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Review Article

Respiratory syncytial virus – the discovery and optimization of orally bioavailable fusion inhibitors

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Abstract

Using a tissue cell culture assay to screen for leads, potent inhibitors of respiratory syncytial virus (RSV) that function early in the virus life cycle were discovered. Structure-activity relationship studies optimized these leads into compounds with antiviral activity in animal models of infection. Guided by *in vitro* measures of cell permeability and metabolic stability in human liver microsomes, 1-cyclopropyl-3-[[1-(4-hydroxybutyl)-1*H*-benzo[*d*]imidazol-2-yl]methyl]-1*H*-imidazo[4,5-*c*]pyridin-2(3*H*)-one (BMS-433771, **12**) was identified as a clinical candidate with potential for the treatment of RSV infections. BMS-433771 is a potent inhibitor of RSV laboratory and clinical strains in cell culture that demonstrates antiviral activity in murine and cotton rat models of infection following oral administration. The characterization of mutations in resistant viruses generated in response to serial passage with increasing concentrations of this class of RSV inhibitor, coupled with mechanistic studies, indicated interference with the virus-host fusion process. A radioaffinity probe was developed which, upon irradiation with light to generate a carbene, specifically labeled the RSV F protein in intact virus in a region mapped to the *N*-terminal heptad repeat element HR-N. *In vitro* labeling experiments conducted with a portion of the HR-N known to be involved in 6-helix bundle formation identified the major site of labeling as tyrosine-198. This finding localized the binding of these RSV inhibitors to a hydrophobic cavity created in the HR-N element after it assembles into a trimeric fusion core that subsequently associates with the *C*-terminal heptad repeats during the final stages of virus and host membrane fusion.

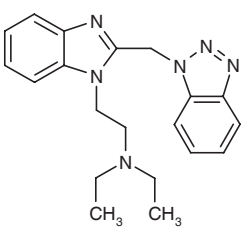
Introduction

Since its original isolation from infected infants in 1957 and subsequent characterization, respiratory syncytial virus (RSV) has emerged as an important human pathogen (1-9). Although it circulates predominantly in the winter season, RSV is also present in the summer months (4, 5). RSV infection is prevalent during the early years of life and virtually all children are infected by their second birthday. However, immune protection is of limited durability and re-infection is common, not only later in childhood but also in adults and the elderly (10-16). Indeed, a recent study that focused specifically on the elderly population estimated annual RSV infection rates of 3-7% in healthy individuals and 4-10% in high-risk groups (14). Since RSV infection is often misdiagnosed as influenza, the disease burden and associated morbidity and mortality are considered to be significantly underestimated (17-21). Recent estimates indicate that mortality due to RSV infection accounts for approximately one-third of that attributed to influenza virus infection, with the elderly and children under 1 year of age the population groups most affected (19). In the majority of individuals, RSV infection is restricted to the upper respiratory tract and recovery is not associated with significant, long-lasting effects. However, progression to a more serious lower respiratory tract infection is particularly problematic in patients with underlying cardiopulmonary problems or compromised immune function (22-27). Indeed, RSV is an important cause of hospitalization for infants with bronchiolitis and a significant problem in bone marrow transplantation units, where fatality rates have been high (24-26).

The development of a protective vaccine for RSV has been particularly challenging, following an early attempt to use formalin-inactivated virus, which was found to exacerbate rather than prevent infection (28, 29). However, the advent of reverse genetics, which allows precise manipulation of the RSV genome, has heightened anticipation that a safe and effective vaccine will be developed (30). In the meantime, a prophylactic approach that relies upon passive immunization with a monoclonal antibody (MAb) directed towards the RSV F protein has proven quite successful. Palivizumab, a humanized MAb market-

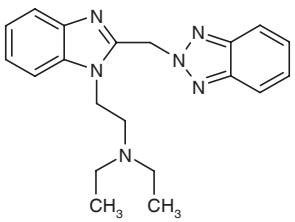
ed in the U.S. in 1998 by MedImmune as Synagis®, is an effective, although expensive, prophylactic agent administered as a series of monthly intramuscular injections to those at significant risk of the consequences of an RSV infection (31, 32). However, due to several factors, Synagis® administration is restricted to high-risk children during their first two winter seasons of life. The only therapeutic agent licensed for the treatment of RSV infection is the nucleoside analogue ribavirin, a compound with a broad spectrum of antiviral activity and an enigmatic mode of action. Ribavirin is administered as an aerosol to treat RSV infections, but the problematic mode of administration, limited efficacy and teratogenicity of the molecule have restricted its widespread application beyond infants considered to be at high risk (33, 34). Consequently, there is a clear medical need for effective therapeutic options that can treat the entire at-risk population, including adults and the elderly.

The dearth of effective therapeutic options for the treatment of RSV in concert with a deeper appreciation of its role in disease pathology has provided an impetus to identify potent and selective antiviral agents with the potential to be developed into clinically useful drugs (35-38). It was against this evolving background that an initiative was implemented at Bristol-Myers Squibb to identify inhibitors of RSV.



1

EC_{50} = 0.47 μM
CC_{50} = 216 μM



2

EC_{50} = 0.22 μM
CC_{50} = 83 μM

Development of antiviral agents for RSV

A tissue culture screen was conducted using the Long A strain replicating in the HEp-2 human epithelial cell line to survey the corporate compound inventory. Substances were evaluated for their ability to protect HEp-2 cells against virus-induced cytopathic effects, and compounds scoring as inhibitors were profiled against a panel of viruses and biochemical assays designed to establish specificity. This process culminated in the identification of a series of disubstituted benzimidazole derivatives as promising lead structures. These compounds, represented by the prototypes **1** and **2**, originated as potential analgesic and antiarrhythmic agents that had resided in the Bristol-Myers Squibb compound archive for over 30 years (39, 40). Both compounds demonstrated potent antiviral activity towards RSV in cell culture, with half-maximal inhibition observed at concentrations an order of magnitude lower than that of ribavirin and without overt cytotoxicity at concentrations over 400-fold higher than their inhibitory activity (ribavirin displayed an EC_{50} of 2.7 μM and a CC_{50} of 34 μM) (41). More importantly, neither compound had demonstrated significant biological activity in an extensive series of screening campaigns conducted over many years, while their structural simplicity and drug-like properties added to their attractiveness as lead structures for optimization (42). Simple time-of-addition experiments established that **2** interfered with replication at an early point in the virus life cycle, and a more detailed analysis ruled out inhibition of attachment of RSV to host cell membranes (43). A temperature-shift experiment in which adsorption of RSV to HEp-2 cells was permitted by incubating at 4 °C for 2 h, followed by the addition of drug and warming of the mixture to 37 °C to allow virus-host cell fusion, established that **2** interfered with the fusion step of the virus entry process (43).

