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Research Paper

RFI-641 inhibits entry of respiratory syncytial virus via interactions with fusion protein

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Abstract

Background: RFI-641, a small dendrimer-like compound, is a potent and selective inhibitor of respiratory syncytial virus (RSV), which is currently a clinical candidate for the treatment of upper and lower respiratory tract infections caused by RSV. RFI-641 inhibits RSV growth with an IC_{50} value of 50 nM and prevents syncytia formation in tissue culture. RSV contains of three surface glycoproteins, a small hydrophobic (SH) protein of unknown function, and attachment (G) and fusion (F) proteins that enable binding and fusion of virus, respectively, with target cells. Because of their role in attachment and fusion, the G and F surface proteins are prominent targets for therapeutic intervention. RFI-641 was previously shown to bind purified preparations of RSV fusion protein. Based on this observation, in conjunction with the biological results, it was speculated that the fusion event might be the target of these inhibitors.

Results: A fusion assay based upon the relief of self-quenching of octadecyl rhodamine R18 was used to determine effects of the inhibitors on binding and fusion of RSV. The results show that RFI-641 inhibits both RSV–cell binding and fusion events. The inhibition of RSV is mediated via binding to the fusion protein on the viral surface. A closely related analog, WAY-158830, which is much less active in the virus-infectivity assay does not inhibit binding and fusion of RSV with Vero cells.

Conclusions: RFI-641, an in vivo active RSV inhibitor, is shown to inhibit both binding and fusion of RSV with cells, events that are early committed steps in RSV entry and pathogenicity. The results described here demonstrate that a non-peptidic, small molecule can inhibit binding and fusion of enveloped virus specifically via interaction with the viral fusion protein.

Keywords: Respiratory syncytial virus; Entry; Attachment; Fusion; Inhibition; Fusion protein; Dequenching assay

1. Introduction

Respiratory syncytial virus (RSV) is a member of the family Paramyxoviridae and the genus Pneumovirus. Human RSV is a major cause of respiratory tract infection in young children and infants and is an important cause of community acquired respiratory infection among hospitalized adults [1]. RSV is reported to cause an estimated 91 000 hospitalizations and 4500 deaths annually in the USA [2,3]. Recent clinical studies have probed the role of RSV infections as the cause of a variety of pathological conditions. It is clear that the current understanding of the RSV interaction with target cells is considerably less than that of influenza, for which RSV infection is frequently confused.

Entry of enveloped viruses into cells occurs via two general routes, both of which are dependent on recognition of specific cell surface receptors and membrane fusion. In certain viruses, such as orthomyxovirus, cell entry is via adsorptive endocytosis, a process in which the virus particle is delivered to the intracellular endosomal compartment [4–7]. Viral membrane fusion is then mediated at slightly acidic pH by the surface glycoproteins [8]. In paramyxoviruses such as RSV and Sendai virus, adsorp-

Abbreviations: FBS, fetal bovine serum; pfu, plaque forming units; PEG, polyethylene glycol; DMEM, Dulbecco's modified Eagle medium; RSV, respiratory syncytial virus

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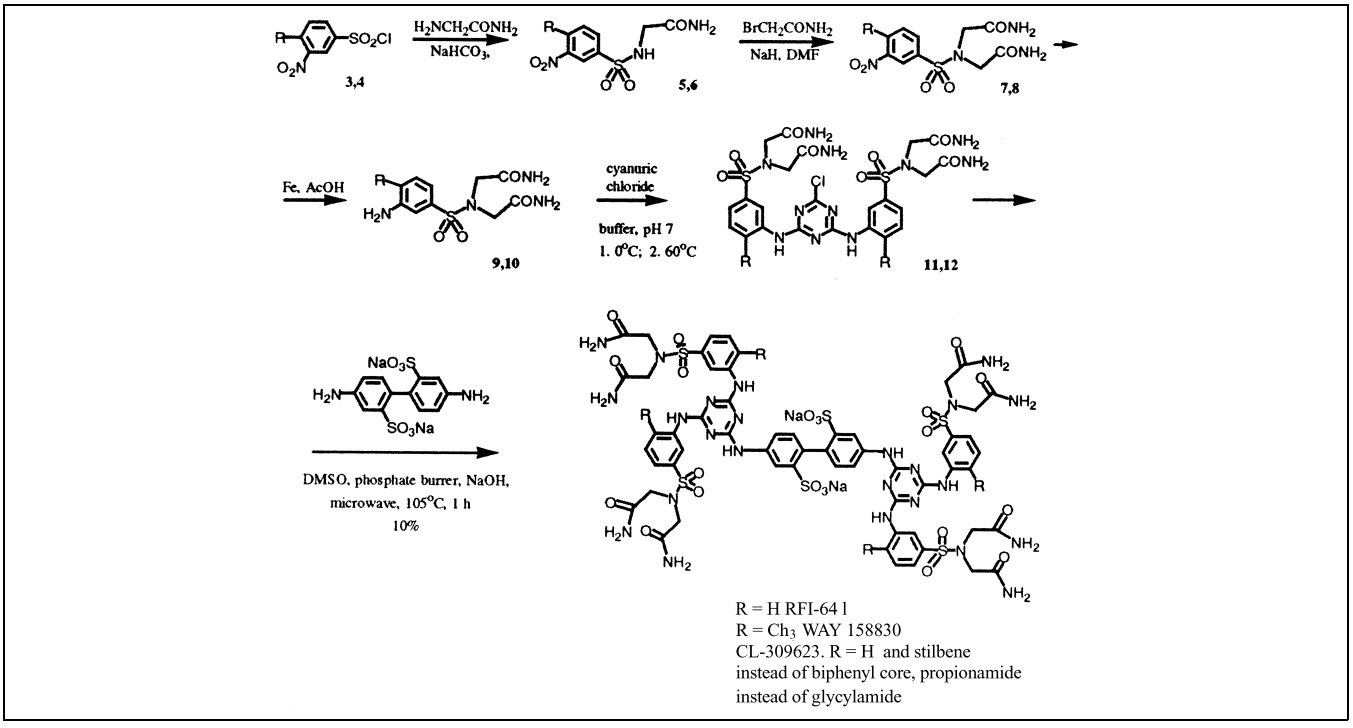
tion and membrane fusion occur at the cell surface at neutral pH thereby allowing direct entry of the viral capsid into the cytoplasm [9,10]. RSV contains three surface glycoproteins as spike-like projections, namely, G (attachment), F (fusion) and SH (small hydrophobic) proteins [11]. The fusion protein in RSV, similar to other paramyxoviruses, is synthesized as a precursor, Fo, which is subsequently activated by cleavage to two fragments F_1 and F_2 that are linked by a disulfide bond. F_2 is highly glycosylated and, similar to other enveloped viruses, appears to be involved in target cell recognition and attachment providing a backup to the G attachment protein.

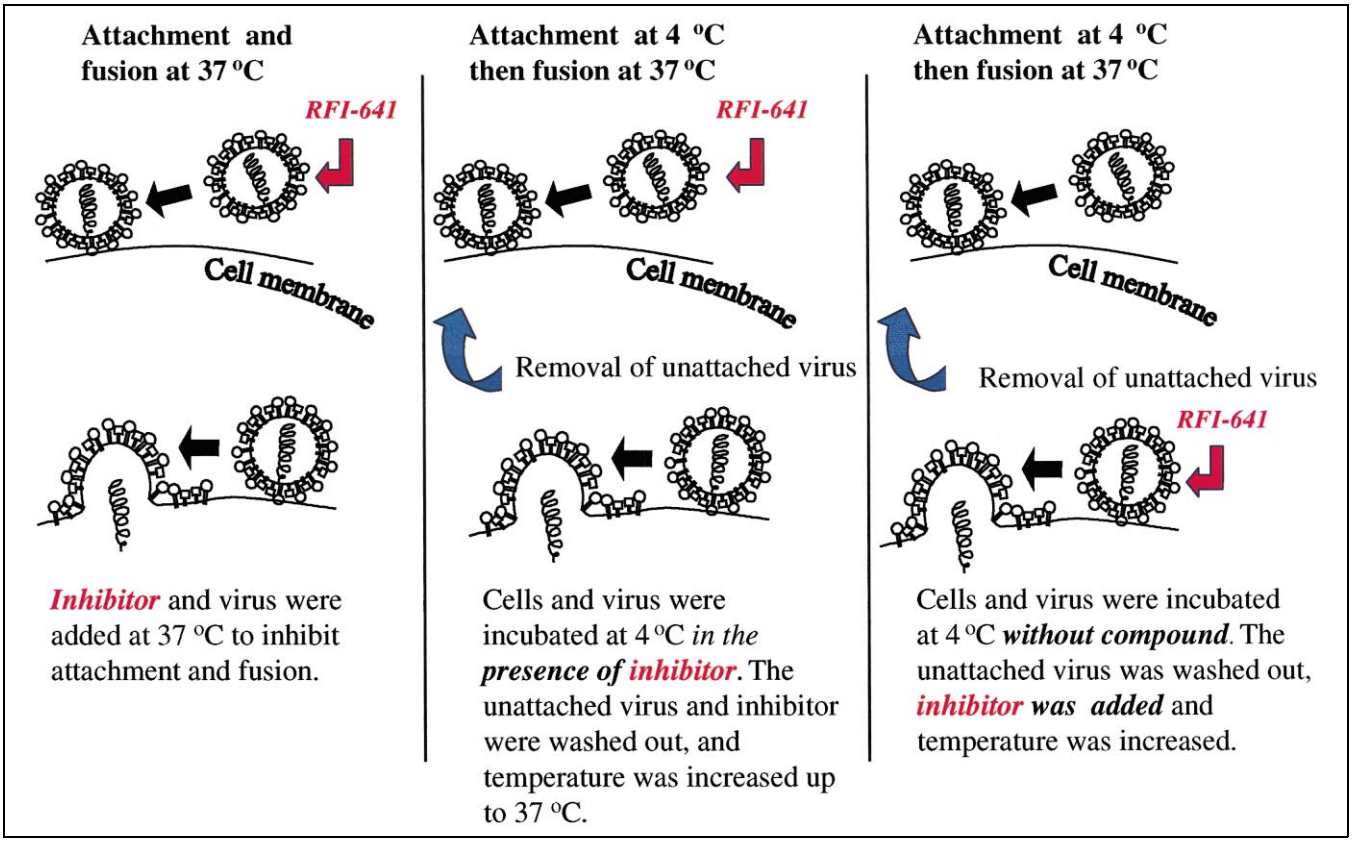
Several years ago a screening program at Wyeth-Ayerst Research identified a potent and specific inhibitor for RSV, the disulfonated stilbene CL-309623, from a diverse 20 000 compound library. After the preparation of some 150 analogs, the biphenyl analog RFI-641 (IC_{50} 50 nM) was chosen for evaluation in the clinic (see structures in Scheme 1 below). The analogs, CL-309623, RFI-641 and WAY-158830, were synthesized as outlined in Scheme 1 [12].

