b</th>
<th>F protein genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP-14637 (A)</td>
<td>5–50</td>
<td>&gt;1,000</td>
<td>1,000</td>
</tr>
<tr>
<td>VP-14637 (B)</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
<td>T400A</td>
</tr>
<tr>
<td>JNJ-2408068 (A)</td>
<td>10–100</td>
<td>4.5</td>
<td>158</td>
</tr>
<tr>
<td>JNJ-2408068 (B)</td>
<td>29</td>
<td>40</td>
<td>E487D</td>
</tr>
<tr>
<td>JNJ-2408068 (C)</td>
<td>25–2,000</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>JNJ-2408068 (D)</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
<td>D486N</td>
</tr>
<tr>
<td>Untreated (A)</td>
<td>d</td>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Untreated (B)</td>
<td>0.75</td>
<td>0.7</td>
<td>WT c</td>
</tr>
</tbody>
</table>

a Viruses selected in the presence of the indicated inhibitor. A, B, C, and D represent independent resistance selections.

b Compound concentration range used during the resistance selection process.

c Fold resistance was determined as the ratio of compound EC50 value against the parent RSV A2 virus relative to its EC50 value against each selected virus.

d Viruses passed in parallel in the absence of an inhibitor.

WT, wild type.

formed. Cells were incubated with [3H]VP-14637 in the presence of various concentrations of unlabeled VP-14637, JNJ-2408068, or T-118. Both VP-14637 and JNJ-2408068 interfered with the binding of [3H]VP-14637 to infected cells at concentrations similar to those inhibiting RSV fusion and replication, suggesting a similar binding mode for the two small molecules (Table 1). In contrast, 20 μM T-118 did not affect [3H]VP-14637 binding to infected cells. T-118 is thought to inhibit RSV fusion by binding to the HR1 domain, thus preventing the heptad repeats from interacting and forming a stable fusion-competent six-helix complex (10).

Drug-resistant RSV variants were selected by culturing the virus for six to eight passages in the presence of increasing concentrations of each inhibitor to further assess their mode of action. Drug susceptibilities of the resultant viruses were determined by the cytopathic antiviral assay, and viral F genes were amplified and sequenced. Two viruses that were independently selected in the presence of VP-14637 showed >1,000-fold reduced susceptibility to both VP-14637 and JNJ-2408068 (Table 2). Similarly, both low and high concentrations of JNJ-2408068 selected for viruses with diminished susceptibility to VP-14637 and JNJ-2408068. Hence, all selected viruses were cross resistant to both inhibitors. Genotypic analysis of the resistant viruses revealed various mutations located either in HR2 or the intervening domain of the F protein (Table 2 and Fig. 2). Although VP-14637 and JNJ-2408068 did not select for the exact same resistance mutations, it is interesting that the mutations in each region were within one to two amino acids of each other. There were no apparent defects in F protein expression or transport to the cell surface for any of the resistant viruses as verified by Western blot and FACS analysis (data not shown).

To determine whether a decreased inhibitor binding to mutant F protein could account for the resistance, the VP-14637 binding assay was performed in CEF cells expressing drug-resistant mutant F proteins by using the MVA-T7 system (6, 20). The binding of [3H]VP-14637 to all F proteins containing the drug resistance mutations, including those selected by JNJ-2408068, was considerably reduced (Fig. 3A). Only the wild-type F protein exhibited efficient binding of VP-14637. FACS analysis confirmed that all of the F proteins were expressed at equivalent levels on the cell surface (data not shown). In addition to the mutations selected in our studies, S398L, a previously reported resistance mutation for JNJ-2408068 (1), also showed reduced binding of [3H]VP-14637 (Fig. 3B). Thus, the reduced activity of both inhibitors against the selected RSV mutants is likely due to their inability to interact with the mutant forms of the F protein. To further investigate the binding mode of VP-14637, several negatively charged amino acids in the HR2 region were substituted by neutral residues. D479N had no apparent effect on VP-14637 binding (Fig. 3B). Interestingly, despite a profound effect of the E487D mutation on the activity and binding of [3H]VP-14637, the E487Q mutant did not show diminished compound binding, suggesting that the length rather than the charge of the amino acid residue affected the compound interaction at this site. Lastly, the binding of [3H]VP-14637 to D489N mutant was greatly diminished, indicating the potential importance of an acidic residue for this interaction. Although no resistance mutations were selected at D489 in our study, D489Y was previously selected by JNJ-2408068 (14).

