Inhibition of Respiratory Syncytial Virus-Host Cell Interactions by Mono- and Diamidines

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Several aromatic mono- and diamidines were found to block cell fusion induced by respiratory syncytial virus. The best inhibitors were able to achieve complete suppression of syncytium formation at a concentration of 1.0 μ M. Inhibition occurred in respiratory syncytial virus-infected HEp-2 and CV-1 cells, but the same inhibitors were ineffective in preventing fusion induced by parainfluenza virus type 3. The fusion inhibitors did not reduce single-cycle virus yields, but did reduce multiple-cycle yields. In addition, the active compounds caused a significant retardation of respiratory syncytial virus penetration. The mechanism by which amidines interfere with respiratory syncytial virus-host cell interactions is unknown, but parallels can be drawn between antiviral activity and the ability of the compounds to inhibit certain trypsin-like proteases.

Respiratory syncytial (RS) virus is a common respiratory pathogen which causes, almost without exception, a well-defined epidemic of respiratory illness every year. Early studies clearly defined the threat of RS virus to pediatric populations, and more recent studies demonstrated the susceptibility to infection of geriatric populations (1). Attempts to develop effective vaccines for RS virus have been unsuccessful (3, 14). In addition, reinfections are a common event, suggesting that naturally acquired immunity does not provide long-lasting protection.

Recently, we have reported that bis(5-amidino-2-benzimidazolyl)methane possesses a significant suppressive effect on the cytopathology and yield of RS virus (5). Since bis(5-amidino-2-benzimidazolyl)methane is the most potent reversible synthetic inhibitor of trypsin so far reported (20), we have examined other inhibitors of trypsin-like enzymes for their influence on RS virus-host cell interactions. In addition, homologs and isosters of the active compounds were included to define the specificity of the inhibitory process. This study has identified several other amidines which modulate the interaction of RS virus with the host cell.

MATERIALS AND METHODS

Virus and cell cultures. HEp-2 and CV-1 cells were propagated in Eagle minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). In experiments involving protease inhibitors, HEp-2 cells were used between passages 370 and 376.

The A₂ strain of RS virus, obtained from R. M. Chanock, was grown in suspensions of HEp-2 cells.

After virus absorption, the infected cells were seeded in 250-ml plastic culture flasks containing MEM plus 10% FBS. When viral cytopathic effects were maximal (72 to 96 h), the flasks were frozen at -70°C . Typical lysates contained 10^7 to 10^8 50% tissue culture infective doses per ml.

All virus stocks and cell lines were determined to be free of mycoplasma contamination by a modification of the fluorochrome technique of Chen (2).

Viral titrations. Virus yields were quantitated by calculating 50% endpoints by the method of Reed and Muench (18). Briefly, 0.05 ml of virus diluted in MEM plus 10% FBS was added to microtiter plates (Linbro Scientific Inc., Hamden, Conn.). This was followed by the addition of 40,000 to 60,000 HEp-2 cells in a volume of 0.05 ml. Plates were incubated at 36°C for 5 to 7 days to allow the development of virus-induced cytopathic effects. Wells demonstrating any signs of RS virus infection were scored as positive for the endpoint calculations. Six or eight wells were used for each dilution. Standard error analysis was done by the method of Pizzi (17).

Protease inhibitors. We have reported the synthesis and physical properties of the following amidino derivatives in previous communications (see Table 1 for structural formulas): compounds 1 to 7 (20); compounds 8 to 11, 15, and 16 (10); compounds 23 and 24 (16); compounds 25 to 28 (9). In addition, compounds 17 to 20 have been previously reported and were supplied to us by May and Baker Ltd. (Dagenham, England). Finally, compounds 12, 13, 14, 21, and 22 were synthesized in our laboratory and have not been previously reported. Compounds 21 and 22 were prepared and purified by a general synthetic procedure for the synthesis of unsymmetrical α , ω -diphenoxyalkane derivatives (12). The nitrile intermediates for compounds 12, 13, and 14 were prepared by a previously described method (4). The nitriles were converted to the corresponding amidines via a modified

TABLE 1. Structure of amidines and their effect on RS virus cytopathology

Comp. no.	Structure ^a	Concentration (μM)			
		100	10	1.0	0.1
I. Bis-Amidinobenzimidazoles					
1		NS ^b	NS	NS	S ^c
2		NS	NS	S	S
3		NS	S	S	S
4		I ^d	NS	S	S
5		T ^e	NS	S	S
6		T	S	S	S
7		I	I	S	S
II. Amidinoindoles and related compounds					
8		NS	NS	S	S
9		T	S	S	S
10		NS	NS	NS	S
11		I	NS	S	S
12		T	S	S	S
13		T	S	S	S
14		T	S	S	S
15		NS	S	S	-
16		S	S	-	-