3-Nitrobenzenesulfonyl chlorides **3, 4** were converted to the corresponding amides **5, 6** by reaction with glycylamide under Shotten–Baumann conditions. The second glycylamido chain was added by reaction with sodium hydride and bromoacetamide in DMF. The nitro group in the compounds **7, 8** was reduced to the amino group by treatment with iron powder in water/acetic acid to give the amino compounds **9, 10**. Next, nucleophilic addition of **9, 10** to cyanuric chloride under controlled conditions led to the disubstituted monochlorotriazines **11, 12** in good yield. Condensation of the substituted triazines **11, 12** with the core part, 1,1′-diaminobiphenyl-3,3′-disulfonic acid, led to RFI-641 and WAY-158830. The individual compounds were isolated by preparative high performance liquid chromatography. The IC_{50} values of RFI-641, WAY-158830 and CL-309623 in the viral growth assay are 50 nM, 25 μM and 150 nM respectively.

The biphenyl analogs and other small dendrimer-like analogs based on stilbene and biphenyl cores were shown to prevent syncytia formation in cell cultures. Evidence for direct binding [12] of the stilbene inhibitor, CL-309623, and the more potent biphenyl analog RFI-641 with a purified preparation of F protein was obtained using a fluorescence binding assay. The goal of the present studies has been to determine if the compounds specifically inhibit attachment of virus to target cells as well as the virus–cell fusion event. Towards this goal we have implemented a fluorescence-dequenching assay [13] to determine the effect of RFI-641 on attachment and fusion against a wild type (A2 strain) and a mutant (*cp-52*) virus which contains only the fusion protein on its surface. The mutant virus is a cold-passaged strain that effectively infects mammalian cells in tissue culture even though it lacks the small hydrophobic (SH) and attachment (G) protein [14]. The fusion protein in the mutant *cp-52* strain has one amino acid substitution at position 218 (Glu to Gly) in the F_2 domain [14]. Since the mutant virus has only one surface glycoprotein, the virus is a good model to study the effect of inhibitors on attachment and fusion. The results of the fluorescence-dequenching assay show that RFI-641 inhibits both virus–cell attachment and fusion events.



Scheme 1.



Scheme 2.

2. Results and discussion

2.1. Inhibition of attachment and fusion of RSV with Vero cells using the fluorescence microscopy method

RSV can infect a wide variety of human and animal cells. The virus can be cultured in Vero cells, HEp-2, HeLa, and CV-1. In this work we chose green monkey epithelial cells (Vero) to study RSV fusion because the virus primarily infects the epithelial cell lining the respiratory tract. Fusion of octadecyl rhodamine R18-labeled RSV with unlabeled Vero cells can be directly observed in a fluorescence microscope as an increase in quantum yield of R18 due to membrane fusion events and the resulting dilution of dye in the merged membrane. The fluorescence microscopy assay is based on a fluorescence-dequenching method previously described [13]. Briefly the assay involves labeling the virus with the rhodamine dye at self-quenching concentrations and then fusing with Vero cells. Upon membrane merger, during viral fusion, the self-quenching is relieved due to dye spread.

RSV binds cells both at 4°C and 37°C but fusion occurs only when the temperature is raised above 18°C. The experimental strategies that were used to determine the effects of RFI-641 on cell entry, namely, the combined events of virus–cell attachment and fusion, virus–cell attachment and virus–cell fusion events alone, are shown in Scheme 2.

To determine qualitatively the effect of inhibitor on combined RSV attachment and fusion events according to Scheme 2, left, above, R18-labeled RSV was added to Vero cells in a 12-well microtiter plate. Vero cells and virus were incubated either in the absence or presence of 2 μM of the inhibitor for 30 min at 37°C. The plate was imaged using a fluorescence microscope after washing out the unattached virus. In this experiment, only those virions that are attached specifically to Vero cells can undergo fusion and any inhibition of attachment by the inhibitor will result in a lesser amount of fusion. On the other hand, any inhibition of fusion alone will also result in diminished dye spread. The inhibitory effects of RFI-641 on fusion and the lack of it in the case of WAY-158830 are shown in the fluorescence images in Fig. 1. Fig. 1A,B correspond to fusion events of wild type and mutant strains of RSV (A2 and *cp-52*) respectively, in the absence of inhibitor. In Fig. 1B, the increase in fluorescence of R18 due to fusion of *cp-52* with cells is significantly less than the fusion of A2 strain shown in Fig. 1A. Since *cp-52* virus lacks the G protein, which directs specific attachment of RSV with cells, the lower extent of R18 fluorescence would be indicative of lower efficiency of fusion in the mutant strain resulting from a fewer number of attached viruses and