An initial survey of structure-activity relationships focused on defining the scope of opportunity to vary the dialkylaminoalkyl side-chain (44). This exercise revealed a broad tolerance for both polar and lipophilic functionality at the chain terminus, provided that it was projected from the heterocycle core by 2 atoms of separation. A sampling of this survey is presented in Figure 1, with the results providing confidence that the physical properties of these molecules would be amenable to modulation without adversely effecting antiviral potency (44). Because the synthetic approach produced small amounts of the 2-substituted benzotriazole derivatives in addition to the 1-isomers, it was also established that the topological relationship of this ring with respect to the substituted benzimidazole moiety was not critical, since, in those cases examined, both isomers were found to be essentially equipotent, as summarized in Figure 2 (44).

In the next phase of the structure-activity survey, substitutes for the benzotriazole moiety were examined with a view towards introducing opportunities to probe both functionalization of the heterocycle element and substitution of the fused phenyl ring. A benzimidazol-2-one was initially selected for this purpose based on its synthetic accessibility and ease of decoration, a tactic that proved to be both informative and productive (45). This heterocycle was quickly established as an advantageous structural element, providing the opportunity to probe a substituent vector unavailable to **1** and producing a series of

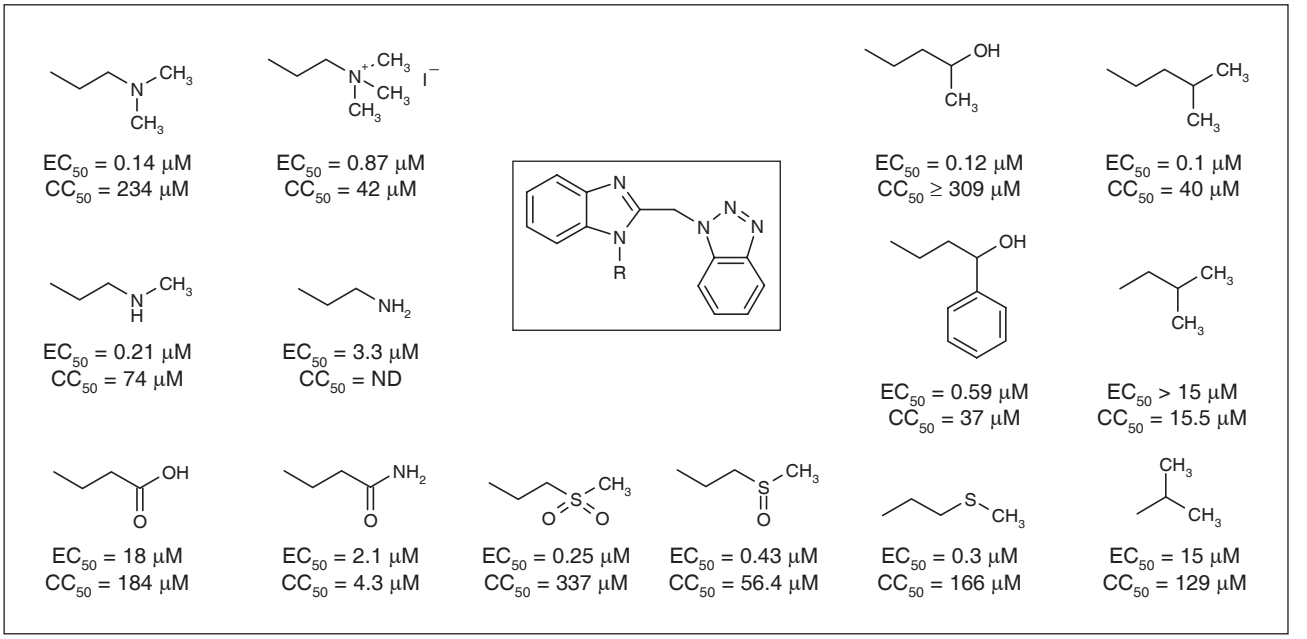


Fig. 1. A synopsis of the structure-activity relationships (SAR) associated with the benzimidazole side-chain of benzotriazole-based inhibitors of respiratory syncytial virus (RSV). EC_{50} indicates the concentration of drug required to inhibit virus replication in HEp-2 cells in cell culture, while CC_{50} is the concentration of drug which is cytotoxic to 50% of uninfected host cells.

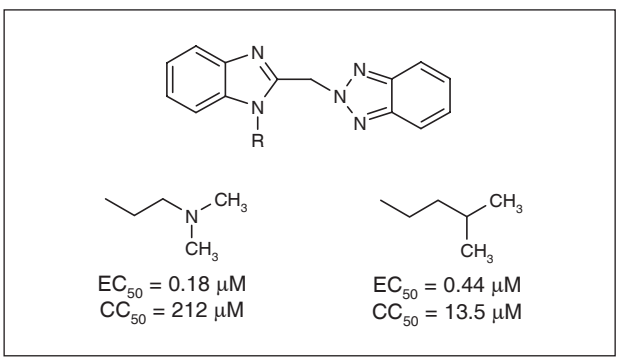
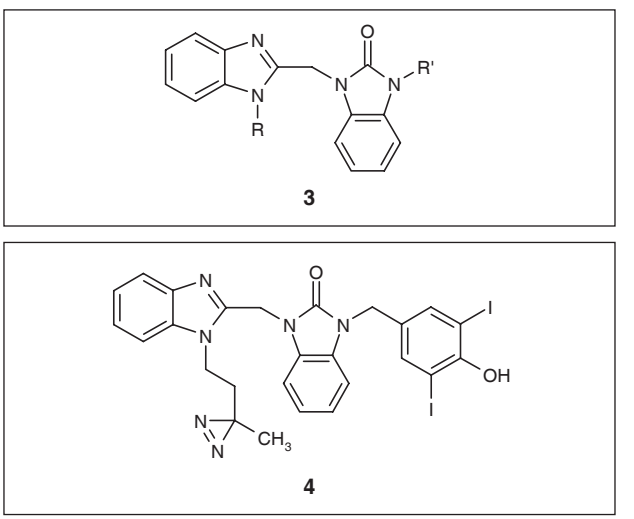


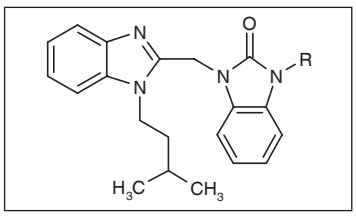
Fig. 2. Respiratory syncytial virus (RSV)-inhibitory activity associated with 2-substituted benzotriazole derivatives. EC_{50} indicates the concentration of drug required to inhibit virus replication in HEp-2 cells in cell culture, while CC_{50} is the concentration of drug which is cytotoxic to 50% of uninfected host cells.



molecules, generically represented by **3**, that demonstrated potent inhibition of RSV *in vitro* (46).

The preliminary query of this chemotype was conducted using an isoamyl side-chain attached to the benzimidazole nucleus (R in **3**), since this moiety combined excellent potency with chemical inertness, permitting application of a wide range of synthetic chemistries. The structure-activity relationships surrounding the benzimidazol-2-one *N*-substituent (R' in **3**) revealed a remarkable tolerance for diverse chemical functionality that could be incorporated in a range of topological presentations (46, 47). A synopsis of these studies is presented in Figure 3 where, of particular note, there is a clear compatibility of acidic functionality with a high level of antiviral activity, providing that the acidic moiety is remote from the core heterocycle. This discovery proved to be of importance in the context of demonstrating antiviral activity *in vivo*, as described in greater detail below (47). Subsequent variation of the benzimidazole side-chain (R in **3**) established correspondence with the SAR for this element in the context of the benzotriazole series and markedly expanded the opportunity to modulate and optimize physical chemical attributes and, ultimately, pharmacokinetic properties, without sacrificing antiviral potency (46, 47).