TABLE 1—Continued

Comp. no.	Structure ^a	Concentration (μM)			
		100	10	1.0	0.1
III. Amidino-substituted α,ω-diphenoxyalkanes					
17		T	T	S	S
18		T	T	S	S
19		T	T	S	S
20		T	T	S	S
21		T	S	-	-
22		T	I	S	-
23		T	I	S	-
24		T	I	S	-
25		I	NS	S	S
26		T	I	S	S
27		T	S	S	S
28		T	NS	S	S

^b Complete inhibition of syncytium development without apparent toxicity.

^c Presence of syncytia.

^d Inhibition of cell growth with or without syncytia.

^e Toxic.

version of the Pinner amidine synthesis (12). The melting points and elemental analyses for the novel inhibitors are shown in Table 2.

Inhibitor studies. HEP-2 cells or CV-1 cells were seeded in well trays (Falcon Plastics, Oxnard, Calif.) to contain approximately 10^6 cells per well at 72 h. RS virus, added at various input multiplicities of infection in 0.2 ml, was adsorbed for 2 h at 36°C. After this period, the wells were rinsed twice with 1 ml of MEM and received either inhibitor-containing or control medium consisting of MEM with 2% FBS and 1% dimethyl sulfoxide. Toxicity and cytopathology were scored at 24, 48, and 72 h after the addition of the

inhibitors. Total virus yields from infected cultures were determined after one freeze-thaw cycle at -70°C.

The effect of the inhibitors on viral penetration was determined as follows: RS virus was serially diluted in MEM containing 2% FBS and 1% dimethyl sulfoxide with or without 50 μM inhibitor. Amounts of 0.05 ml were added to wells of microtiter plates containing monolayers of HEP-2 cells. Virus was adsorbed for 1.5 h at 36°C, at which time the inoculum was removed. Horse anti-RS virus serum (Flow Laboratories, McLean, Va.) diluted 1:10 in MEM was then added to the appropriate wells. After an additional 1 h of incubation at 36°C, all monolayers were rinsed and overlaid

TABLE 2. *Physical data on novel amidine derivatives*

Compound no.	Formula	Melting point (°C)	Analysis (calculated/found), %		
			C	N	H
12	C ₁₁ H ₁₁ N ₃ O·HCl·0.25H ₂ O	301 to 303	54.55/54.64	17.35/17.31	5.20/5.03
13	C ₁₁ H ₈ N ₃ OF ₃ ·HCl	>300	44.84/44.97	14.26/14.17	3.08/3.00
14	C ₁₆ H ₁₃ N ₃ O·HCl	>300	64.11/63.99	14.02/13.92	4.71/4.54
21	C ₁₉ H ₂₄ N ₂ O ₂ ·HCl	290 to 292	65.41/65.32	8.03/8.29	7.22/7.02
22	C ₂₁ H ₂₈ N ₂ O ₂ ·HCl·0.5H ₂ O	100 to 102	65.35/65.58	7.25/7.34	7.83/8.07

with 0.1 ml of MEM plus 5% FBS. Titers were determined at 5 to 7 days.

RESULTS

The effect of amidino compounds on cell viability. To separate the cytotoxic properties of the amidino compounds from specific antiviral activities, HEP-2 cell cultures were examined for toxicity after incubation with the compounds for 24, 48, and 72 h. Toxicity was judged by microscopic examination of the treated monolayers or by trypan blue exclusion tests. Many of the compounds tested were clearly toxic for HEP-2 cells at a concentration of 100 μ M (Table 1). Four compounds (compounds 17, 18, 19, and 20) exhibited toxicity at 10 μ M. In addition to showing toxicity at high concentrations, eight compounds inhibited cell growth without overt toxicity at lower concentrations. In Table 3 cell growth is compared in the presence of a 10 μ M concentration of compounds 1, 10, and 22. Cells treated with compound 22, a representative of the growth-inhibitory group, did not exhibit any apparent growth during the 72 h of incubation when compared with control cells or cells treated with compounds 1 and 10. Although these cells did not undergo proliferation, they remained viable as judged by trypan blue exclusion.