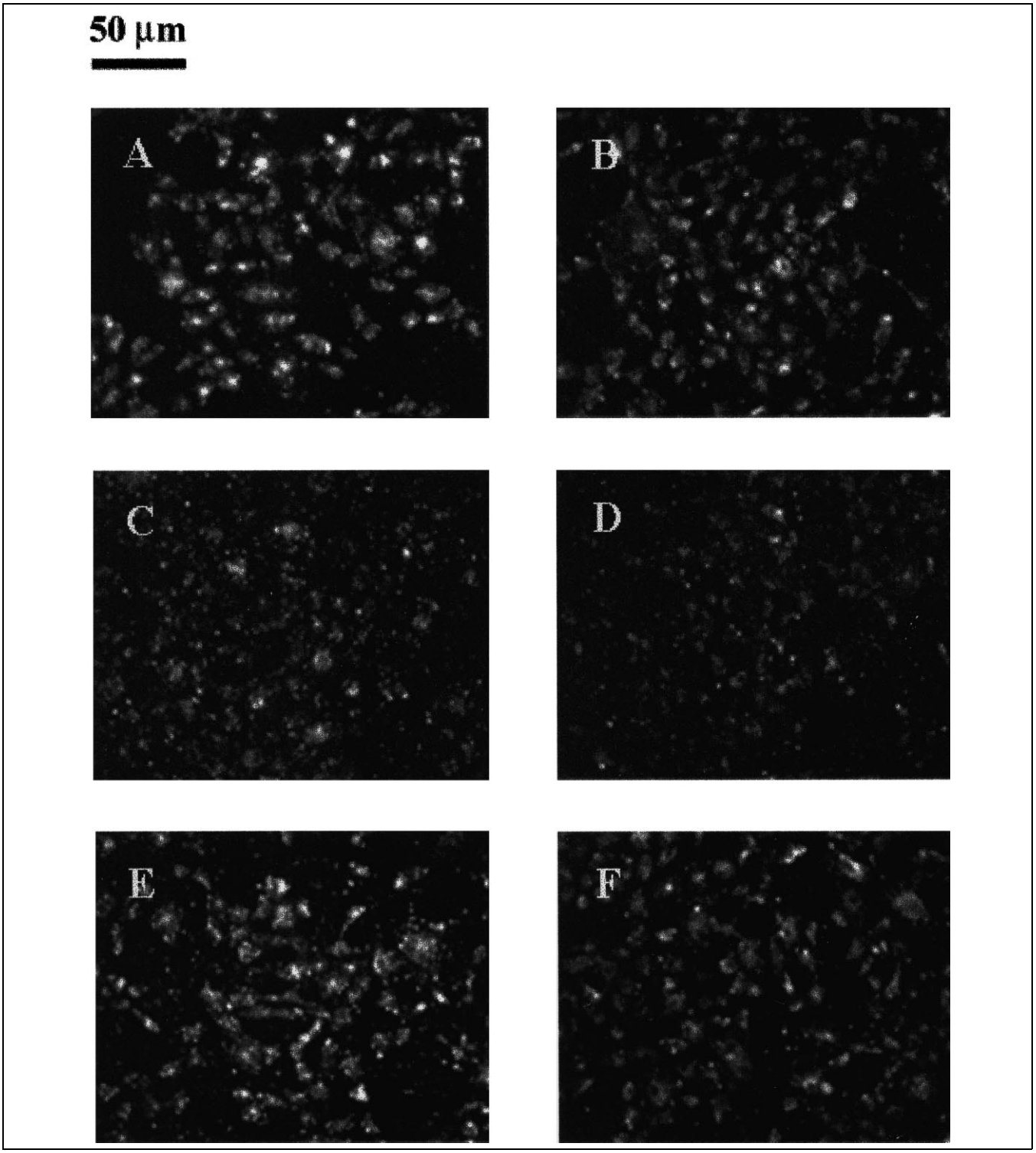


Fig. 1. Effect of RFI-641 and WAY-158830 on RSV attachment and fusion events at 37°C using the fluorescence microscopy method. Virus and inhibitor were added to a 12-well Corning™ plate containing adherent Vero cells in MEM at 37°C. The inhibitor, cell and virus mixture was incubated for 30 min at 37°C. After removal of the unattached virus, cells were imaged using a fluorescence microscope. A and B correspond to fusion of A2 and *cp-52* strains with Vero cells in the absence of inhibitor respectively. C and D correspond to A2 and *cp-52* strains in the presence of 2 μM of RFI-641 respectively. E and F are due to fusion of A2 and *cp-52* strains in the presence of 2 μM WAY-158830 respectively.

direct inhibition of fusion. The fluorescence images shown in Fig. 1C,D correspond to attachment and fusion of the viruses in the presence of 2 μM of RFI-641. The fluorescence of R18 due to fusion, shown in Fig. 1C,D, is significantly quenched compared to those in Fig. 1A,B; thus the lack of increase in R18 fluorescence could be due to inhibition of virus–cell attachment and/or fusion. The inhibitor alone does not interact with the R18 dye and there-

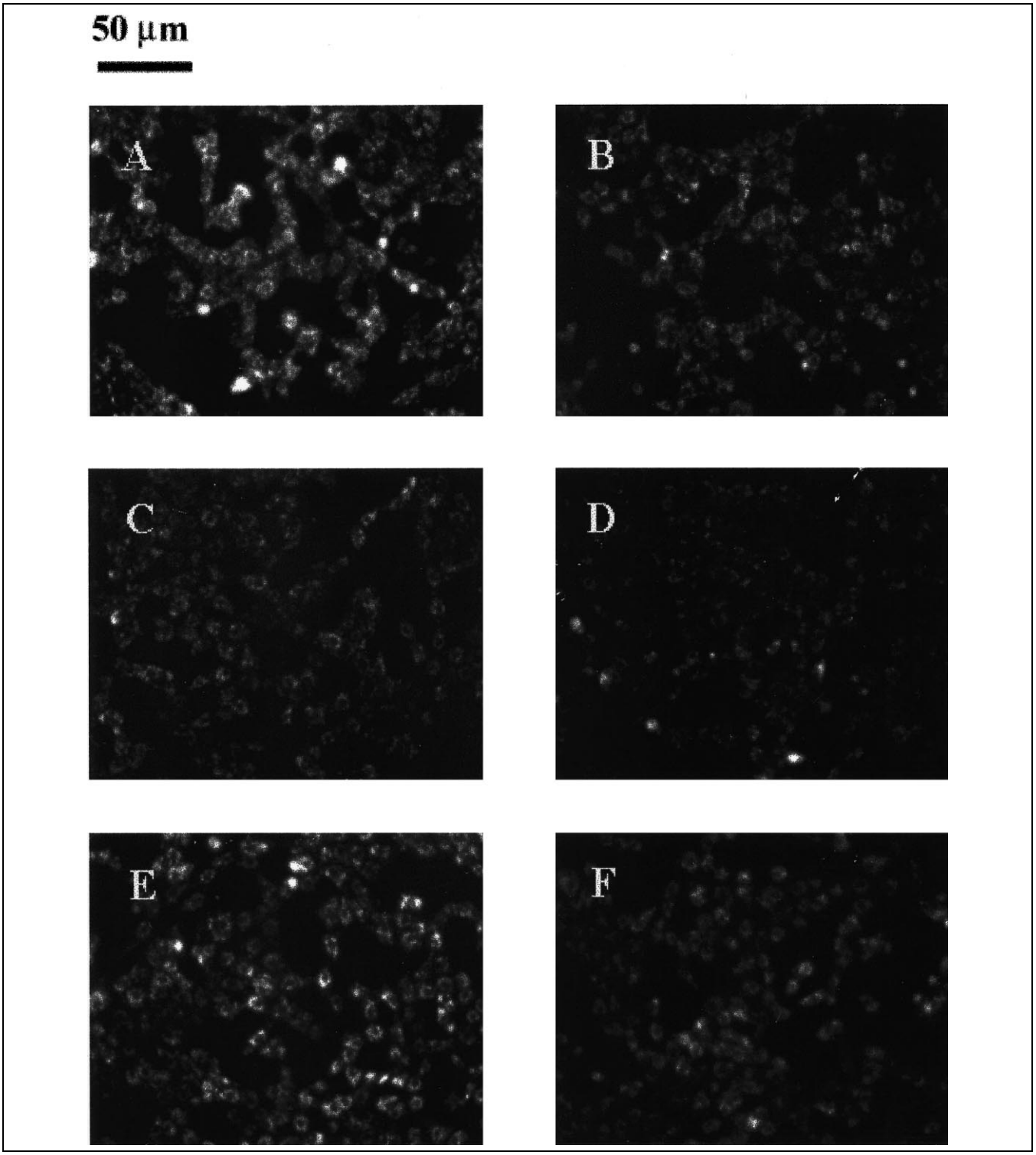


Fig. 2. Effect of RFI-641 and WAY-158830 on RSV–cell attachment at 4°C using the fluorescence microscopy method. Labeled virus was allowed to bind to Vero cells grown on a 12-well Corning™ plate at 4°C. Inhibitor was added to cells immediately before the addition of virus. After incubation at 4°C for 30 min, unattached virus particles were removed and cells were incubated at 37°C for 30 min. Cells were imaged using a fluorescence microscope at ambient temperature. A – A2 strain in the absence of inhibitor; B – *cp-52* strain in the absence of inhibitor; C – A2 strain with 2 μM RFI-641; D – *cp-52* strain with 2 μM RFI-641; E – A2 virus with 2 μM WAY-158830; F – *cp-52* virus with 2 μM WAY-158830.

fore has no direct effect on the quantum yield of the fluorescence of the dye.

The structurally related analog WAY-158830 consisting of a methyl group in the peripheral benzenesulfonamides (see Scheme 1 for structures) was also tested in the fusion assay as a negative control. WAY-158830, which has weak inhibitory activity in the virus growth assay (IC_{50} values of WAY-158830 and RFI-641 are 25 μM and 50 nM respec-

tively [12]), is also less selective against a panel of viruses. Fig. 2E,F are due to fusion of labeled virus with cells in the presence of the inactive analog, WAY-158830. WAY-158830 inhibits virus fusion weakly as evidenced by relief of R18 self-quenching. In this instance, the extent of self-quenching is comparable to the control and therefore the fluorescence is significantly higher than those observed in the presence of RFI-641.

As mentioned above, it has recently been shown that the cold-passaged mutant virus *cp-52* infects mammalian cells in tissue cultures [14] even though it lacks the attachment protein. This observation suggests that the fusion protein is capable of mediating attachment on its own. Since the fluorescence microscopy results of viral fusion described above have shown that RFI-641 blocks the interaction (binding and/or fusion) of RSV with Vero cells, the next step was to quantify the inhibition of attachment and fusion. However, the molecular recognition aspects of RSV–cell interactions and fusion are uncertain, unlike the case of retroviruses such as HIV and also for influenza where cell surface receptors and fusion mechanisms are fairly well understood. Therefore, a 'temperature-shift' experiment was set up in an attempt to separate virus–cell attachment from fusion events [15,16]. This experiment is based on our previous observations that have shown that RSV can be co-incubated with target cells at low temperatures, namely 4°C, without fusion, and when the temperature is raised to 37°C the virus becomes fusion competent and viable in a tissue culture assay [17].

To determine the effect of inhibitor on fusion alone (Fig. 2) according to Scheme 2, right, above, the virus was pre-incubated with Vero cells at 4°C to enable attachment without triggering fusion. After this attachment step, the unattached virus was removed and inhibitor added to the virus–cell complex prior to triggering fusion at 37°C. The inhibitory effects of RFI-641 on fusion and the lack of it in the presence of WAY-158830 are qualitatively similar to the results described in Fig. 1.

Fig. 3 shows the effect of inhibitors on virus–cell attachment at 4°C, again using the fluorescence microscopy method, and the strategy shown in Scheme 2, middle, above. In this experiment, labeled virus was added to Vero cells at 4°C and the inhibitor was added immediately before addition of virus. To enable attachment, the virus, cells and inhibitor mixture was incubated at 4°C for 30 min. At the end of the binding step, the medium containing unattached virus and compound was removed and the temperature increased to 37°C to induce fusion. Based on this assay, RFI-641 inhibits virus–cell attachment (Fig. 3C,D) whereas WAY-158830 does not significantly inhibit attachment at 2 μM (Fig. 3E,F).