Another important compound to emerge from this aspect of the SAR survey was the diazirine derivative **4**, a potent RSV inhibitor that, when labeled with ^{125}-iodine, proved to be an effective photoaffinity probe (46, 48). A



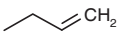
	— CONHPh	— SO_2CH_3	— $CH_2p\text{-}C_6H_4SO_2NMe_2$	— CONHEt
EC_{50} = 0.004 μM CC_{50} = 25 μM	EC_{50} = 0.041 μM CC_{50} = 22 μM	EC_{50} = 0.007 μM CC_{50} = 16 μM	EC_{50} = 0.004 μM CC_{50} ≥ 152 μM	EC_{50} = 0.052 μM CC_{50} = 19 μM
— CH_2CO_2H	— H		— CH_2CH_3	— $CH_2p\text{-}C_6H_4CN$
EC_{50} = 0.163 μM CC_{50} = 194.3 μM	EC_{50} = 0.057 μM CC_{50} = 61 μM	EC_{50} = 0.016 μM CC_{50} = 13 μM	EC_{50} = 0.01 μM CC_{50} = 18 μM	EC_{50} = 0.012 μM CC_{50} = 5.6 μM
— $CH_2CH_2NMe_2$	— $CH_2CH_2CO_2H$	— $(CH_2)_4CO_2H$	— $CH_2CH_2CH_2NMe_2$	— $CH_2p\text{-}C_6H_4OH$
EC_{50} = 0.400 μM CC_{50} = 10 μM	EC_{50} = 0.015 μM CC_{50} = 102.1 μM	EC_{50} = 0.008 μM CC_{50} = 16.5 μM	EC_{50} = 0.292 μM CC_{50} = 5.3 μM	EC_{50} = 0.018 μM CC_{50} = 59.9 μM

Fig. 3. A synopsis of the structure-activity relationships (SAR) associated with the benzimidazol-2-one side-chain of inhibitors of respiratory syncytial virus (RSV). EC_{50} indicates the concentration of drug required to inhibit virus replication in HEp-2 cells in cell culture, while CC_{50} is the concentration of drug which is cytotoxic to 50% of uninfected host cells.

series of experiments conducted with this tool molecule, described in detail below, have provided crucial insights into the mode of action of this class of RSV fusion inhibitors.

The antiviral potency associated with the benzimidazol-2-one series coupled with their synthetic plasticity led to their adoption as the structural theme for further study. At this point in the program, a key objective was to demonstrate antiviral activity *in vivo* in a model of RSV infection. Several animal species have been examined as hosts of RSV infection, but none fully reproduce the spectrum of disease observed in humans (49, 50). The cotton rat and BALB/c mouse have been established as permissive hosts for RSV following intranasal administration of virus, with titers typically reaching a maximum 4 days after inoculation (49-54). These models have been used widely to evaluate RSV inhibitors *in vivo* and the cotton rat was used to establish the efficacy of monoclonal antibodies and ribavirin as a prelude to clinical studies (55-57). Both the cotton rat and BALB/c mouse models of RSV infection were established in-house and used to evaluate inhibitors using several drug dosing paradigms, including oral, intraperitoneal and subcutaneous administration, the latter designed to facilitate a slow dissolution of drug to prolong exposure. However, this proved to be a significant challenge with early compounds in the series, and it was decided to explore the potential of topical drug delivery to cotton rats using a small particle aerosol (SPA) in order to establish antiviral activity *in vivo* (57, 58). The formulation of compounds for SPA delivery demands high aqueous solubility and attention was focused on the series of carboxylic acid derivatives described above, many of which demonstrated aqueous solubility in excess of 10 mg/ml. Several compounds were identified that demonstrated antiviral activity in this setting, summarized in Figure 4, where the concentration reported is the concentration of the drug in the aerosol solution (47). The phosphonic acid **5** and the aspartate derivative **7** demonstrated robust efficacy when administered by SPA at concentrations of 2 and 1.35 mg/ml, respectively, and **7** was similarly effective when the aerosol concentration was reduced to 0.2 mg/ml (47). The oxadiazolone **6** was subsequently examined in the BALB/c mouse model of infection, administered subcutaneously at 120 mg/kg b.i.d. beginning 1 h postinfection, where it reproducibly reduced RSV titers in lung homogenates (47). This result was important because it established the potential of these RSV fusion inhibitors to demonstrate antiviral activity following systemic administration, indicating successful delivery of drug to lung tissue.

Encouraged by the successful result with **6**, the basic dimethylamine derivative **9** was selected for examination in the BALB/c model, recognizing that the carboxylic acid **10** would very likely be formed *in vivo* as a metabolite. Subcutaneous administration of **9** at a dose of 50 mg/kg b.i.d. to BALB/c mice infected with RSV reproducibly reduced viral titers measured on day 4 postinoculation (47). In a subsequent experiment, **9** was administered orally to infected mice under the same dosing protocol and also proved to be effective, providing the first orally active compound in the program (47).

