Novel and Specific Respiratory Syncytial Virus Inhibitors That Target Virus Fusion

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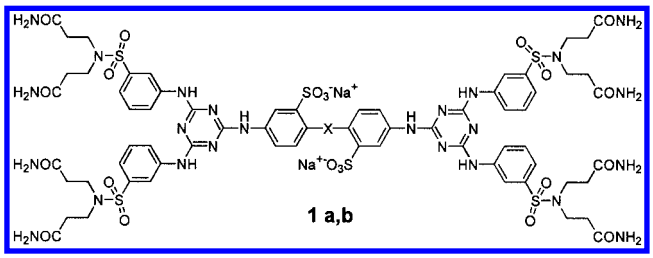
Human respiratory syncytial virus (RSV) is a major cause of respiratory tract infections in young children and infants and is an important cause of community-acquired respiratory infection among hospitalized adults.[1] A member of the paramyxovirus group of enveloped viruses, RSV is reported to cause an estimated 91000 hospitalizations and 4500 deaths annually in the United States.[2] Ribavirin is currently used for therapy although its efficacy has been questioned.[3] In certain high-risk infants, however, hyperimmune γ-globulin is administered prophylactically. Due to the lack of effective treatment, a screening program was initiated at Wyeth-Ayerst to look for inhibitors of RSV. A remarkably active lead, **1a** (IC_{50} 0.15 μM), was identified from a 20000 compound library with a high-throughput screen using a whole virus cell-based assay. We describe here the synthesis and SAR of a novel class of RSV inhibitors based on the stilbene **1a** and the somewhat more active biphenyl analogue **1b** (CL387626; IC_{50} 0.05 μM). This biphenyl analogue has also shown very promising antiviral activity against RSV in cotton rats.[4] Unpublished biological studies indicate these inhibitors target the viral fusion event but not attachment, and strongly suggest that the 70K viral fusion glycoprotein (F)[5] is the site of action. Corroborative evidence for this conclusion comes from the fact that the agents also inhibit infectivity of a mutant RSV virus, *cp*-52, which lacks the attachment (G) and small hydrophobic (SH) surface proteins,[6] with an effect similar to that observed with the wt RSV. Furthermore, sequence analysis of the F gene of viruses resistant to **1a** and **1b** demonstrated amino acid changes in highly conserved regions of the F_1 domain (unpublished results). In support of this, we present preliminary biophysical characterization of drug interactions with F protein by fluorescence spectroscopy and analytical ultracentrifugation.

Synthesis. Two general methods of inhibitor synthesis were used (Schemes 1 and 2), depending on the particular substitution patterns desired. Cyanuric chloride (**2**) was the key precursor. Displacement of the first chlorine atom occurred readily at 0 °C within a few minutes, whereas the second required 3 h at about 50 °C. Substitution of the third chlorine required 100 °C for 24−36 h. The stepwise reaction of cyanuric chloride with amines allows wide latitude to introduce separately into the triazine ring different functional groups.



The first method (Scheme 1) depicts the stepwise condensation of dianionic derivatives **3**, corresponding to the central part of the inhibitors, with 2 mol of cyanuric chloride (**2**) followed by the addition of the amino-containing derivatives **4a**, **b**. These reactions were carried out in organic−aqueous media in the presence of phosphate buffer at pH 7.0. It is possible to stop the reaction at an intermediate stage, e.g., dichloro derivative **6**, and to isolate the corresponding product.

Scheme 2 depicts inhibitor construction starting from the peripheral substituents rather than from the central core. The reaction of cyanuric chloride (**2**) with amino derivative **4a** at 0 °C and then **4b** at 50 °C provided the uncharged intermediates **7** and **8**, respectively. Reaction of the monochlorotriazine **8** with the disodium salt of the dianionic core compounds **3** was carried out at 100−115 °C in organic media in the presence of a tertiary amine and provided the target compounds **1a**−**o**.

Results. SAR. The antiviral activity of a number of analogues against RSV, HCMV, and HSV is listed in Tables 1 and 2. This novel class of inhibitors consists of two parts: the dianionic core and four benzenesulfonamides to which are appended eight arms terminating in functional groups capable of being hydrogen bond acceptors or donors. These two parts are connected by two triazine radicals. Early in these investigations it became clear that at least three of the benzenesulfonamide appendages are necessary not only for biological activity but also for binding to the isolated F protein in vitro as demonstrated by fluorescence binding studies. The specificity and potency of the inhibitors for RSV relative to other viruses is remarkable. Indeed, these agents are some 2−3 orders of magnitude less active against other viruses such as HCMV and HSV (e.g., **1b** exhibits an IC_{50} of 0.05 μM against RSV, 25 μM against HCMV, and 175 μM against HSV). The negatively charged core is critical based on the activity of **1a**, **1b**, and **1f** compared to those analogues without the negative charge such as **1e**, **1g**, and **1h** (Table 1**)**. Rigidity around the central core is important as the dihydrostilbene derivative **1c** is essentially inactive. The sulfonamide grouping is also necessary because analogues with amido linkages (**1n**, Table 2) are inactive.