RSV attachment is reported to be mediated through the SH and G glycoproteins via heparin moieties on the target cell surface [18,19]. The fact that the *cp-52* mutant lacking the SH and G proteins is infectious and is capable of attachment and fusion (results of fusion assays shown here) indicates the existence of an alternative F-mediated pathway for RSV attachment. Hypothetically, within a wild type population some viruses may use this F-mediated pathway for virus entry. We propose that RFI-641 inhibits these attachment and fusion events by interaction with fusion protein in the wild type and mutant virus strains.

2.2. R18 fusion assay using Vero cells in suspension

2.2.1. RSV binding to cells in the absence of inhibitor

To differentiate the early events of virus binding and fusion that are specifically blocked by inhibitor and to quantify such effects, the fusion assay was implemented using cells in suspension. The R18 dye spread assay has been used with cells in suspension by many other investigators and is a commonly used fusion assay for the influenza and Sendai viruses and RSV [15,16] as well. In the attachment experiment, virus was incubated with cells either at 37°C (Fig. 4aA) or at 4°C (Fig. 4aB). The attached virus was sedimented by centrifugation and SDS detergent added to the pellet to relieve self-quenching by complete randomization of the dye. The extent of attached virus was estimated as a ratio of the labeled virus in the pellet relative to the total amount of virus added prior to centrifugation. The estimate of viral particles is based upon randomization of the R18 dye in the presence of detergent. At 37°C (Fig. 4aA) the increase in fluorescence scores as fusion but it does not account for the total number of virus particles attached to cells. The total virus particles will be proportional to the fluorescence intensity only when the R18 dye is completely randomized and dequenched in solution.

To determine if aggregated virus would co-sediment with bound virus in experiments such as the ones shown in Fig. 4a, the following control experiment was performed. Labeled virus was centrifuged at 4°C in the absence of cells, and SDS was added to the supernatant to estimate the virus in this fraction after randomization of dye. The amount of virus in the supernatant was estimated to be within 2% of the total because the standard centrifugation conditions did not pellet free or aggregated virus. The result of this control experiment enabled the quantification of the bound virus without interference from free virus particles.

In a typical attachment experiment, less than 10% of the total number of the A2 virus and about half as much of the *cp-52* virus are bound to Vero cells at 37°C (Fig. 4aA). The same experiment at 4°C showed that about 20% of the wild type strain and the same amount of the mutant strain are attached to cells as shown in Fig. 4aB. Thus it is evident that nearly twice as much virus is bound at 4°C than at 37°C. Nevertheless, the results show that the *cp-52* mutant virus binds cells albeit weakly at 37°C, via the fusion protein, despite the lack of G protein. The extent of binding of 20% at the lower temperature is comparable

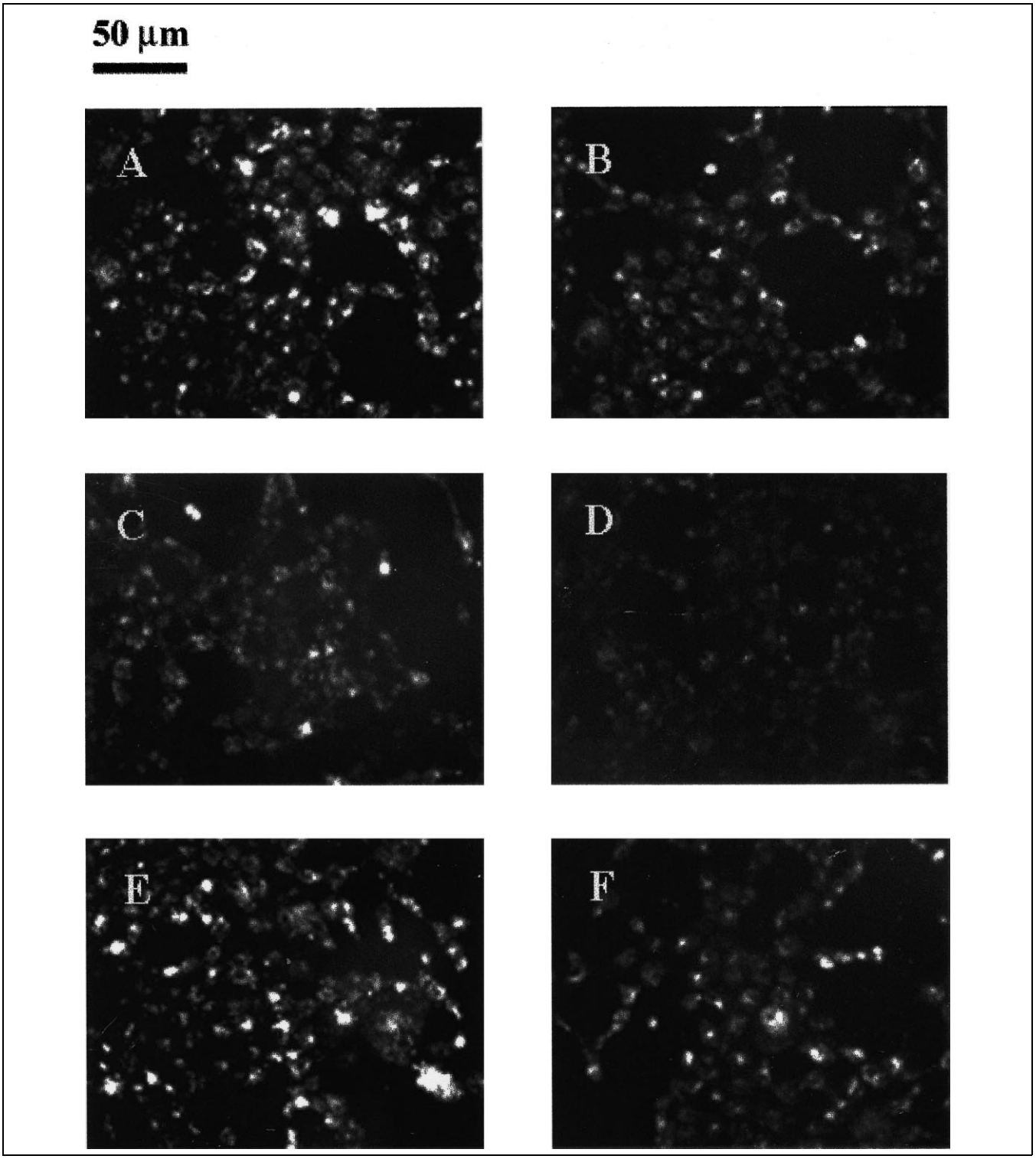


Fig. 3. Effect of RFI-641 and WAY-158830 on RSV–cell fusion at 37°C using the fluorescence microscopy method. Labeled virus was allowed to bind to Vero cells on a 12-well Corning™ plate at 4°C. After incubation at 4°C for 30 min, unattached virus particles were removed. The plate was incubated at 37°C for 30 min to induce fusion. The cells were imaged in the fluorescent microscope at ambient temperature following fusion. A and B – fusion of A2 and *cp-52* strains respectively in the absence of inhibitor; C and D – fusion of A2 virus and *cp-52* strains in the presence of 2 μM RFI-641; E and F – A2 and *cp-52* strains respectively in the presence of 2 μM WAY-158830.

to a reported 30% binding observed for Sendai virus [20–22].

To determine the stability of the virus–cell complex and the percentage of virus that would spontaneously dissociate from cells (in the absence of inhibitor) in a fusion experiment (Scheme 2, right), the virus was pre-bound to cells at 4°C and then transferred to 37°C after removing the unattached or free virus. The results shown in Fig. 4aC