Effect of amidino compounds on the cytopathic effects of RS virus. With paramyxoviruses, infectivity and the ability to induce cell fusion are coordinately expressed (19). With this in mind, we decided to initially use the presence or absence of syncytium formation to score the impact of the amidino compounds on the replication of RS virus. The amidino compounds were added to the HEP-2 cell cultures after viral adsorption and penetration (2 h postinfection). Therefore, any inhibitor-related modification of the cytopathic effects must have had its origin in the alteration of the later stages of virus replication. All compounds were tested in serial 10-fold dilutions, and syncytium formation was evaluated by inspection of infected cultures at low microscopic power.

As reported previously (5), bis(5-amidino-2-benzimidazolyl)methane (compound 1) is a potent inhibitor of RS virus-induced cell fusion. The specificity of the inhibition of fusion by

TABLE 3. *Growth of HEP-2 cells treated with amidines^a*

Growth conditions	Cell harvest at time (h):		
	24	48	96
Control ^b	5.4×10^{5c}	1.0×10^6	1.4×10^6
Compound 1 ^d	5.7×10^5	1.0×10^6	1.5×10^6
Compound 10 ^d	6.2×10^5	1.0×10^6	1.2×10^6
Compound 22 ^d	4.5×10^5	5.7×10^5	4.5×10^5

^a HEP-2 cells were seeded in multiwell plates and incubated at 36°C. Cells were harvested by using trypsin-ethylenediaminetetraacetate. Hemacytometer counts were made on cells suspended in trypan blue-phosphate-buffered saline.

^b MEM plus 5% FBS.

^c Cells per well.

^d MEM plus 5% FBS plus selected amidine at 10 μ M.

compound 1 was tested by examining a series of homologs. Compound 2 differed from compound 1 only by the presence of a second hydrocarbon group between the heterocyclic rings, but this additional group reduced the potency of compound 2 by at least 10-fold. A third hydrocarbon group reduced the potency even further (compound 3). Further lengthening of the chain to five and six carbons recouped some of the activity, but also increased toxicity (compounds 4 and 5). Replacement of the central hydrocarbon chain by a benzene ring or ring closure of the amidine moieties led to a loss of syncytial inhibitory activity.

A second interesting compound which exhibited both antiprotease activity and inhibition of cell fusion is 5-amidinoindole (compound 8). Toxicity was not demonstrated at 100 μ M, and cell fusion inhibition was evident at 10 μ M (Table 1). The exacting structure-activity relationships are clearly evident for this inhibitor. Moving the amidino group from the 5 position on the heterocyclic ring to the 6 position (compound 9) completely abolished the ability to inhibit RS virus-induced cell fusion. Substitutions at the 3 position also eliminated the cell fusion inhibition (compounds 12, 13, and 14). However, the introduction of a methyl group at the 1 position (compound 10) increased the potency by a factor of 10 and made the inhibitor equivalent to compound 1. Surprisingly, the ad-

dition of a 4-amidinobenzyl group at the 1 position (compound 11) did not alter the activity from that of the unsubstituted parent compound. On the other hand, substitution of an oxygen for the nitrogen at the 1 position (5-amidinobenzofuran, compound 16) eliminated the inhibitory activity. Completing this series, 5-amidinobenzimidazole (compound 15) was 10 times less active than the corresponding indole derivative (compound 8) and 100 times less active than the bis-5-amidino-benzimidazole derivative (compound 1).

The final group of compounds examined is represented by a series of mono- or diamidino-substituted α , ω -diphenoxyalkanes. Included in this group are pentamidine (compound 18) and compound 25, both of which are potent inhibitors of trypsin-like enzymes (6-9). The major feature of this series of compounds was their considerable level of toxicity, though it is evident that toxicity was reduced by 1 order of magnitude when one of the amidino moieties was removed or replaced by a nitro group. The only compounds which exhibited cell fusion inhibition in the absence of toxicity were compounds 25 and 28 which are characterized by the presence of a hydroxyl group on the central dioxyalkane chain. Even within this series of compounds, the activity was dependent on rather narrow molecular specifications. For example, placement of the amidino group into the 3 position (compound 27) rather than the 4 position (compound 25) resulted in a loss of all inhibitory activity.