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Scheme 1

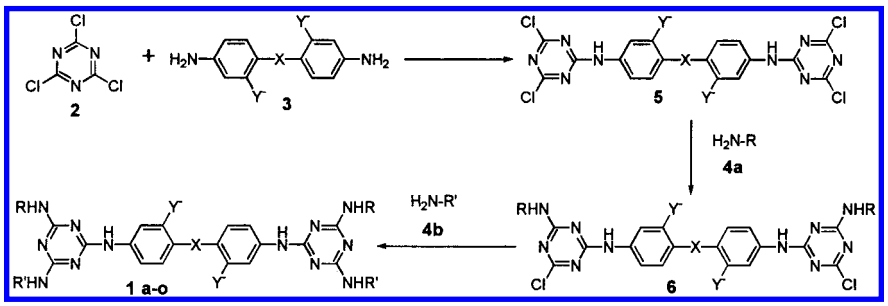
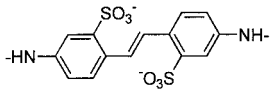
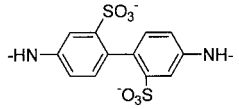
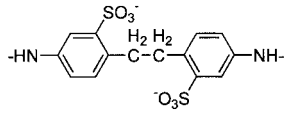
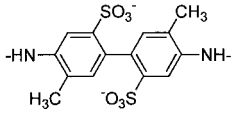
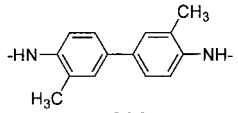
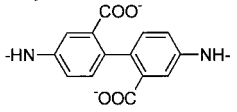
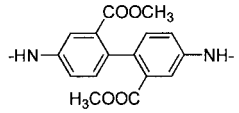
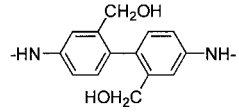
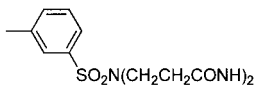


Table 1. Effect of Varying the Anionic Core on Activity and Specificity of RSV Inhibitors **1a**−**h***

		IC$_{50}$ (μM) [a]		
		HRSV [b]	HCMV [c]	HSV [d]
1a		0.15	>25	5.5
1b		0.05	18.5	>25
1c		22.5	>25	ND [e]
1d		0.25	20.5	20
1e		>25	>25	25
1f		0.35	0.9	7.5
1g		>25	>25	ND
1h		>25	>25	>25

* R = R '= 

[a] IC$_{50}$; concentration inhibiting growth by 50%. The compounds were tested at 10 concentrations differing by 2-fold dilutions. Each concentration was tested at least in duplicate (SD <10%) and the IC$_{50}$ values given in Tables 1 and 2 represent the average values. Moreover, each compound was tested at least twice in two different weeks, and to be accepted, the calculated IC$_{50}$ could not be more than 2-fold different or the compounds were retested. [b] HRSV; human respiratory syncytial virus, strain A2. Human foreskin fibroblasts were seeded at 4×10^4/well in 96-well dishes on day 0. On day 1, the compound was added and serially diluted. Virus (160 plaque-forming units in 50 μL) was then added. After 4 days of incubation at 37 °C, the extent of virus growth was measured using an ELISA. [c] HCMV; human cytomegalovirus, strain Ad169. The conditions were as in b, except that the virus used was engineered to encode β-glucuronidase and virus growth was followed by measuring the amount of β-glucuronidase in the cell culture on day 4. [d] HSV; herpes simplex 1, strain Patton. The conditions were as in b, except that African green monkey kidney cells (Vero) were used and virus growth was measured 28 h after infection using an ELISA. [e] ND; not done.

Functional groups capable of acting as hydrogen bond donors or acceptors around the exterior, such as hydroxyls or amide groupings, are required as shown by the inactivity of analogues that terminate in methyl groups such as **1l**. A meta orientation on the benzenesulfonamide groupings (**1b**, Table 2) is surprisingly