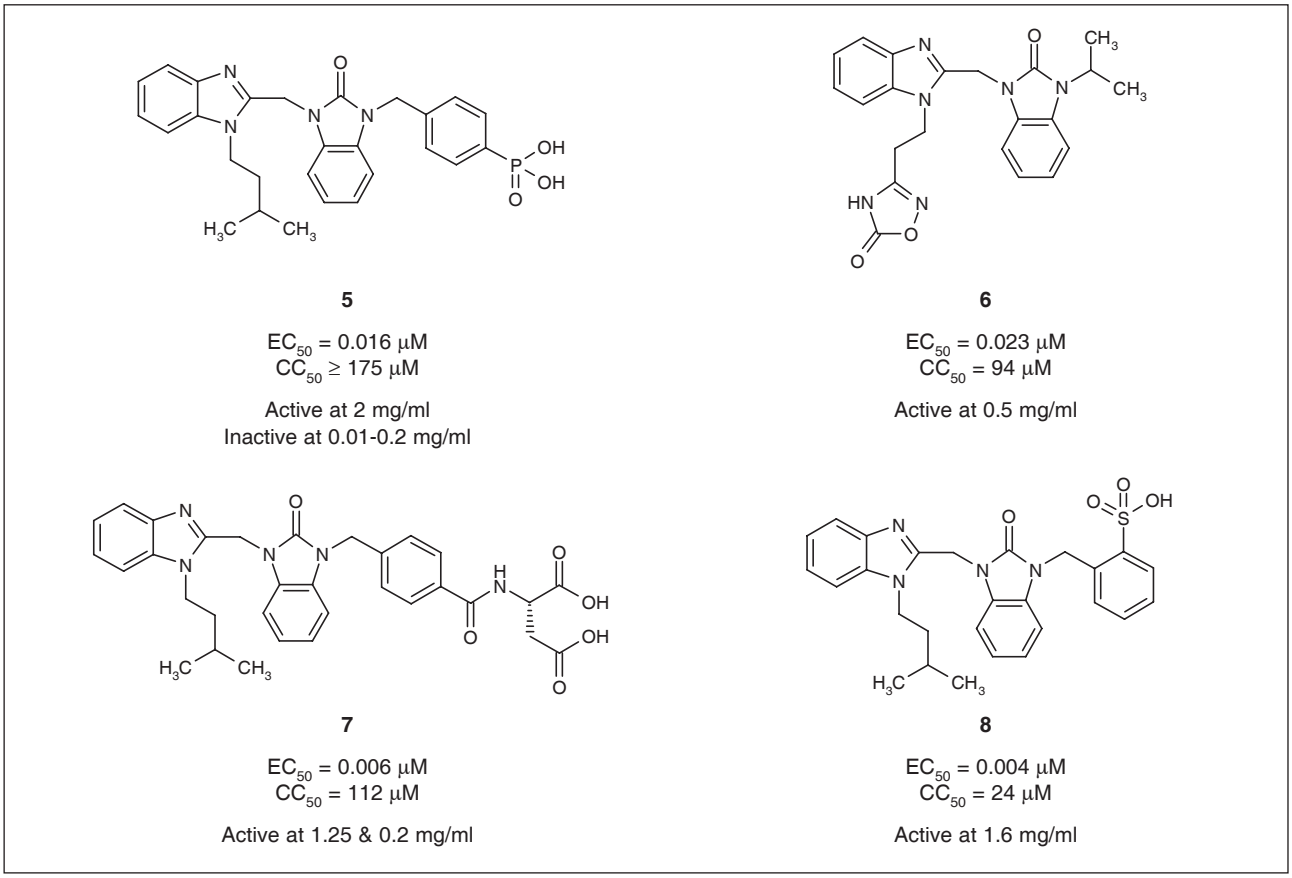
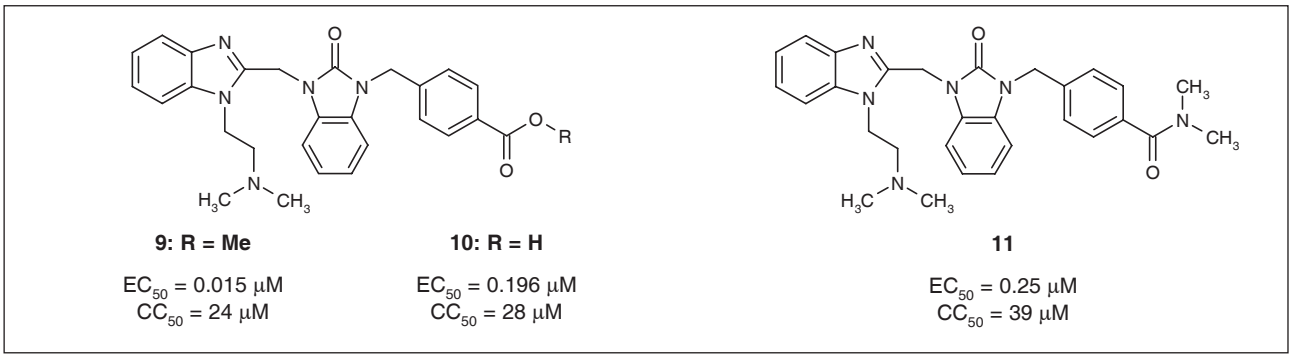


Fig. 4. Compounds active in the cotton rat model of respiratory syncytial virus (RSV) infection with the Long strain following delivery of drug by small particle aerosol (SPA). The concentration of the drug in the aerosol solution that provided a significant reduction in RSV titers in rat lungs is reported below each structure. Infected cotton rats were continuously exposed to drug-containing aerosol for 21 h a day for 4 consecutive days beginning 1 h after virus inoculation.

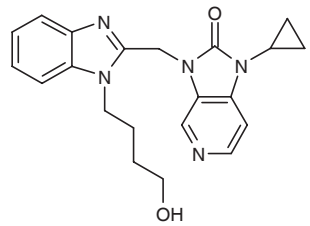


The acid **10** was indeed observed to be readily produced *in vivo* and oral administration of this compound to mice at a dose of 50 mg/kg b.i.d. produced a small reduction in RSV titers, although this was not statistically significant. In anticipation of enhanced metabolic stability, the dimethylamide derivative **11** was the next example selected for *in vivo* evaluation, and this compound produced significant reductions in viral titers after doses of 50 and 15 mg/kg when administered p.o. on a b.i.d. dosing regimen (47).

While the amide moiety of **11** appeared not to be cleaved to the carboxylic acid in mouse and human liver microsomal preparations, the molecule was found to be extensively metabolized by oxidative pathways. Demethylation of the nitrogen atoms of both the amine and amide and hydroxylation of both heterocyclic rings were identified as the major metabolic pathways (47). This observation defined a clear objective, and attention was focused on enhancing metabolic stability while maintaining absorption, a process guided by evaluating

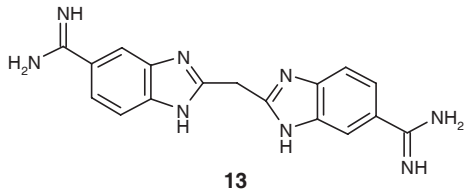
compounds for both stability in liver microsomes and permeability across a Caco-2 monolayer, *in vitro* assays that proved to be predictive of *in vivo* properties. The introduction of a nitrogen heteroatom into the 6-position of the benzimidazol-2-one ring provided one solution to that metabolically labile site, fortunately without introducing a potential liability associated with cytochrome P-450 (CYP450) inhibition (59, 60). A small substituent attached to the cyclic urea nitrogen, attractive from a physicochemical perspective, further advanced the chemotype, with the cyclopropyl moiety providing an effective combination of metabolic stability and potency. The structural refinement was completed with optimization of the terminus of the benzimidazole side-chain, where a primary alcohol moiety satisfied the targeted criteria, leading to the identification of BMS-433771 (**12**) as a clinical candidate (41, 61, 62).