**DISCUSSION**

By using a RSV-induced cell fusion assay we have confirmed that JNJ-2408068, like VP-14637, inhibits fusion at similar drug concentrations that prevent RSV replication in a standard antiviral assay. In addition, we have shown that these same concentrations of JNJ-2408068 can block the binding of VP-14637 to RSV-infected cells, suggesting an overlapping mode of interaction for both compounds. The observations that mutant viruses selected in the presence of either drug were cross resistant to both compounds and that F proteins containing resistance mutations selected by either VP-14637 or JNJ-2408068 could no longer bind VP-14637 further support the conclusion that the two inhibitors act via a similar mechanism.
Structural analysis of the F protein core formed by HR1 and HR2 peptides has revealed a hydrophobic cavity formed by six amino acids from two adjacent HR1 repeats. This pocket can presumably accommodate two hydrophobic residues from HR2 (F483 and F488) during the process of six-helix formation (Fig. 4A) (24). VP-14637 selected for a resistance mutation at F488, and the other HR2 mutations selected by both VP-14637 and JNJ-2408068 (E487 and D486) were in close proximity. Therefore, this hydrophobic pocket might potentially be a site for VP-14637 and JNJ-2408068 binding, and the various substitutions in and around F488 may change these interactions without impairing the F protein function. Photoaffinity labeling experiments with a structural analog of another recently identified RSV fusion inhibitor BMS-433771 showed that this compound reacted specifically with Y198 of HR1 repeat, one of the amino acids that form this hydrophobic cavity (4a), providing direct evidence that the pocket is indeed capable of accommodating a potent RSV-specific inhibitor.

We applied molecular modeling techniques to assess the feasibility of VP-14637 and JNJ-2408068 binding to the F hydrophobic pocket. Docking studies identified several possible binding modes for these two compounds. Figure 4B shows a representative binding mode for JNJ-2408068. Similar binding modes were obtained for VP-14637 (Fig. 4C). These results are consistent with the observed inhibition of VP-14637 binding by JNJ-2408068.

Even though the binding of a small-molecule inhibitor to the hydrophobic pocket may prevent the formation of a fusion-competent HR1 and HR2 helical bundle, it would probably not eliminate all interactions between HR1 and HR2 peptides. Structural analysis indicates that significant interactions between HR1 and HR2 are possible even in the presence of inhibitor bound to the hydrophobic pocket. A region encompassing approximately 37 HR2 residues (L481-N517) is involved in the interaction with the HR1 inner core trimer. Interactions between the HR1 trimer and approximately 12 of those 37 HR2 residues (L481-I492) would be affected by the binding of an inhibitor to the hydrophobic pocket, leaving 25 amino acid residues from HR2 (S493-N517) still available for the interaction with the HR1 trimer. The interaction between HR1 and HR2 can be envisioned as a zipper that can close all the way in the absence of an inhibitor. However, with the inhibitor bound, the zipper can only go approximately two thirds of the way. The disruption of the last one third of the
HR1/HR2 interaction is likely sufficient to prevent the formation of a fusion-competent conformation of F protein, resulting in a potent inhibition of virus replication.