Figures 1 and 2 illustrate the changes in RS virus cytopathic effects produced by the fusion inhibitors. Uninfected control HEp-2 cell cultures showed some single rounded cells on the monolayer (Fig. 1a), whereas virus-infected cells (multiplicity of infection, 1 to 2; 40 h postinfection) had formed syncytia of various sizes (Fig. 1b). Treatment of RS virus-infected cells with a 1.0 μ M concentration of compound 1 resulted in the abolition of fusion (Fig. 1c), to the extent that infected monolayers did not appear different from uninfected controls. With longer periods of incubation, foci of infected cells were evident, but they consisted of clusters of non-fused cells. Figure 2 shows a similar blocking effect with CV-1 cells, indicating that fusion inhibition was not restricted to HEp-2 cells. It should be noted that none of the compounds tested was able to inhibit fusion induced by parainfluenza virus type 3 (data not shown).

Effect of amidino compounds on the yields of RS virus. Contrary to our expectations, the suppression of RS virus-induced cell fusion by amidino compounds added after penetration had no detrimental effect on essentially single-cycle yields of RS virus (data not shown).

This was somewhat puzzling in light of the drastic reduction in cytopathology in treated cultures. Next, we examined the effects of compounds 1 and 10 on multiple-cycle growth of RS virus. HEp-2 cultures were infected at a multiplicity of infection of 0.001. After adsorption of the virus, medium was added containing compounds 1 or 10 (50 μ M). Both compounds were able to reduce the yield of RS virus at all time points (Table 4). After 120 h of incubation, the control monolayer exhibited 4+ cytopathic effects with most of the cells forming large, detached syncytia. Treated monolayers showed scattered foci of rounded cells and numerous single rounded cells.

Effect of amidino compounds on the penetration of RS virus. Previously, we demonstrated that compound 1 inhibited the penetration of RS virus (5). Having now identified additional compounds which block RS virus-induced cell fusion, we chose two of these agents, compounds 8 and 10, and compared them with compound 1 for their ability to inhibit RS penetration. This was achieved by determining the ability of RS virus-specific antiserum to neutralize adsorbed virus. This approach was used employing a modification of the standard 50% tissue culture infective dose assay. Briefly, RS virus was serially diluted in MEM with or without a 50 μ M concentration of either compound 1, 8, or 10. A 0.05-ml sample of each 10-fold dilution was added to eight preformed HEp-2 cell monolayers in microtiter plates. After a 1.5-h adsorption period at 36°C, the inoculum was removed, and 0.05 ml of either MEM or MEM containing a 1:10 dilution of RS virus antiserum was added. After an additional 1 h of incubation at 36°C, the monolayers were rinsed twice and overlaid with 0.1 ml of MEM plus 5% FBS. Titers were determined at 5 to 7 days.

Attachment of the virus was not inhibited by the fusion inhibitors, because the final titers of all assays without antiserum treatment were identical (Table 5). Since the inoculum was removed at the same time in all groups, a block in adsorption would have been detected in the absence of antiserum. The addition of RS virus-specific antiserum reduced the final titers in the assays containing the fusion inhibitors, but not in the control. These data indicate that the three fusion inhibitors exert a similar effect on the penetration of RS virus.

DISCUSSION

This investigation has demonstrated a strong inhibitory activity of several aromatic mono- and diamidines against cell fusion induced by RS virus. The expression of this property was not dependent on a single type of host cell, but

FIG. 1. Inhibition of RS virus-induced cell fusion of HEP-2 cells by compound 1. HEP-2 cells infected with RS virus (multiplicity of infection, 1 to 2) were

occurred with HEP-2 as well as CV-1 cells. The suppressive effect was virus specific, however, and did not extend to cell fusion induced by P-3 virus. Though the compounds did not interfere with virus adsorption or with replication per se, they did retard or block penetration and did reduce the yield from multiple growth cycles of RS virus. As a possible explanation of these findings, we postulate that the inhibitors impeded the fusion of the virions with the cell membrane when added to the monolayers together with the virus, and that they block fusion from within when added after the adsorption and penetration period. It also follows that under the latter condition any newly formed and released virions would be hindered from infecting adjacent cells, and that spread under a multiple-cycle growth pattern would therefore be restricted. This was well demonstrated morphologically by the focal limitation of cytopathic effects at low multiplicities of infection by RS virus.

The mechanism by which amidines block RS virus-related activities is uncertain at this time, but two possible explanations are apparent. The strong cationic nature of amidines in combination with their considerable potential for hydrophobic bonding endows them with a strong affinity for cell membrane systems and raises the question of whether this property could have formed the basis for the antifusion effect without regard to any fortuitously associated antiprotease characteristics (11, 13, 21). All such membrane-directed activity would seem rather nonspecific and would explain neither the considerable differential in strength between structurally very similar inhibitory compounds nor the selective inhibition of only RS virus. However, the specificity and selectivity of the inhibitors could be the result of competition between a singular component of the RS virus and amidines for a well-defined binding site on the cell membrane. The binding site can be thought of as being necessary for penetration and fusion, but not for the initial adsorption process. In this manner, the inhibition of RS virus by amidines could be similar to the inhibition of measles virus by a synthetic tripeptide (15).