Scheme 2

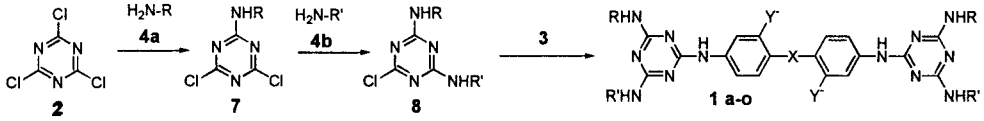
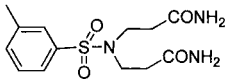
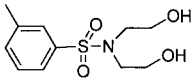
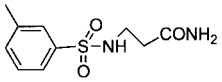
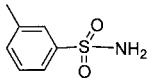
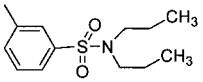
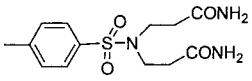
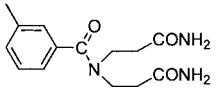
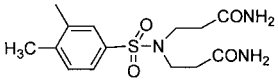
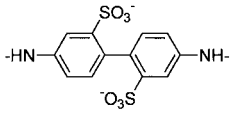


Table 2. Effect of Varying the Peripheral Groups on Activity and Specificity of RSV Inhibitors **1b,i-o***

	R = R'	IC_{50} (μM) [a] HRSV [b]	HCMV [c]	HSV [d]
1b		0.05	18,5	>25
1i		0.09	2.3	14.7
1j		0.24	1.2	>25
1k		1.7	1.2	8.8
1l		>25	>25	>25
1m		2.9	14.7	17.7
1n		>25	ND [e]	ND
1o		>25	ND	ND

* Anionic core: 

[a–e] See corresponding footnotes in Table 1.

intolerant of alteration as the para analogue, **1m**, is 60-fold less active against RSV than **1b**. This appears to relate to multivalent hydrogen bond interactions between the terminal functional groupings of the inhibitors and F protein which are highly complementary and directional.

Direct Evidence for Binding of RSV Inhibitors with F Protein Using Fluorescence Spectroscopy. Fluoresence spectroscopy was used to determine if the inhibitors which were identified in the antiviral screen bound specifically to purified F protein.[7] Insight into the nature and strength of the association between inhibitors and F protein target was obtained by observing changes in the intrinsic fluorescence of inhibitor in the presence of protein (Figure 1). Both the stilbene and biphenyl analogues (**1a**,**b**) are strong fluorophores, and binding to F protein caused an increase in the emission intensity at 450 and 375 nm, respectively. The enhancement in the fluorescence intensity was accompanied by significant band narrowing and blue shift in the emission spectrum compared to the fluorophore alone in buffer. These spectral changes were simulated by placing the inhibitor in mixtures of solvents of lower dielectric constants such as *p*-dioxane and water. These results suggest a change from a polar to a less polar microenvironment of the inhibitors on binding to the F protein. Analogues of **1a** and **1b** which are inactive in the biological assay, such as those lacking sulfonates, did not bind F protein in the fluorescence assay.

An estimated K_D value of 0.1 μM for the initial binding of **1a** or **1b** to F protein was derived from two sets of fluorescence titrations of inhibitor with F protein (data not shown). In the first set, the protein concentration was fixed at 0.05, 0.5, and 1 μM and the inhibitor titrated into the protein solution. In the second set, a reverse titration was carried out with fixed inhibitor concentration (0.05 μM) and the protein titrated into the inhibitor solutions. On the basis of these results,