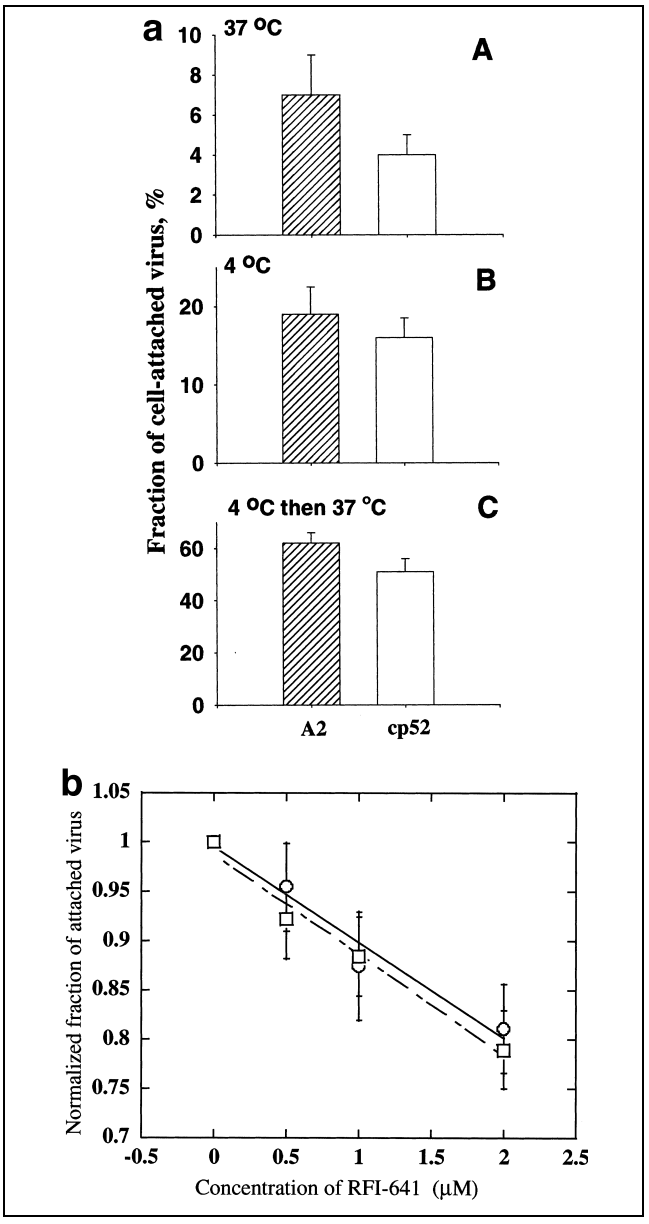


Fig. 4. a: Estimation of attachment of RSV to Vero cells at 4°C and 37°C using cells in suspension. Virus was initially added to Vero cells in suspension in the absence of inhibitor. The cells were centrifuged to separate unattached virus. The detergent was added to pellet and supernatant and the fluorescence of octadecyl rhodamine R18 measured at the maximum wavelength of 560/580 nm. The percentage of attached virus was calculated from the ratio of fluorescence of attached virus in pellet to total fluorescence in pellet and supernatant. A – extent of binding of labeled virus at 37°C. B – extent of binding of labeled virus at 4°C. C – extent of dissociation of labeled virus at 37°C. b: Effect of RFI-641 on attachment of A2 and *cp-52* to Vero cells at 37°C. Virus was initially added to Vero cells in suspension at 37°C. Inhibitor was added to cells just before addition of virus. After 30 min incubation of mixture at 37°C, unattached virus was separated by centrifugation. 2% sodium dodecyl sulfate (SDS) was added to pellet consisting of cells with attached and fused virus and to supernatant containing unattached virus. Fluorescence of R18 was measured as described above. Fraction of attached virus in the presence of 0.5, 1 or 2 μM of RFI-641 was calculated as a ratio of fluorescence of pellet to total fluorescence of pellet and supernatant as described above for *cp-52* (□) and A2 (○) strains.

would suggest that approximately 40% of the bound virus dissociated from the cells, under these experimental conditions. The decrease in virus binding shown in Fig. 4aC corresponds approximately to the difference in binding observed at 37°C and 4°C in Fig. 4aA,B respectively. The reversibility of binding in the temperature-shift experiment in Fig. 4aC is due to the establishment of a new equilibrium of the bound virus with unattached virus and cells. The new equilibrium at 37°C represents a fewer number of binding sites for RSV on Vero cells. The experiment that measures fusion event alone (as in Scheme 2, right) would, therefore, underestimate the amount of attached virus. In the case of influenza virus, a similar result was observed where it was shown that an increase in temperature decreases the number of binding sites [23]. Since fusion is triggered at 37°C in RSV, the lower binding efficiency at this temperature could result from loss of potential binding sites due to virus–cell fusion or due to conformational changes in the fusion protein.

2.2.2. Inhibition of RSV–cell attachment by RFI-641

The effect of RFI-641 on virus binding to cells was investigated at 37°C according to the method described above in the legend to Fig. 4aA. In this experiment, the inhibitor was added to cells immediately followed by the addition of virus. After incubating this complex for 30 min, the labeled virus in the centrifuged pellet was estimated by adding detergent. Results in Fig. 4b show a dose response of inhibition of attachment both of wild type and mutant viruses in the presence of RFI-641. RFI-641 progressively inhibits virus–cell attachment with a maximum value of 20% at 2 μM. Of the total virus added to Vero cells, 20% less virus is attached in the presence of the inhibitor than in its absence. A similar experiment at 4°C could not be quantified due to virus aggregation issues as evidenced by preliminary ultracentrifugation studies (data not shown). In the attachment inhibition experiments both *cp-52* and wild type A2 virus strains are inhibited to a similar extent due to the interaction of the inhibitor with fusion protein.

The reversible association of RFI-641 with virus was demonstrated in the virus–cell lipid mixing assays and in the biological plaque reduction assays as described below. In the first assay, labeled virus was pre-incubated with RFI-641 in phosphate-buffered saline (PBS) buffer, at 37°C for 5 min. The virus–inhibitor mixture was diluted 50-fold into a cuvette containing Vero cells to determine the extent of dequenching of R18 dye. The extent of dequenching due to lipid mixing is identical for inhibitor-treated virus and untreated virus particles. Alternatively, the virus–inhibitor mixture was diluted 50-fold into media and then added to Vero cells in a 12-well plate at 37°C and the resultant dye spread due to fusion was imaged using the fluorescence microscopy method. The extent of fusion of virus pre-mixed with inhibitor is identical to fusion of untreated virus in both the experiments. Similarly, in the

second assay pre-incubation of virus with inhibitor followed by removal of inhibitor or dilution of virus did not block infectivity in plaque assays. The results of the experiments described here show that the biological activity and lipid mixing activity are fully resumed upon dilution of virus–inhibitor mixture or after removing inhibitor in a washing step. These results and those shown in Fig. 4 show that RFI-641 reversibly associates with virus and inhibits virus–cell attachment.

2.2.3. *Kinetics of RSV–cell fusion*

The results of the R18-dequenching assays described above using Vero cells either in suspension or attached to a plate provide direct evidence for inhibition of attachment and fusion due to inhibitor binding to the fusion protein. To gain further insight into the nature of the inhibition, namely effects on the kinetics and extent of fusion, dye spread due to virus–cell fusion was monitored continuously as a function of time after pre-binding the labeled virus to cells at 4°C (according to Scheme 2, right). In this experiment, inhibitor was added to the virus–cell complex immediately before inducing fusion. After removing unattached virus particles by centrifugation, the virus–cell complex formed at 4°C was combined with inhibitor in a cuvette at 37°C. The kinetics (Fig. 5A) and extent of fusion (Fig. 5B) were evaluated as a function of the concentration of the inhibitor. The rate of fusion levels off and reaches steady state values after approximately 5 min in both virus strains due to maximal increase in fluorescence signal after fusion of the attached virus. The fusion rates of *cp-52* and A2 virus strains are nearly identical (Fig. 5A) because only the fusion protein is involved in the membrane merger event. In the presence of RFI-641, the rate of fusion as well as the normalized extent of dequenching in R18 fluorescence are progressively inhibited, until the rate reaches a value of zero (Fig. 5A). These results suggest that RFI-641 inhibits virus–cell fusion efficiently. The potency of fusion inhibition, 50% at approximately 100 nM in the *cp-52* strain and 250 nM in the wild type strain (Fig. 5B), is in agreement with the infectivity assays.

Since only 20% of the input virus is attached to Vero cells under the stated conditions of the experiment, the extent of fusion shown in Fig. 5A in the absence of inhibitor is equivalent to a fusion efficiency of nearly 50%. In other words, only 10% of the input virus undergoes fusion with cells. Although this number appears small, the estimated number of RSV particles fusing with cells at this concentration is comparable to the extent of fusion of Sendai virus [20].

In order to quantify the effect of inhibitor on the combined events of attachment and fusion (according to Scheme 2, left) using the above described continuous fusion assay, the inhibitor was co-incubated with virus and Vero cells at 37°C without removing the unattached virus particles. In Fig. 6A, the rate of fusion of the A2 virus is

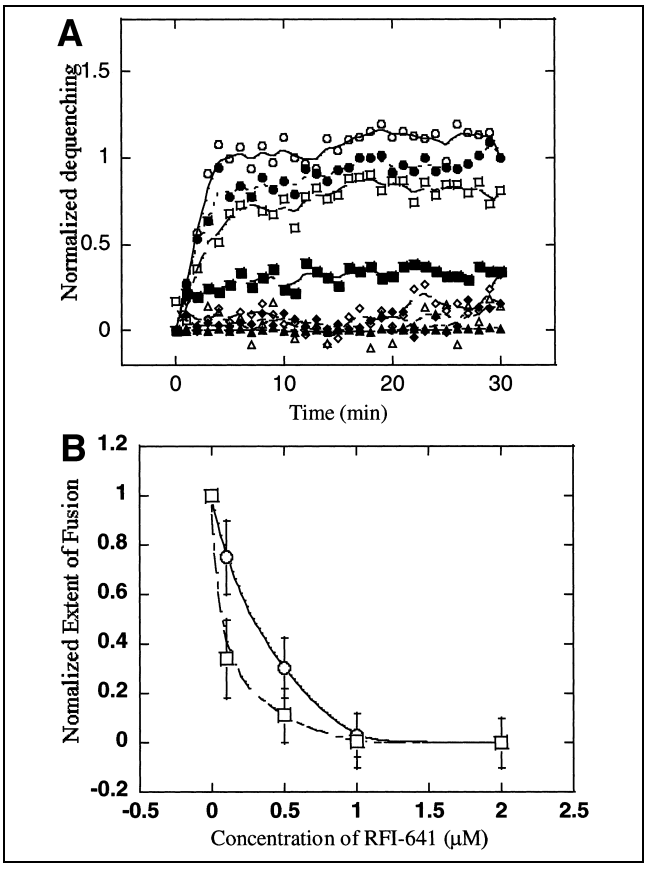


Fig. 5. A: Kinetics of fusion of *cp-52* virus after pre-attachment to cells at 4°C. Labeled virus was incubated with suspension of Vero cells at 4°C for 30 min. Unattached virus was removed after centrifugation. The pellet containing virus-bound cells was resuspended in 50 μl of PBS and the suspension was injected into a cuvette containing 450 μl of PBS at 37°C. Inhibitor was added before injection of cell/virus suspension. Fluorescence of R18 was measured at the emission maximum in 1 min time intervals. Dequenching (DQ) was calculated as described in Section 4. This value was normalized relative to the final DQ value at 30 min for the respective virus in the absence of inhibitor. Kinetics of fusion of A2 strain in the presence of 0 μM (○), 0.1 μM (□), 0.5 μM (◇) and 1 μM (△) of RFI-641 respectively. Kinetic traces of fusion of *cp-52* strain in the presence of 0 μM (●), 0.1 μM (■), 0.5 μM (◆) and 1 μM (▲) of RFI-641 respectively. B: Effect of RFI-641 on extent of fusion of A2 and *cp-52* strains with Vero cells after pre-attachment of virus at 4°C. The extent of dequenching due to fusion was estimated in the absence and presence of inhibitor and the value in the presence of inhibitor was expressed as a ratio relative to the value in its absence. The normalized values for A2 strain (○) and *cp-52* virus strain (□) in the presence of 0, 0.1 μM, 0.5 μM and 2 μM of RFI-641 are shown.

