Inhibition of Respiratory Syncytical Virus-Host Cell Interaction by Aminomethylenebenzimidazole Derivatives

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Several α-aminomethylenebenzimidazole-2-acetonitriles were found to block cell fusion induced by respiratory syncytial virus (RSV). Inhibition of occured in RSV-infected HeLa cell, and also the same inhibitors were effective in preventing fusion induced by parainfluenza virus type 3 (PFLUV-3) and measles virus. EC_{50} values of ECABN against RSV, PFLUV-3, and measles virus were 4.4, 15, and 24 μg/ml, respectively.

Respiratory syncytial virus (RSV) 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 RSV to pediatric populations, and more recent studies demonstrated the susceptibility to infection of geriatric populations (1). Attempts to develop effective vaccines for RSV have been unsuccessful (2, 3). In addition, reinfections are a common event, suggesting that naturally aquired immunity does not provide long-lasting protection.

Recently, author has reported that pyridobenzimidazoles posses a siginificant suppresive effect on the cytopathology and yield of RSV (4). Autor has examined other inhibitors like open-ring compounds of pyridobenzimidazle such as α-aminomethylene-benzimidazole-2-acetonitrile derivatives. In addition, homologes and isosters of the active compounds were included to define the specificity of the inhibitory process. This study has identified several other benzimidazoles which modulate the interaction of RSV with the host cell.

Materials And Methods

Virus and cell culture

HeLa cells were propagated in Eagle minimal essential medium (MEM) supplemented with 10% new born calf serum (NCS). In experiments involving protease inhibitors, HeLa cells were used between passages 370 and 376.

The long strain of RS virus, obtained from Sendai National Hospital, was grown in suspensions of HeLa cells. After virus absorption, the infected cells were seeded in 250-ml plastic culture flasks containing MEM plus 10% NCS. 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. RFLUV-3 (C243 strain) was a laboratory strain which was passed more than 10 times in hens embroynated eggs and 3 times in MDCK cells. The measles virus Sugiyama strain is widely used as the standard measles strain in Japan and was cultured in VERO cells.

All virus stocks and cell lines were determined to be free of mycloplasma contamination by a modification of the fluorochrome technique of Chen (5).

Viral titurations

Virus yields were quantitated by calculating 50% endpoints by the method of Reed and Muench (6). Briefly, 0.05 ml of virus diluted in MEM plus 10% NCS was added to microtiter plates (Falcon Plastics). This was followed by the addition of 40,000 to 60,000 HeLa cells in a volume of 0.05 ml. Plates were incubated at 35°C for 5 to 7 days to allow the development of virus-induced cytopathic effects. Wells demonstrating any signs of RSV infection were scored as posi-

tive for the endpoint calculations. Vero cells were used for the PFLUV-3 assays; they were cultured with MEM supplemented with 10% NCS and antibiotics. As a maintenance medium for MDCK cells, MEM supplemented with 0.2% bovine albumin and crystallized trypsin (2.5 μg/ml) (Sigma) was used. The growth medium of VERO cells consisted of MEM supplemented with 15% newborn bovine serum, 100 U of penicillin G/ml, and 100 μg of streptomycin/ml. The maintenance medium consisted of MEM supplemented with 2% fetal calf serum and antibiotics at the same concentrations as in the growth medium.

Chemicals

Aminomethylenebenzimidazoles were prepared from the reaction of benzimidazole-2-acetonitrile with amino derivatives in the presence of triethyl othoformate. The products were purified by the usual manner.

Antiviral Assays

HeLa cells were seeded in well trays 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 35°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% NCS and 1% dimethyl sulfoxide. Toxicity and pathology 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. Enumeration of the foci formed by PFLUV-3 was confirmed by haemadsorption of guinea pig erythrocytes to infected cells at 4°C for 1 h before fixation. An average number of plaque was calculated and the compound concentration required to inhibit the number of plaques or haemadsorbing foci to 50% of the control value was estimated as the 50% effective concentration (EC50).