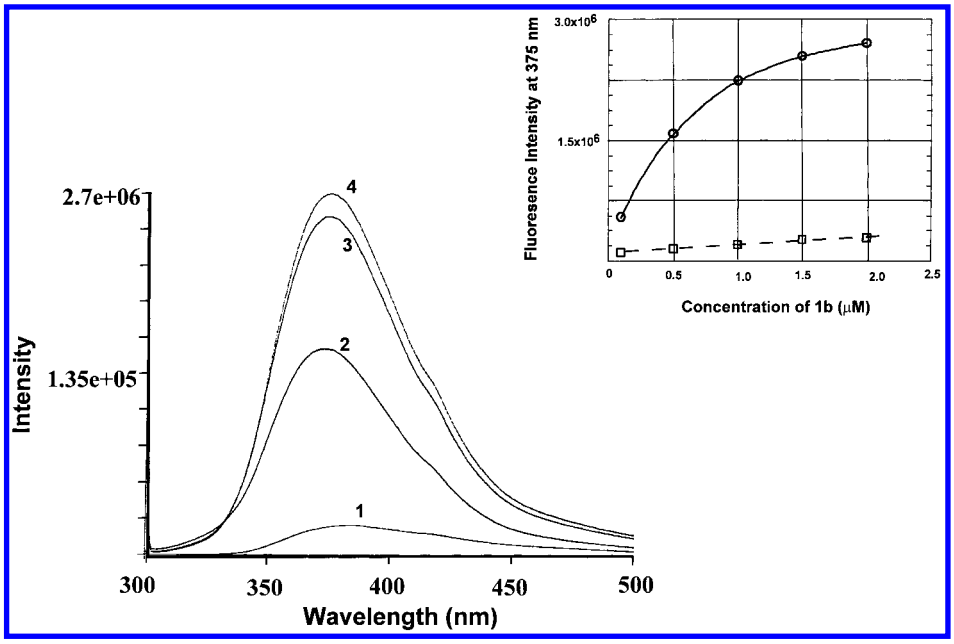


Figure 1. Fluorescence spectra of the interaction of inhibitor **1b** with F protein. Emission spectra of **1b** were scanned in the absence and presence of F protein (1 μM), with the excitation monochromator at 295 nm: curve 1, the fluorescence of **1b** alone; 2, F protein with 0.5 μM **1b**; 3, F protein with 1.5 μM **1b**; 4, F protein with 2.0 μM **1b**. Inset: solid curve, binding isotherm of F protein and **1b** obtained by plotting the fluorescence intensity as a function of inhibitor concentration; dashed curve, fluorescence of **1b** alone at various concentrations.

we conclude that there is an initial tight binding event (K_D ~0.1 μM) followed by multiple, weaker binding events.

The fluorescence experiment shown in Figure 1 was carried out under conditions where the protein concentration (1 μM) was greater than the K_D value to demonstrate that low concentrations of inhibitor are sufficient to bind F protein. Taken together, the above fluorescence results indicate multiple binding events due to heterogeneity of the aggregated protein (see below) which precludes determination of a discrete binding constant. The estimated K_D is based on a 70K monomeric F protein,[5] and therefore, the apparent K_D could be significantly lower because of the oligomeric nature of the F protein. Given the premise that an oligomeric state of the F protein on the surface of the virion is essential for biological activity,[8] the estimated K_D of 0.1 μM derived here for **1b** is consistent with the IC_{50} (0.05 μM) obtained from the biological assay.

Inhibitor Binding to and Disassembly of F Protein Using Analytical Ultracentrifugation. Because initial dynamic light scattering experiments suggested the purified F protein[7] to be highly aggregated, analytical ultracentrifugation was used to investigate its oligomerization state and the effect of inhibitor binding on the hydrodynamic properties of the inhibitor/F protein complex. Sedimentation velocity results indicated that in the range 0.5−8 μM the F protein was aggregated with an average Svedberg constant (*s*) of 13, suggesting perhaps at least a dodecameric species. Inhibitor binding to F protein resulted in the disassembly of the 13*s* oligomer to one of 8.95*s*, consistent with a shift in molecular weight to a tetrameric species. The inactive (IC_{50} >25 μM) analogue **1o**, with a methyl group para to the sulfonamide and ortho to the linking amino groupings, also caused disassembly of the F protein to a similar aggregation state. Thus the impact of the disaggregation by inhibitor on the functional state of the F protein in the viral membrane is unclear because the effective concentration and oligomerization state of the F protein on the virion surface is currently not well characterized.

Discussion. A recent review[9] summarizes inhibitors of RSV replication which include adsorption inhibitors such as polysulfates, polysulfonates, and polyoxometalates. Virus-cell fusion inhibitors include bis(5-amidino-2-benzimidazolyl)methane and pyridobenzoazoles such as 4-cyano-2-benzimide-1-oxo-1,5-dihydropyrido[1,2-*a*]-benzimidazole. The target of these two fusion inhibitors is unclear although it is speculated to be cellular endoproteases involved in the cleavage of the inactive F_0 precursor to the active form consisting of two disulfide-linked subunits, F_1 and F_2.[9] None of these agents, however, bear any structural resemblance to the RSV inhibitors described above. The SAR and biophysical measurements described here for **1a** and **1b** are consistent with the importance of multivalent hydrogen bonding, electrostatic, and hydrophobic interactions in the inhibitor/F protein association. Thus the polyamide groupings radiating from the exterior of the inhibitor can form extensive polyfunctional hydrogen bonding networks with the carbohydrate side chains, peptide backbone and side chain functional groups of the F protein. The anionic core of the inhibitors can make electrostatic contacts with nearby cationic patches on the peptide backbone. Photoaffinity labeling studies are underway to determine where these inhibitors bind to the F protein.

In conclusion, it appears that these inhibitors act by binding to the F protein and thus block viral fusion and infectivity although a precise understanding of this interaction is unclear. RSV antiviral activity and specificity seems best correlated to the multivalency and side chain orientations of the inhibitor to maximize binding to the F protein.

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(7) The F protein used in these biophysical studies was isolated from Vero cell cultures infected with A2 strains of RSV and purified by ion exchange and hydroxyapatite chromatography. A stock solution was prepared in pH 7.0 phosphate (50 mM) buffered saline (150 mM NaCl) containing 0.01% Triton X-100. The purity is greater than 90% F protein by SDS/PAGE under nonreducing conditions and by Western blot analysis. Neutralizing antibodies to RSV were elicited in a Swiss Webster mouse model by this protein preparation indicating that at least some of the binding epitopes are present and thus is a suitable system for characterization of drug/target interaction.

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