significantly slower than the corresponding fusion rates of the attached viruses alone (see Fig. 5A). The rate of fusion is slower because virus binding and fusion are occurring concurrently, whereas in the fusion alone experiment (Fig. 5A) the rate is proportional to the concentration of pre-bound virus only. Also, the fusion rates of *cp-52* and A2 virus strains are nearly identical (Fig. 6A,B) because only the fusion protein is involved in the membrane merger event. RFI-641 inhibits attachment and fusion more effectively in *cp-52* than in the wild type virus (Fig. 6C) due to interaction with fusion protein only. Here again WAY-

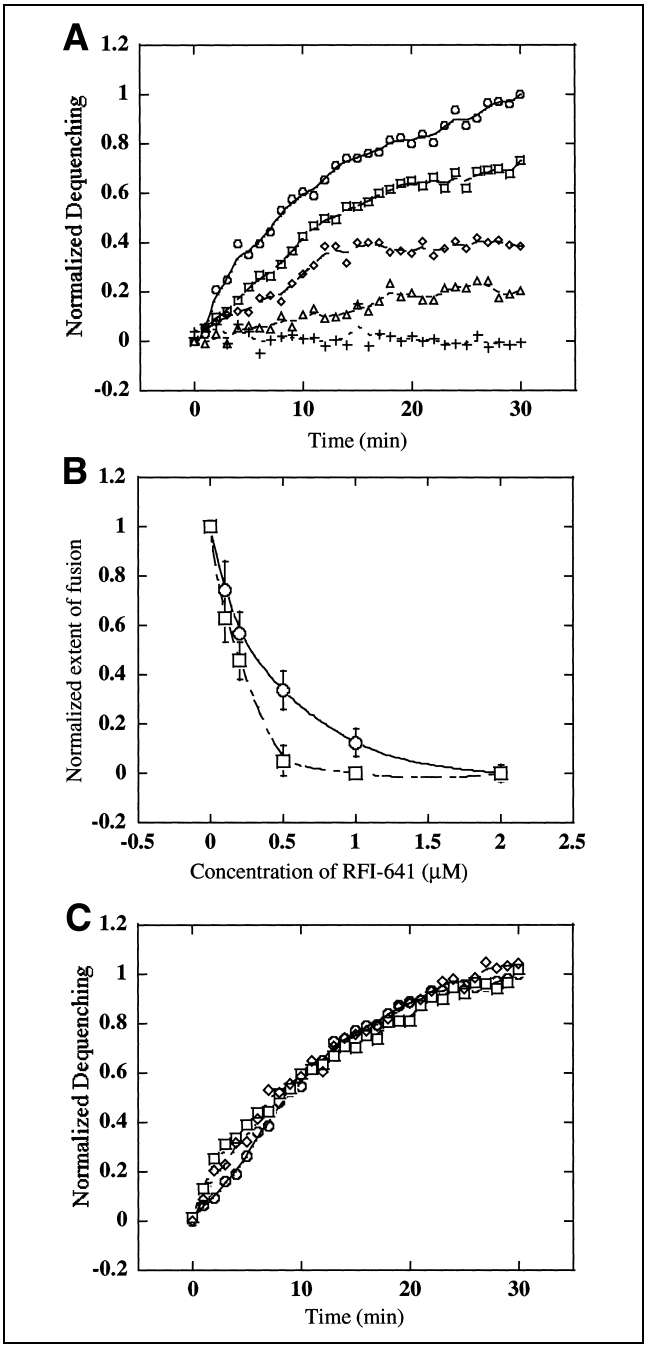


Fig. 6. A: Effect of RFI-641 on binding and fusion of A2 strain at 37°C using the continuous fusion assay. Aliquots of A2 strain of virus were injected into Vero cells in suspension. RFI-641 was added to the cells immediately before the addition of virus. The extent of dequenching was measured at 1 min time intervals as previously described. The dequenching value in the presence of inhibitor at 0.1 μM (○), 0.2 μM (□), 0.5 μM (◇), 1 μM (△) and 2 μM (+) was expressed as a ratio relative to the dequenching value in its absence. B: Effect of RFI-641 on attachment and fusion events of A2 and *cp-52* strains. The extent of dequenching under the stated experimental conditions was estimated according to the method described in the legend to A. The normalized values for A2 strain (○) and *cp-52* virus strain (□) in the presence of 0, 0.1 μM, 0.5 μM and 2 μM of RFI-641 are shown. C: Effect of WAY-158830 on binding and fusion of A2 virus strain. The experimental conditions are identical to those described for RFI-641 in A. The fusion assay was performed in the absence (○) and presence of 2 μM (□) or 5 μM (◇) of the inhibitor.

158830 is inactive in the fusion assay apparently because it does not inhibit attachment or fusion events (Fig. 6C). On the basis of the fusion and attachment experiments, it would appear that RFI-641 inhibits virus fusion more than the attachment event. However, in the fusion experiment, the ratio of inhibitor to virus is higher compared to the attachment experiment, which would result in a greater extent of inhibition of fusion.

Since the inhibitory effects described here directly implicate fusion protein as the primary target, binding experiments were done with virus using a stilbene analog, CL-309623, due to its desirable fluorescence properties, namely, emission maximum at long wavelengths and enhancement in quantum yield in the presence of fusion protein. The emission maximum of CL-309623 is centered at 460 nm and the quantum yield increases upon interaction with fusion protein [12]. In addition to these desirable fluorescence properties, CL-309623 inhibits virus growth with an IC_{50} value of 150 nM which is only three-fold weaker than RFI-641. This analog also inhibits virus attachment and fusion efficiently in the R18 fusion assay as shown in Fig. 7a. Biologically, CL-309623 and RFI-641 are identical in their mechanism of action except for the difference in potency of inhibition of RSV. CL-309623 and RFI-641 cross-react and inhibit resistant virus strains cultivated against each of these two compounds (data not shown). Due to the equivalent response of CL-309623 and RFI-641 in the in vitro assays, CL-309623 is a good candidate for the virus binding studies. The fluorescence spectra of the inhibitor in the absence and presence of mutant and wild type virus strains at 15°C are shown in Fig. 7b. In the presence of virus (equivalent to 5 μg of total protein, including envelope proteins and proteins in the nucleocapsid), the fluorescence of CL-309623 is significantly enhanced. Using the molecular weight of 70 000 for the fusion protein, the concentration of fusion protein on the virus surface would correspond to less than 150 nM. On the basis of the fluorescence results in Fig. 7bA, it appears that the interaction is more pronounced with *cp-52* than with the wild type virus, possibly due to a higher concentration of fusion protein in the mutant *cp-52* virus than in the wild type strain. Fig. 7bB shows the changes in the fluorescence of the inhibitor in the presence of fusion protein. It is important to note that the binding site that is responsible for the increase in quantum yield of the CL-309623 is present both in the *cp-52* virus as well as in the isolated fusion protein (see [12] for the isolation and purification of fusion protein). These results are consistent with direct interaction of the inhibitor with a pre-fusogenic conformation of the protein. The nature of the interactions with a fusogenic state of the protein is not known because such a state may only be stabilized by the target membrane during viral fusion. For the first time it is shown here that selective interactions of the inhibitor with fusion protein are responsible for the observed inhibition of entry of RSV into cells.