Because of the relatively small size and shallowness of the hydrophobic pocket, it is unlikely that the binding of an inhibitor to this pocket alone can result in such potent antiviral activity. A possible scenario is that HR2 is bound to the HR1 trimer through anchoring interactions of 25 residues (S493-N517, approximately 70% of the “zipper” length) with the remaining 12 HR2 residues (L481-I492) acting as a cover over the hydrophobic pocket and the bound inhibitor, effectively enhancing the interaction between the bound inhibitor and the HR1/HR2 complex (Fig. 5A). This could explain why the resistance mutations found within the six-helix bundle (positions 486 to 488) were all located in the HR2 peptide and not in the HR1 hydrophobic pocket. Amino acids 486 to 488 are among the residues that form the putative inhibitor cover and therefore could make direct contacts with the bound inhibitor (Fig. 5B). Mutations at these positions would then directly affect interactions with the inhibitor, resulting in altered virus susceptibility. This model would also be consistent with the inability of the HR2-derived peptide T-118 to inhibit VP-14637 binding.

The role of F protein residues 398 to 400 in the interaction with inhibitors is not clear since no specific function has been determined yet for the intervening domain between HR1 and HR2. A homology model of the RSV F protein derived from the crystal structure of the NDV fusion protein suggests that these residues would be located in a predicted immunoglobulin-like β-sandwich domain on the surface of the F protein (3, 17). Since the intervening domain has to undergo a dramatic conformational change during the process of viral fusion, mutations located in this region may affect the kinetics of this conformational change. It may result in a shortening of the time window during which the inhibition-sensitive conformation of F protein is available for the inhibitor to bind. Alternatively, the intervening sequences might be involved in an early transient conformation of F protein present prior to the heptad repeat interaction. The small molecules might be acting at this earlier metastable state instead of directly preventing the formation of the six-helix fusion core. However, experimental evidence to support this type of interaction has yet to be generated.

In addition to RSV, multiple other fusogenic viruses rely on the formation of HR1/HR2 helical bundle within the structure of their fusion protein as the mechanism that allows for their efficient entry into a host cell. Among these, gp41 of human immunodeficiency virus type 1 (HIV-1) is both functionally and structurally equivalent to RSV F protein, i.e., it also contains an inner coiled coil formed by three N-terminal HR1 helices, which interact with three C-terminal HR2 helices to form a stable six-helix bundle that drives the HIV-1 fusion process. Similar to RSV HR1 helical trimer, there is a pocket present in the HR1 coiled coil of gp41 (2). Even though both pockets are hydrophobic in nature, they accommodate different HR2 amino acid residues—two Trp residues in the case of gp41 and two Phe residues in RSV F protein. A comparative structural analysis suggested that the RSV pocket is deeper and better defined whereas the pocket present in HIV-1 gp41 is slightly larger but shallower and more open. This might be one of the reasons why, despite extensive screening efforts, no promising small-molecule gp41 inhibitors have been identified thus far. When we tested VP-14637 and JNJ-2408068 against HIV-1, no antiviral activity was observed at concentrations as high as 10 μM, a result consistent with the observed differences in the two hydrophobic pockets.

**FIG. 4.** (A) Hydrophobic cavity in RSV F protein formed by the HR1 inner core trimer. Surface representation of the cavity is shown in cyan. HR1 heptad repeats are represented by purple ribbons, and the HR2 helix is shown as a yellow ribbon. Three HR2 residues that bind the pocket (Phe483, Phe488, and Ile492) and other key HR2 residues mutated in resistant viruses (Glu 486, Glu 487, Asp 489) are shown in green sticks. (B) A potential binding mode of JNJ-2408068 to the HR1 hydrophobic cavity. (C) A potential binding mode of VP-14637 to the HR1 hydrophobic cavity.
in the size and shape of the pocket present in RSV F protein and that in HIV-1 gp41.

In summary, VP-14637 and JNJ-2408068 are two small molecules from the growing collection of RSV inhibitors that inhibit viral fusion by interacting with the F protein in a similar manner despite their apparent structural dissimilarity. Further detailed understanding of the mechanisms of interaction between these inhibitors and the F protein should facilitate the design of a new generation of RSV fusion inhibitors.

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REFERENCES