The antiviral activity of BMS-433771 *in vivo* was established in both the BALB/c mouse and cotton rat models of RSV infection, where it was effective in reducing virus titers in lung homogenates when administered 1 h prior to intranasal inoculation with virus (61). Perhaps reflecting fundamental aspects associated with virus replication and pathology in these models, neither of which fully recapitulates the spectrum of disease observed in humans infected with RSV, a single dose of drug delivered prior to virus inoculation was found to be as effective as a multiple-dose regimen in which BMS-433771 was administered for 4 days on a b.i.d. schedule (61). This observation ultimately allowed for a simpler protocol for *in vivo* efficacy studies, in which a single dose of drug was administered p.o. 1 h prior to inoculation with virus. Using this dosing paradigm, a dose-efficacy relationship was established in both species, with the maximal effect of a > 1 $\log_{10}$ reduction in viral titers observed at doses > 5 mg/kg in BALB/c mice and > 50 mg/kg in the cotton rat. The higher dose requirements in the cotton rat appear to reflect pharmacodynamic differences between the two species, since the cotton rat is a more permissive host for RSV infection than the BALB/c mouse (61). That the antiviral effect of BMS-433771 *in vivo* reflected inhibition of RSV fusion was established by infecting BALB/c mice with a Long strain of RSV incorporating a K394R substitution in the F protein, a change that confers resistance to BMS-433771 *in vitro*. Doses of 50 mg/kg of BMS-433771 administered b.i.d. beginning 1 h prior to inoculation with this mutant virus were ineffective at reducing virus titers in lung homogenates harvested 4 days later (61). Moreover, in BALB/c mice immunosuppressed by treatment with cyclophosphamide, BMS-433771 retained full efficacy against wild-type virus, indicating that the antiviral activity *in vivo* was not dependent on an intact immune system. However, attempts to demonstrate therapeutic efficacy were not successful, perhaps reflecting unique aspects associated with the pathogenesis of RSV infection in rodents and consistent with the finding that a single dose of drug was as effective *in vivo* as multiple doses administered over 4 days (61).



12 (BMS-433771)

EC_{50} = 0.010 μM
$CC_{50} \geq$ 218 μM



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Mechanistic insights and implications

The generation of viruses resistant to this class of RSV fusion inhibitors allowed for mapping of the substitutions responsible for resistance, the results of which strongly implicated the RSV F protein as the biochemical target (41). Mutations conferring resistance to BMS-433771 included K394R, found within the cysteine-rich region of the F1 subunit of the F protein, and D489Y, a residue located in the heptad repeat associated with the carboxy (*C*) terminus (41). These viruses demonstrated over 1,000-fold reduced sensitivity to BMS-433771. Interestingly, resistant RSV emerging in response to increasing doses of BABIM (**13**), a bis-benzimidazole derivative that has demonstrated RSV-inhibitory activity and which is also a potent inhibitor of a range of trypsin-like proteases, incorporated an F140I substitution in the fusion peptide region of the F protein. Virus expressing the F140I substitution was also found to be cross-resistant to BMS-433771, suggesting similarity in their modes of action (41, 63-66).

More detailed insights into the mechanism of action of BMS-433771 evolved through the application and analysis of photoaffinity labeling experiments employing the diazirine derivative **4** (48, 67). Irradiation of diazirines with ultraviolet light liberates nitrogen and generates a highly reactive carbene moiety that is capable of inserting into many of the bonds found in amino acid residues of proteins (68). Conducting this kind of experiment with [^{125}I]-labeled **4** in the presence of virus resulted in the covalent labeling of only the F1 subunit of the fusion protein (67). Labeling was inhibited in a concentration-dependent fashion by incubation in the presence of BMS-433771, establishing correspondence of the binding sites of the two molecules. Using the covalently linked radiolabel as a guide, cyanogen bromide digestion of the F1 subunit produced a smaller, 11-13-kDa radiolabeled fragment consisting of

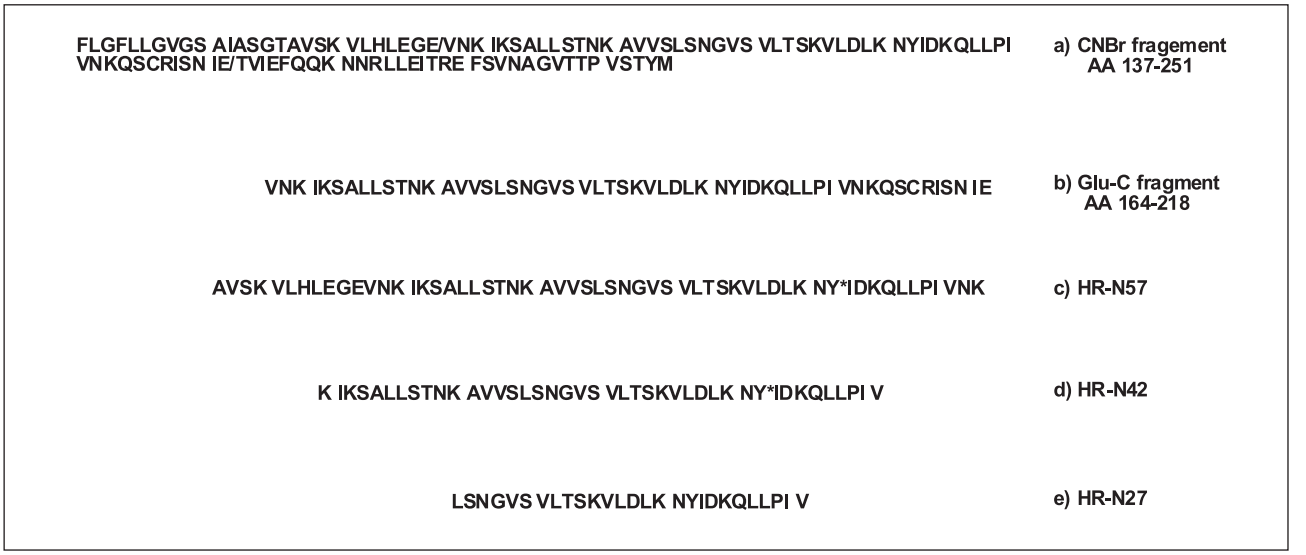


Fig. 5. Respiratory syncytial virus (RSV) F protein fragments labeled with [^{125}I]-**4** after digestion with CNBr (**a**) and proteolysis with Glu-C (**b**). The identity of the labeled fragments in (**a**) and (**b**) was determined through size determination and sequencing from the amino terminus. The HR-N57 (**c**) and HR-N42 (**d**) peptides were used in biochemical labeling experiments to identify Tyr198 as the predominant amino acid labeled by [^{125}I]-4, while (**e**) is the structure of HR-N27, the short peptide sequence that fails to adopt a helical structure. The labeled tyrosine is marked by an asterisk in (**c**) and (**d**).