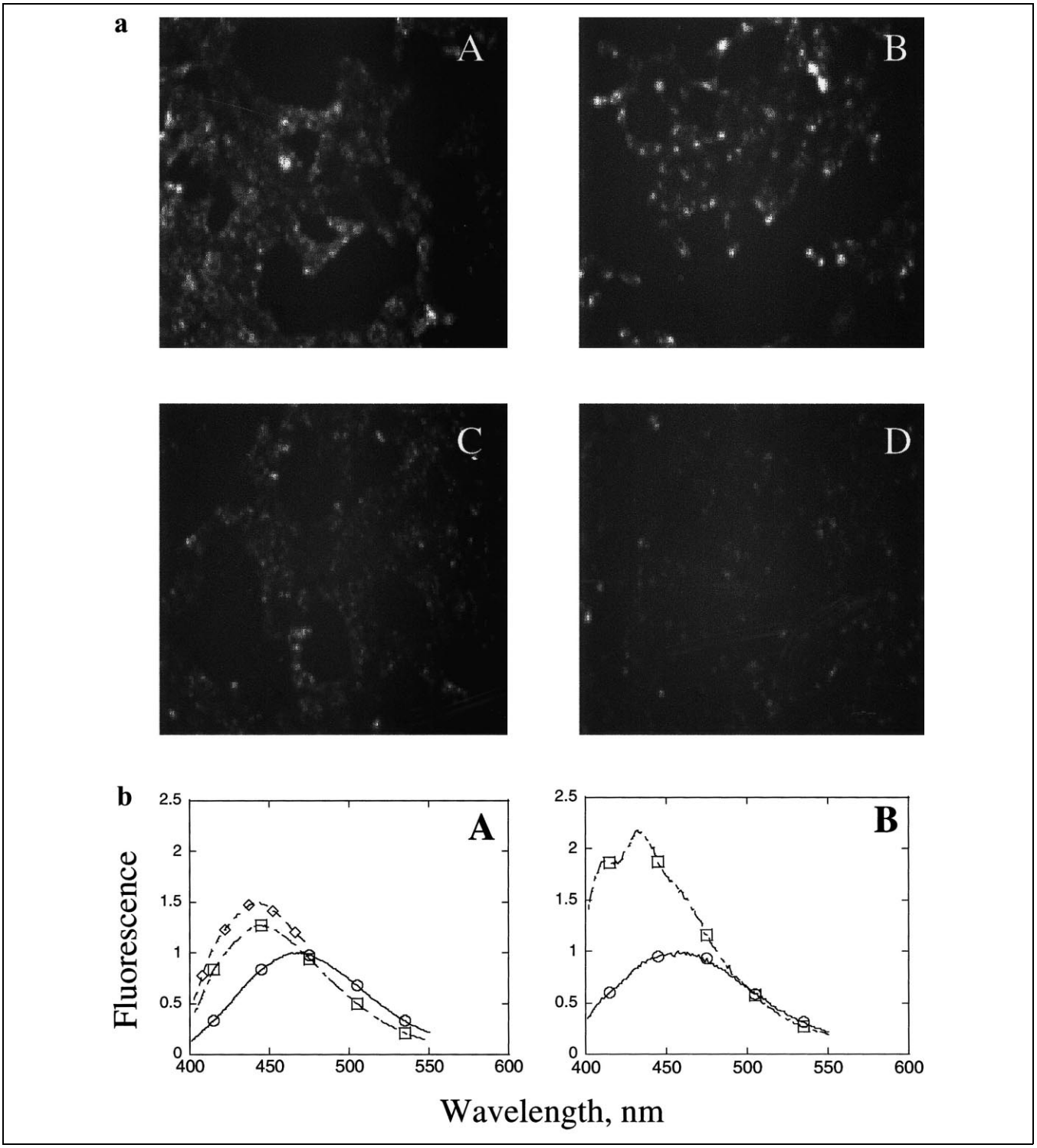


Fig. 7. a: Effect of CL-309623 on RSV–cell fusion at 37°C using the fluorescence microscopy method. The experimental conditions are identical to those described in the legend to Fig. 3. A and B – fusion of A2 and *cp-52* strains respectively in the absence of inhibitor; C and D – fusion of A2 virus and *cp-52* strains in the presence of 2 μM CL-309623. b: A: Normalized fluorescence spectra of CL-309623 in the absence and presence of A2 and *cp-52* virus strains at 15°C. Emission spectra of 200 nM of CL-309623 in the absence of virus (○) and in the presence of 5 μg of either the A2 (□) or the *cp-52* (◇) virus strains. Emission spectra were obtained using an excitation maximum of 335 nm. The fluorescence intensity of the inhibitor in the presence of the virus was normalized relative to the fluorescence of the free inhibitor. B: Normalized fluorescence spectra of CL-309623 in the absence (○) and presence (□) of 1 μM purified fusion protein at 15°C. The experimental conditions are identical to those described in A. Fluorescence intensity of CL-309623 in the presence of fusion protein was normalized relative to the fluorescence of the free inhibitor.

On the basis of the above results, it is evident that blockage of virus–cell attachment and fusion is responsible for the observed antiviral activity of RFI-641 and that a significant portion of the effect has to do with inhibition of fusion. The effect of inhibitor on virus–cell binding is significantly higher in the fluorescence microscopy plate assays than the 20% observed in the suspension experiments. The difference in the extent of inhibition in the two assays could be attributed to differences in the morphology of the Vero cells attached to the plate in the microscopy experiments versus the cells in suspension in the fluorometric assays. Differences in the medium in which the cells are suspended, MEM versus PBS, could be yet another possibility. The physical state of the cells attached to the plate versus those in suspension could be significantly different in such a way that interactions with virus are altered in the two states. In any event, the observed inhibition of virus entry observed in both assays appears to be due to specific interactions of RFI-641 with the viral fusion protein. Similarly, in the biological plaque reduction assay, a 20% reduction of virus–cell binding was observed (see Section 4).

Photoaffinity labeling experiments suggest that the target of this series of compounds is the F_1 subunit of the fusion protein. This experiment showed that analogs of RFI-641 are bound predominantly (70%) to the F_1 subunit of the fusion protein with the remainder labeling the F_2 subunit [24]. Since the F_2 fragment is likely involved in attachment, the result might be interpreted as binding at the interface of the F_1 and F_2 fragments such that cross-linking occurs to a greater extent on the F_1 domain of the fusion protein. However, inhibitor binding within or adjacent to the heptad repeat sequences that are primarily involved in conformational changes in other fusion proteins cannot be excluded.

The lack of inhibition in the presence of WAY-158830 deserves comment. This could be due to lower binding affinity to the fusion protein on the virus and/or due to a non-inhibitory binding orientation. Thus RFI-641 mediates inhibition of virus entry via one or more of the following mechanisms: (i) inhibition of binding or displacement of bound virus from cells due to specific interactions of the inhibitor with fusion protein, (ii) allosteric inhibition of fusion protein thereby blocking the formation of fusion-active structures and apposition of the viral and cell membranes. Since the biological activity is mediated by interactions with fusion protein, inhibition of conformational changes of the fusion protein could be causally linked to the inhibitory mechanism.

3. Significance

Human RSV is considered to be the leading cause of upper and lower respiratory tract disease in infants and young children. It is also a common pathogen in immunocompromised adults and in the elderly. A novel class of specific inhibitors of RSV was discovered at Wyeth-Ayerst through a screening program of which RFI-641 is currently a clinical candidate. While previous biological results suggested that the fusion event was inhibited, there was no direct evidence for this mechanism of action. In this work, we provide direct evidence that RFI-641 inhibits entry of RSV into host cells via inhibition of attachment and fusion events. RFI-641 binds to the fusion protein on the viral surface, probably to a pre-fusogenic conformation. It also appears to inhibit formation of an intermediate structure along the pathway to viral fusion, thereby inhibiting virus attachment and fusion. The results reported here provide opportunities to design novel inhibitory agents of RSV due to a better understanding of molecular events of RSV–cell interactions.

4. Materials and methods

The inhibitors were synthesized as disodium salts. The inhibitors were dissolved in 100% DMSO as a 1 mM stock solution. Working stock solutions of the inhibitor were prepared by diluting the master stock solution into water to the required final concentrations.

4.1. Cells

African green monkey kidney Vero (epithelial) cells were obtained from American Type Culture Collection (ATCC). The cells were maintained in Dulbecco's modified Eagle media (DMEM), (Gibco/BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U of penicillin and 100 ng of streptomycin per ml. The cells were routinely maintained and grown at 37°C in a 5% CO_2 atmosphere.

4.2. Cell viability

The viability of infected and uninfected released Vero cells was determined by the trypan blue exclusion method as 96–98%.

4.3. Viruses

Human RSV A2 strain (wild type) was obtained from Wyeth-Lederle Vaccines, Pearl River, NY, USA. *cp-52* RSV cold-passaged mutant was kindly provided by Valerie Randolph from Wyeth-Lederle Vaccines, Pearl River, NY, USA. Mutant RSV *cp-52*, that lacks the G and SH genes, is a strain B candidate vaccine. This *cp-52* virus contains the fusion (F) protein as its only glycoprotein and is infectious in cultured cells [14].

4.4. Virus purification

Purification of RSV virions was done as previously described [25] with some modifications. Virus stock solutions were prepared by infecting Vero cells in roller bottles at a low multiplicity of infection, which is typically less than one plaque forming unit

(pfu) per cell. Infected cells were incubated at 37°C for 3–4 days until extensive syncytium formation occurred. One volume of freezing medium (MEM, 10% FBS, 1× sucrose phosphate glutamate buffer, 25 mM HEPES) was added and the bottles with virus were quickly frozen in dry ice–methanol. The infected cells were thawed quickly in a 37°C water bath, sonicated for 2 min in a Branson 1210 sonicator and Dounce-homogenized just prior to beginning the purification procedure. Cells were pelleted by centrifugation at $5000\times g$ at 4°C for 20 min. Polyethylene glycol (PEG) 6000 was added to supernatant to a final concentration of 6.5% (w/v) and the solution was stirred at 4°C for 1.5 h. The virus was collected by centrifugation of the PEG solution at $5000\times g$ at 4°C for 1 h and the pellet resuspended in NTE buffer (100 mM NaCl, 100 mM Tris–HCl, pH 7.5 and 10 mM EDTA) by Dounce homogenization. The resuspended virus was then added to a tube containing two concentration layers of sucrose in NTE buffer. Eight ml of 40% sucrose was layered on the top of the tube and 1 ml of 60% sucrose at the bottom. Upon adding virus the tube was spun at 25 000 rpm in an SW41 Ti rotor at 4°C for 2 h. The virus band at the interface of 40 and 60% sucrose was harvested, resuspended in 20% sucrose in NTE buffer and centrifuged on a 30–60% sucrose gradient at 30 000 rpm in an SW41 Ti rotor at 4°C for 2 h. The virus band was collected and stored as 50 µl aliquots, at −70°C.