The ability of the amidines to inhibit a wide variety of arginine- or lysine-directed, i.e., trypsin-like, proteases would also suggest that such an antiproteolytic property might be of prime importance. Support for this possibility comes from known structure-activity relationships for

incubated for 40 h at 36°C in MEM plus 2% FBS plus 1% dimethyl sulfoxide with or without compound 1; a, uninfected control; b, infected control; c, infected cells plus 1.0 μ M compound 1; $\times 250$.

FIG. 2. Inhibition of RS virus-induced cell fusion of CV-1 cells by compounds 1 and 10. CV-1 cells infected with RS virus (multiplicity of infection, 0.1) were incubated for 72 h at 36°C in MEM plus 2% FBS

TABLE 4. Effect of amidines on multiple-cycle growth yields of RS virus^a

Growth conditions	Virus harvest at time (h):			
	48	72	96	120
Control ^b	5.5 ^c	6.3	7.0	7.5
Compound 1 ^d	4.7	4.9	6.0	6.0
Compound 10 ^d	4.0	5.5	6.1	6.5

^a HEp-2 cell monolayers were infected with RS virus at multiplicity of infection of 0.001. Cultures were incubated at 36°C until harvested.

^b MEM plus 2% FBS plus 1% dimethyl sulfoxide.

^c Log₁₀ 50% tissue culture infection doses per milliliter.

^d MEM plus 2% FBS plus 1% dimethyl sulfoxide plus selected amidine at 50 μM.

TABLE 5. Effect of amidines on RS virus penetration of HEp-2 cells^a

Inoculation conditions	Virus titer	
	No AB ^b	With Ab ^b
Control ^c	5.9 (0.31) ^d	5.5 (0.24)
Compound 1 ^c	5.9 (0.19)	4.3 (0.31)
Compound 8 ^c	5.8 (0.25)	4.6 (0.26)
Compound 10 ^c	5.8 (0.25)	4.9 (0.29)

^a Serial 10-fold dilutions of RS virus were added to HEp-2 cell monolayers in microtiter plates. After 1.5 h of incubation at 36°C, the inoculum was removed. Medium with or without RS antiserum was then added for 1 h at 36°C.

^b Ab, Horse anti-RS serum diluted 1:10.

^c MEM plus 2% FBS plus 1% dimethyl sulfoxide.

^d Log₁₀ 50% tissue culture infection doses (standard error).

^e MEM plus 2% FBS plus 1% dimethyl sulfoxide plus selected amidine at 50 μM.

the compounds investigated. Thus, we had previously demonstrated for several proteases that α , ω -bis(5-amidinobenzimidazolyl)alkanes show an early inhibitory maximum at a carbon bridge chain length of one, a minimum at a length of three, and a second maximum at a length of six or eight (20). It would appear more than coincidental that the same inhibitory pattern held also for the anti-RS virus effectiveness. A similar parallelism extended to the small molecular heterocycles in which 5-amidinoindole (compound 8) is a potent inhibitor of enzymes, such as thrombin, kallikrein, and factor Xa, whereas 5-amidinobenzimidazole (compound 15) and 5-amidinobenzofuran (compound 16) are considerably less active (10). Last, it is of interest that compound 25 represents a strong antagonist of

plus 1% dimethyl sulfoxide with or without amidines. Cells were fixed with methanol and stained with Giemsa; a, infected control; b, infected cells plus 10 μM compound 1; c, infected cells plus 10 μM compound 10; $\times 250$.

the plasminogen activator urokinase, and that its full antiprotease activity depends on the correct position of the amidino groups and on the absence of halogen substitution on the benzene rings (9). These were the same factors which determined the antiviral potency of the agent, and the close correlation between the two inhibitory activities raises the question of whether the putative protease target of the inhibitors may not be a plasminogen activator. Conceivably, such an enzyme could serve to activate plasminogen contained in the serum, and the active plasmin, in turn, could be involved in the fusion process by altering the RS virus envelope or the host cell membrane. Studies are continuing to more clearly define the structure-function relationships of the active compounds. In addition, we are trying to determine the pattern of RS virus-induced proteins in infected cells so that we can assess the impact of the amidines on the RS virus replication cycle.

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