amino acids 137-251 (Fig. 5a). This 115-residue sequence encompasses elements of both the fusion peptide and the *N*-terminal heptad repeat. Proteolytic cleavage of labeled F1 protein using the endoprotease Glu-C (*Staphylococcus aureus* V8 protease), which specifically cleaves peptides at the carboxyl moiety of glutamate residues, produced a 5-7-kDa radiolabeled fragment comprising amino acids 164-218, further narrowing the site of labeling to the *N*-terminal heptad repeat sequence (Fig. 5b). With the labeling of the F1 subunit narrowed to a region that had previously been studied under biochemical conditions, subsequent experiments were conducted using synthetic peptides (67). In a reaction promoted by the presence of the RSV fusion inhibitor RFI-641 (**14**) (50 μM), a compound that binds to a different site of the RSV fusion peptide, or, to a lesser extent, 20% trifluoroethanol, the heptad repeat peptide HR-N57 (Fig. 5c) that comprises the RSV fusion core was labeled by **4** (67, 69-74). The role of both RFI-641 and trifluoroethanol is considered to be stabilization of the helical trimeric form of the heptad repeat peptide, thereby promoting inhibitor binding (67). Labeling under these circumstances was blocked in the presence of BMS-433771, suggesting specificity for this reaction. A shorter peptide, HR-N42, which contains the *N*-terminal heptad repeat but is truncated largely at the amino terminus (Fig. 5d), was also covalently labeled by **4**. However, a much shorter F peptide fragment, designated HR-N27 (Fig. 5e), was not labeled under identical conditions. This could be explained by the markedly reduced propensity of this peptide to adopt an α-helical structure, as determined by CD spectroscopy. Sequencing of the labeled HR-N42 peptide coupled with mass spectroscopic analysis of fragments revealed that Tyr198 was the amino acid predominantly labeled by the carbene moiety of labeled **4**, although several adjacent residues were also observed to have incorporated radioactivity (67). This result localized the binding of these RSV fusion inhibitors to a hydrophobic cavity formed in the *N*-terminal heptad repeat element that accommodates Phe483, Phe488 and Ile492 of the *C*-terminus heptad repeat in the assembled fusion core, as observed in the X-ray crystallographic structure depicted in Figure 6 (69).

Computer-aided docking and molecular dynamics simulations were used to evaluate a series of potential binding modes for these RSV inhibitors in the hydrophobic cavity, with the 100 best scoring possibilities clustered into 6 distinct binding modes (67). Additional molecular dynamics analysis using these six poses as the starting point identified two binding postulates in which the test compound bound solely to the hydrophobic pocket, with preference ultimately given to the pose in which the diazirine moiety is proximal to Tyr198. This hypothesis, depicted in Figure 7, places the benzimidazol-2-one heterocycle of **4** in the site that is normally occupied by Phe488 after the *N*- and *C*-terminal heptad repeats have assembled to form the 6-helix bundle associated with membrane fusion. By extrapolation, the pyridine element of BMS-433771 would be buried deep into the groove created by the juxtaposition of two of the HR-N helices and adjacent to Leu195. In this disposition, the diiodophenol moiety of **4** effectively mimics Ile492 of the *C*-terminal helix, while the benzimidazole heterocycle is accommodated in the pocket occupied by Phe483. Small variations in this mode of binding provide a potential explanation for the observation that adjacent amino acids are also labeled by the photoaffinity probe (67).

An understanding of the mechanism by which paramyxoviruses and other viruses with type 1 fusion pro-

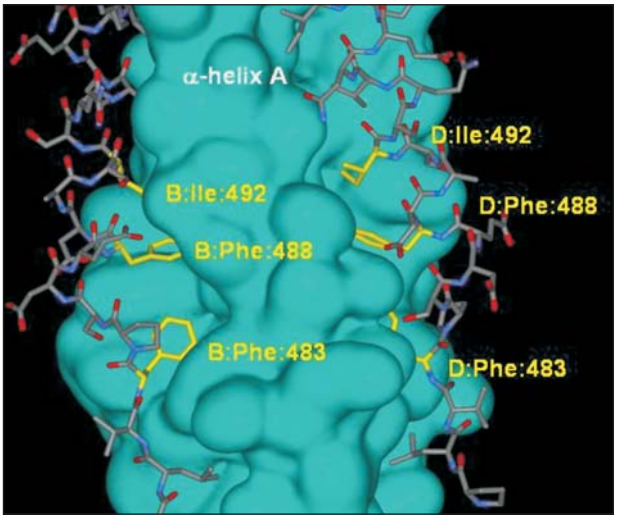


Fig. 6. Details of the association of the key residues of the HR-C (stick display) with the trimeric assembly of HR-N (turquoise surface) of the RSV F protein in its fusion-competent state. The side-chains of the key residues Phe483, Phe488 and Ile492 of HR-C that associate with the hydrophobic pocket of HR-N are highlighted in yellow.

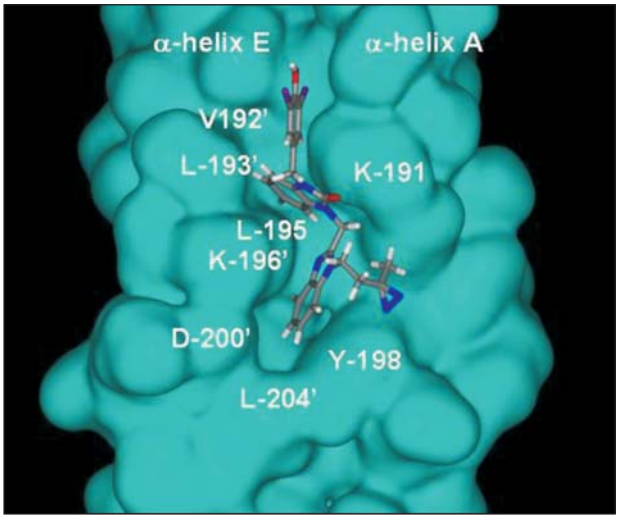
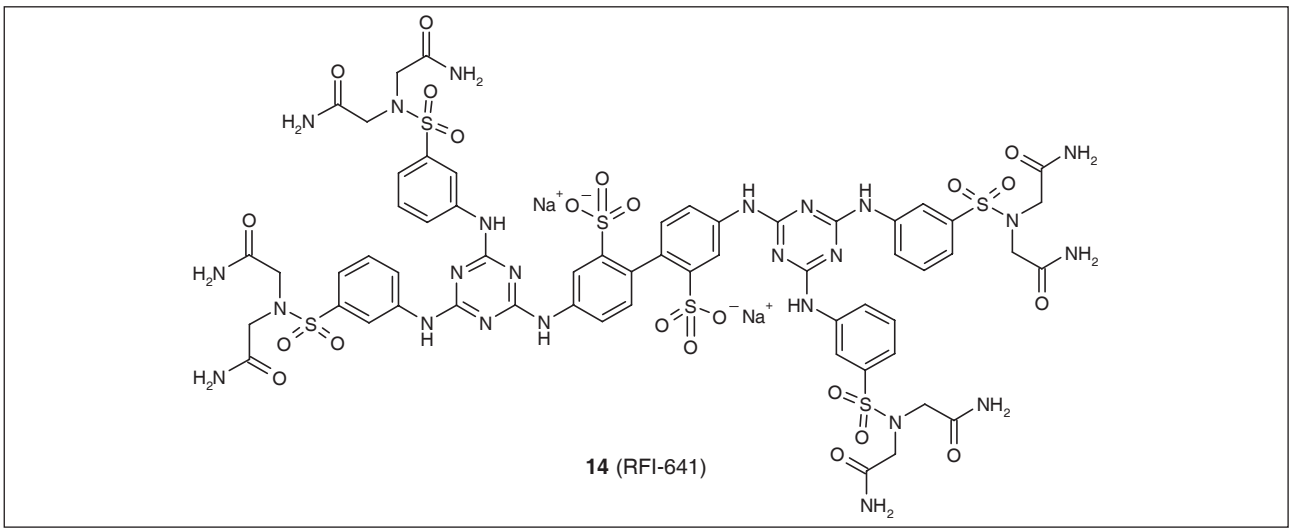


Fig. 7. Model of compound **4** docked into the proposed binding site in the trimeric assembly of the HR-N peptide of the respiratory syncytial virus (RSV) F protein. The amino acids of the F protein that line the binding site are noted. Y-198 is the amino acid residue predominantly labeled by **4**. The view in this Figure is looking directly down into the hydrophobic cavity and is different from the perspective presented in Figure 6, which presents a more oblique view of the pocket.