4.5. Plaque assay

Viral titer was measured by a plaque assay. Serial, 10-fold dilutions of RSV A2 strain in DMEM with 2% FBS were used to infect Vero cell monolayers on 6-well plates in duplicates for 1.5 h at room temperature. After virus adsorption the inoculum was removed and infected cells were overlaid with 0.5% agarose containing 1× MEM with 2% FBS and incubated at 37°C. Plaques developed after 6 days and were fixed by treatment with 10% trichloroacetic acid for 15 min followed by 15 min staining in 0.5% crystal violet in 80% methanol–PBS. RSV titers as determined by plaque assay were: 2×10^7 pfu/ml for wild type A2 strain, and 2×10^6 pfu/ml for the *cp-52* mutant. The effect of inhibitor on virus–cell binding event was also measured by plaque assay. Approximately 200–300 pfu of the virus were combined with Vero cell monolayers on a 6-well plate in the presence of 2 µM of inhibitor, and incubated at 4°C for 30 min. After virus adsorption to cells in the presence of inhibitor, the inoculum was removed and the cells were washed with MEM. The infected cells were overlaid with 0.5% agarose containing MEM and incubated at 37°C and viral plaques estimated as described above.

4.6. Virus labeling procedure

The purified virus was labeled with the lipid probe octadecyl rhodamine B chloride, R18 (Molecular Probes Inc., Eugene, OR, USA), according to the procedure described previously [13] with slight modifications. Virus (200 µg of total protein) in 300 µl MEM was incubated with 5 µg of R18 added as an ethanolic solution (5 ml). RSV and R18 were incubated at room temperature for 1 h in the dark at ambient temperature. The labeled virus was separated from unincorporated R18 probe by gel filtration on a G-25 Sephadex microspin column (Amika Corporation, Columbia, MD, USA). The column was spun at $1500\times g$ for 4 min. The concentration of total protein in the virus preparation was determined using a bicinchoninic acid assay kit (Pierce, Rockford, IL, USA) and the procedure provided by the supplier.

4.7. CL-309623 binding to RSV and fusion protein

An aliquot of virus (2 µg equivalent in terms of total protein) was added into a 500 µl cuvette containing 1 µM of CL-309623 in PBS, pH 7.4. The virus–inhibitor mixture was incubated for 3 min with magnetic stirring. The fluorescence spectra of the inhibitor were recorded on a FluoroMax-2 (Jobin-Yvon Spex, Edison, NJ, USA) spectrofluorometer using excitation wavelength of 335 nm and emission spectral range of 400 nm to 550 nm. Fusion protein purified from RSV (see [12] for purification procedure) was diluted to a final concentration of 1 µM of protein and 0.001% of the detergent Triton X-100. In a control experiment an identical concentration of the detergent was added to PBS buffer to obtain fluorescence emission of CL-309623 in the buffer. The sample compartment was maintained at a constant temperature using a circulating water bath (Grant Instruments, Barrington, UK).

4.8. Evaluation of RSV binding and fusion by fluorescent microscopy

Vero cells in a 12-well plate containing 500 µl of MEM (∼60–70% confluent or $\sim10^6$ cells per well) were washed twice using MEM. Labeled RSV (2 µg of total protein) was added to each well to yield approximately 5×10^9 virus particles based upon estimations that used Sendai virus. The virus–cell ratio was approximately 5000 particles per cell [16].

To determine the effect of inhibitor on attachment of virus to cells, virus and inhibitor were added to each well at 4°C. Plates were incubated at 4°C for 30 min in the dark with gentle rocking. Unbound virus was removed by washing twice with ice-cold MEM. The plates were then transferred to an incubator at 37°C and incubated for 30 min to induce fusion.

To determine the effect of inhibitor on the fusion event, a 2 µg aliquot of the labeled virus was added to cells at 4°C and after incubation for 30 min in the absence of inhibitor, unattached virus was removed by washing with ice-cold MEM at 4°C. A 10 µl aliquot of a 100 µM stock solution of either RFI-641 or WAY-158830 was added to the bound virus in the wells. In the control well containing cells and attached virus, but lacking inhibitor, an equivalent volume of buffer was added. Approximately 1 min later the plate was incubated at 37°C for 30 min.

To evaluate the effect of compound on attachment and fusion events, labeled virus (2 µg) was incubated with cells similar to the procedure described for the fusion alone experiment at 37°C. After incubating at 37°C for 30 min, the unattached and unfused virus was removed by washing the cells twice with MEM at ambient temperature.

The plates containing the labeled virus attached or fused to Vero cells were imaged using a Nikon Eclipse TE200 microscope consisting of a Spot® Digital camera. The exposure time was preset to be exactly the same for each well.

4.9. RSV binding to cells in suspension

The virus cell binding assays were performed as follows: Vero cells in monolayers attached to the microtiter plate were released by incubation with 500 μl of PBS buffer in the absence of calcium and magnesium for 10 min at 37°C rather than by trypsinizing the cells. The Vero cells were resuspended in 500 μl of PBS and equilibrated to a final temperature of either 4 or 37°C. The labeled virus (equivalent to 2 μg of total protein) was added to the cells at 4 or 37°C. After incubation for 30 min, the cells with attached virus were centrifuged at $1500\times g$ for 3 min. The supernatant was pooled and collected separately. The pellet was resuspended in 500 μl of PBS. 2% SDS was added to both the supernatant and pellet fractions. The fluorescence intensity of R18 was measured at the maximum excitation and emission wavelengths of 565 and 585 nm respectively. Percentage of bound fraction was calculated as a ratio of R18 fluorescence in pellet relative to total fluorescence. The fraction of virus particles bound to cells was expressed as follows:

$$\%\ \text{of binding} = (F_{\text{pellet}}/(F_{\text{pellet}} + F_{\text{supernatant}}))\times 100\%$$

where F_{pellet} – fluorescence of R18 in pellet, $F_{\text{supernatant}}$ – fluorescence of R18 in supernatant.

In a separate experiment, virus pre-bound to cells at 4°C in a total volume of 500 μl was centrifuged to separate the unattached virus in the supernatant fraction. The pellet containing the attached virus was resuspended in 50 μl of PBS buffer. The 50 μl suspension was then added to a cuvette containing 450 μl of prewarmed solution of PBS buffer at 37°C and incubated for 30 min. The virus–cell mixture was centrifuged once again to pellet the attached and fused virus at $1500\times g$ for 3 min. 2% SDS was added to the pellet to randomize the R18 dye according to the procedure described above.

4.10. Spectrofluorometric measurements of RSV fusion by R18-dequenching assay

The fusion of R18-labeled RSV was monitored as reported earlier [15] with slight modifications. The aliquot of virus (2 μg of protein) was added directly into a cuvette containing $\sim 10^6$ cells, either in the presence or absence of an appropriate concentration of inhibitor. The inhibitor was typically added to cells 1 min before the addition of virus. After brief mixing and equilibration time (approximately 1 min later) the fluorescence of R18 at 560/585 nm excitation/emission wavelength (excitation and emission slits of 2/2 nm) was recorded at time intervals of 1 min. 2% SDS was added into the suspension to maximize R18 fluorescence. SDS detergent rather than Triton X-100 was used to randomize R18 dye because in the presence of Triton X-100 the background fluorescence was significantly higher. The virus–cell mixture was incubated with SDS for 2 min at 37°C. The final value of R18 fluorescence was measured at the emission maximum of the dye as described above.

To estimate kinetics of fusion of virus pre-bound to a cell, a slightly modified version of the R18-dequenching assay was used. The labeled virus was incubated with the cell suspension at 4°C with gentle rocking for 45 min. The cells with attached virus were then pelleted by centrifugation at $1500\times g$ for 3 min. After removing the supernatant, 50 μl of PBS was added to the pellet. The resuspended mixture in PBS was then added into a cuvette prewarmed to 37°C, either in the presence or absence of inhibitor. Inhibitor was added immediately before (no more than 1 min) addition of virus. Time-dependent changes in the fluorescence of R18 due to viral fusion were monitored as described above for 30 min. At the end of the 30 min period, 2% SDS was added to the cuvette to completely randomize the dye. Extent of dequenching (DQ) value was calculated as described previously by other investigators [26].

$$\text{DQ} = (F_{\text{f}} - F_0)/(F_{\text{det}} - F_0)\times 100\%$$

Where F_{f} – fluorescence intensity after incubation with target cells at 37°C, F_0 – initial fluorescence of virus–cell suspension after incubation at 4°C, F_{det} – fluorescence intensity after addition of detergent (maximum R18 dilution).

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