14 (RFI-641)

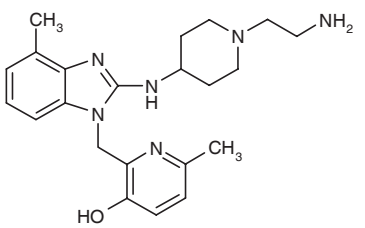
teins, in which the *N*-terminus is extracellular and the *C*-terminus intracellular, enter host cells has emerged in recent years (75-77). Analysis of the influenza hemagglutinin protein has yielded the most detailed insights, providing a mechanistic paradigm that has been extrapolated to many other viruses, including the retroviruses HIV-1 and Moloney murine leukemia virus, Ebola virus, the SARS coronavirus and paramyxoviruses, including RSV, parainfluenza virus 5 (SV5) and human parainfluenza viruses (75-79). In general, the fusion proteins of these viruses are deployed on the viral membrane in a metastable form following protease-mediated cleavage at the *N*-terminal side of the fusion peptide. The fusion peptide is a sequence of 10-12 hydrophobic amino acids that exhibits remarkable similarity in composition across several virus families (80). Activation of viral fusion proteins leads to a conformational rearrangement, in which the fusion peptide is inserted into the host cell membrane with concomitant assembly of the *N*-terminal heptad repeat into a trimeric core. Activation can be promoted by low pH, as exemplified by influenza, the engagement of a co-receptor, as observed with HIV-1, or triggers that remain to be elucidated, as is the case with many viruses, including the paramyxovirus family. The assembled *N*-terminal heptad repeat moiety subsequently associates with the *C*-terminal heptad repeat to form the 6-helix bundle core that is characteristic of the post-fusion conformation, a process considered to draw the virus and host

cell membranes into close proximity as a prelude to membrane convergence.

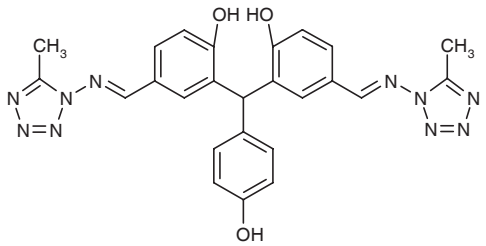
Obtaining interpretable structural data for fusion proteins from paramyxoviruses has proven to be considerably more difficult than for other viruses, possibly a consequence of inherent fragility of the fusion proteins. It is only very recently that critical insights have been obtained that provide some illumination of the mechanism of paramyxovirus F protein action (81-89). Particularly informative is the recently elucidated crystal structure of the SV5 F protein in the prefusion conformation, which provides an interesting contrast with the structures of the human parainfluenza 3 and Newcastle disease virus fusion proteins, both of which appear to have adopted a post-fusion conformation, despite being uncleaved at the protease cleavage site essential for activation (82, 87, 89). The trimeric SV5 F structure reveals the HR-C element to be associated in a parallel fashion, whilst the HR-N heptad repeats are incorporated into the head of the protein, distal from the virus membrane, and both discrete and disordered when compared to their final form (89). The model developed to explain the fusion process based on these comparisons envisages a dissociation of the HR-C with subsequent exposure of the fusion peptide. Movement of the F protein in this fashion allows the fusion peptide to project into the host cell membrane in a process that occurs concomitantly with the assembly of the HR-N element into a trimeric, coiled coil structure. This intermediate bridges the virus and host cell membranes and sets the stage for association of HR-C with HR-N, drawing the membranes together in association to form the 6-helix bundle arrangement that is thought to be closely linked to both membrane fusion and pore formation.

Discordant with the crystallographic data of the closely related SV5 F protein, the experimental observation with the carbene derived from **4** is that it is able to label the RSV fusion protein in intact, infectious virus. This implies that in the native, metastable, pre-fusion state, the RSV F1 peptide *N*-terminal helices are assembled into a trimeric form that is similar, if not identical, to that observed in the post-fusion core structure. Exposure of RSV to these inhibitors does not appear to result in an irreversible change in the F protein, since infectivity of the virus can be straightforwardly restored after incubation with BMS-433771 or structurally related compounds by simply washing away the inhibitor (43). Peptides based on the HR-N sequence of paramyxoviruses inhibit fusion when added to virus prior to F protein activation by proteolytic cleavage, but these compounds are ineffective when added after proteolysis. This experiment suggests that association of HR-N with HR-C is possible in the native state, but not after proteolytic activation. In contrast, HR-C peptides are inhibitory irrespective of the time of addition. Taken together with the propensity of paramyxovirus fusion proteins to adopt a post-fusion conformation when crystallized, the data suggest that the structure of the paramyxovirus fusion protein in its proteolytically activated, metastable form is different from that observed in the uncleaved state. Proteolytic activation presumably allows the fusion protein to partially rearrange by progressing along the trajectory towards a fusion-competent state, perhaps with some dissociation of the HR-C complex. The actual metastable form may have the HR-N partially or fully assembled but without overt exposure of the fusion peptide. This model provides an explanation for the poor inhibitory efficacy of HR-N peptides when added after F protein cleavage and the labeling of native virus by **4**, and also suggests that paramyxovirus fusion proteins are exquisitely poised for activation, consistent with the observation that RSV lacking the G-protein can enter cells with comparable efficiency to native virus (93-96).

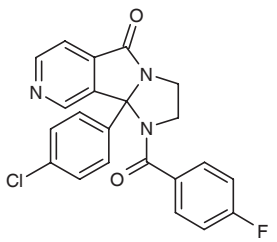
While these studies provided compelling insight into the identity of the binding site for this class of RSV fusion inhibitors, the precise mechanism by which BMS-433771 and its homologues interrupt the virus fusion process in the activation pathway remains to be completely elucidated. Nevertheless, aspects of the binding model were probed in a predictive fashion with the design of analogues capable of establishing and exploiting additional interactions within the pocket. In the preferred pose, the C-5 and C-6 atoms of the benzimidazole ring are in close proximity to Asp200, providing a particularly interesting opportunity to take advantage of a potential salt bridge interaction with the introduction of complementary functionality. Indeed, the installation of basic moieties at C-5 of the benzimidazole heterocycle provided compounds with increased potency, most effectively demonstrated when evaluated against RSV selected to be resistant to inhibitors lacking a C-5 substituent. In particular, an amidine moiety at C-5 affords a potent RSV inhibitor with excellent activity towards virus resistant to BMS-433771, while installation of a carboxylate at this site gave a compound devoid of significant antiviral activity (97). This result allowed a deeper appreciation of the similarity of the silhouettes cast by this chemotype and BABIM, particularly striking for benzotriazole **2**. Taken in conjunction with the observed cross-resistance profiles, BABIM may act in a similar fashion, engaging in an interaction with Asp200 that could compensate for the absence of the side-chain element so important to the potency of **2** and its congeners. More recently, two structurally distinct RSV fusion inhibitors, JNJ-2408068 (**15**) and VP-14637 (**16**), have also been postulated to bind to this pocket, based on analysis of mechanistic and resistance mapping studies (98-100). Both of these compounds have demonstrat-



15 (JNJ-2408068)

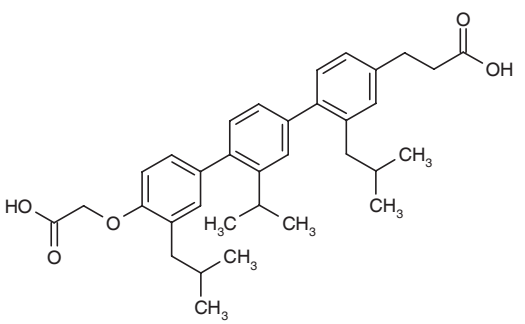


16 (VP-14637)



17

EC_{50} = 100-250 ng/ml



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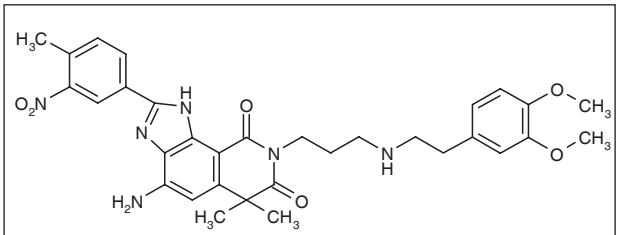

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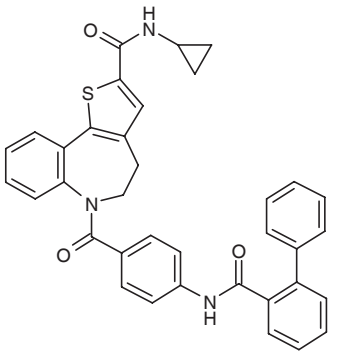
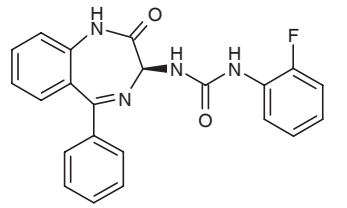


20 (YM-53403)



21 (A-60444)

ed efficacy in rodent models of RSV infection following topical administration as SPAs (101, 102).

Adding further to the structural diversity of RSV fusion inhibitors, Biota recently disclosed a series of imidazo[2,1-*a*]isoindol-5(9b*H*)-one derivatives that are being co-developed with MedImmune (103). A representative compound, **17**, inhibited the RSV A2 strain in HEp2 cells in culture with an EC_{50} of 100-250 ng/ml and was active in a fusion assay with an IC_{50} of < 750 ng/ml. These compounds are also claimed to attenuate RSV infection of cotton rats when administered at a dose of 100 mg/kg 2 h prior to virus inoculation (103).

While there appear to be several structurally distinct chemotypes capable of interfering with 6-helix bundle formation of the RSV F protein, inhibition of other viruses by this mechanism appears to be more challenging, presumably a function of the nature of individual binding sites and HR-C and HR-N interactions (79). A platform that appears to offer some promise of generality has emerged from a study of scaffolds capable of projecting the amino acid side-chains in a fashion that mimics the periodicity and topography associated with helical peptides (104). The substituted terphenyl derivative **18**, in which the three branched alkane substituents are designed after consideration of elements of the HIV gp41 *N*- and *C*-terminal heptad repeats, interfered with 6-helix bundle formation and inhibited HIV-1-mediated cell-cell fusion with an EC_{50} of 15.7 mg/ml (104).

Although many inhibitors of RSV that have been identified by broad screening campaigns target the fusion protein, considerable progress has recently been made towards the characterization and optimization of potent and selective inhibitors that target other proteins of importance in the replication cycle. Two structurally distinct inhibitors of the RSV nucleoside polymerase (L gene product) have recently been described (104-106). The 1*H*-imidazo[4,5-*h*]isoquinoline-7,9(6*H*,8*H*)-dione **19** is a potent antiviral agent in cell culture (EC_{50} = 21 nM) that reduced RSV titers in the lungs of BALB/c mice following topical (intranasal) administration (105, 106). Mutations

conferring resistance to **19** were mapped to Glu1269Asp, Ile1381Ser and Leu1421Phe changes in the RSV L gene and detailed mechanistic analysis suggested that the compound interfered with guanylation of viral RNA, leading to premature termination of viral RNA transcripts (106). YM-53403 (**20**) inhibits RSV A and B strains in a plaque reduction assay with an EC_{50} of 200 nM, with resistance conferred by a Tyr1631His change in the polymerase gene (107).

Arrow Therapeutics' A-60444 (**21**) is the most advanced RSV inhibitor, having successfully completed phase I clinical studies and is currently being examined in phase II studies, where the focus is on establishing efficacy in treating bone marrow transplant recipients with RSV infection (108, 109). A-60444 is orally bioavailable and this benzodiazepine-based chemotype appears to act by interfering with the N protein of the virus, allowing for the potential to productively combine with RSV fusion inhibitors (109, 110). An analysis of the interaction between A-60444 and BMS-433771 *in vitro* indicated synergistic inhibitory activity, whereas combination of A-60444 and ribavirin produced only an additive effect (111).

Another mode of RSV inhibition that is gaining prominence is the use of antisense RNA and short interfering RNA sequences (siRNA) that can be delivered topically (112-116). RB-1034 is a leading 2-5A antisense-based inhibitor of RSV and is between 50- and 100-fold more potent than ribavirin *in vitro*, while exhibiting antiviral activity in mice, cotton rats and African green monkeys following intranasal administration or by aerosol (114-116). Inhibition of RSV replication *in vivo* by siRNA oligonucleotides that target the P or NS1 genes has recently been demonstrated in BALB/c mice following intranasal administration, and Alnylam Pharmaceuticals has initiated phase I clinical studies with ALN-RSV01 for the treatment of RSV infection (117-119).

Conclusions

The discovery and optimization of a series of small-molecule inhibitors of RSV fusion led to the identification of BMS-433771 as an orally bioavailable compound with a pharmacokinetic and safety profile suitable for clinical evaluation. To date, BMS-433771 is the only orally active RSV fusion inhibitor disclosed in the literature that has undergone extensive characterization. Experiments with a photoaffinity probe have illuminated aspects of the mechanism of action of this chemotype, which acts by interfering with the formation of the 6-helix bundle structure of the F protein, an essential step in virus entry.

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