Inhibitory effects of the compounds on viral antigen synthesis and syncytium formation were monitored by counting the numbers of antigen positive foci or cells in a syncytium after immunofluorescent staining in Lak-Tek chamber slide (8 chambers, Nunc Inc., Naperville, IL, USA) and incubated at 37°C in 5% CO_2. When the cell sheets had become confluent, approximately 50 PFU of RSV or PFLUV-3 were inoculated in each chamber (HeLa and Vero cells, respectively). Serial 4-fold dilutions of the compounds in maintenance medium with 0.7% methylcellulose were added to the chambers (in triplicate) and the cultures were incubated at 35°C in a CO_2 incubator. At 36 h (RSV), 24 h (PFLUV-3), or 5 d (measles) after infection, the maintenance medium was withdrawn, the cells were washed twice with phosphate buffered saline (PBS, pH 7.2), and fixed with acetone for 10 min at room temperature. The fixed cells were stained with fluorescein isocyanate (FITC)-conjugated rabbit antibodies against RSNV, PFLUV-3, or measles for 30 min at 37°C. After staining, the cells were washed with PBS and mounted with 20% glycerol in PBS. Immunofluorescence was analysed under a fluorescent microscope (Nikon Optiphot + EFD2, Nikon Industiral Co., Tokyo, Japan).

Cytotoxicity test

HeLa, Vero, and MDCK cells were seeded, at either 0.2×10^5 or 1.0×10^5 cells per well in 24-well tissue culture plate (Falcon 3047) and allowed to adhere to the plate at 37°C for 24 h in glowth medium. If seeded at the higher inoculum, the cells became confluent after 24 h, but with the lower inoculum, they did not become cofluent. The medium was then replaced by fresh maintenance medium (high cell inoculum) or glowth medium (low cell inoculum) containing different concentrations of the test compound in triplicate wells for each concentration. After 3 d incubation at 35°C in maintenance medium (high cell inoculum) or 37°C in glowth medium (low cell inoculum) in 5% CO_2, the cells were dispersed with 0.1% trypsin containing 0.02% EDTA, and the number of viable cells was determined in an

haemocytometer.

The number of viable cells was also monitored by the MTT method (Pauwels et al., 1988). The cells seede in 96-well plates (Falcon 3702) at 0.2×10^4 od 1.0×10^4 cells per well were incubated at 37°C for 24 h. Maintenace medium, culture condition, and concentration of the test compounds were exactly the same for each high and low cell inoculum as for the viable cell counting method. To this end, the ability of the cells to incorporate 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was examined with a spectrophotometer. Cytotoxicity of the compounds was expressed as CC_{50}, which corresponds to the concentration required to reduce the number of viable cells or MTT absorbance to 50% of the control (untreated cells). The selectivity index of each compound was determined as the ratio of the CC_{50} for cell viability to the EC_{50} for virus replication.

Results and Discussion

Ten α-aminomethylenebenzimidazole-2-acetonitriles were examined for their inhibitory activity against RSV, PFLUV-3, and measles. From these compounds, 5 compounds emerged as selective anti-RSV: α-ethoxycarbonylamino-methylenebenzimidazole-2-acetonitrile (ECABN), α-tetrazolylamino-methylene-benzimidazole-2 acetonitorile (TEZABN), α-(1, 3, 4-triazolyl-2-aminomethylene) benzimidazole-2-acetonitrile (TRZABN), α-(3-nitrophenylaminomethylene)-benzimidazole-2-acetonitrile (NPABN), and α-(3-chlorophenylaminomethylene)-benzimidazole-2-acetonitrile (CPABN). ECABN emerged as the most potent antiviral agent: it proved active against RSV, PFLUV-3, and measles. Namely, the EC_{50} of ECABN for the RS strains were 4.4 μg/ml; for PFLUV-3, 15 μg/ml; for measles, 24 μg/ml. TEZABN, TRZABN, NPABN, and CPABN were inhibitory to RSV at a 5-fold, 3-fold, 4-fold, and 7-fold higher concentration than ECABN, respectively. Of the five compounds, TRZABN was the least cytotoxic. TEZABN and CPABN were effective against RSV, and were not inhibitory to PFLUV-3 and measles. The highest selectivity index (SI) was noted with ECABN, against RSV (SI: 2.2) in HeLa cells at 35°C. The SI values of TEZABN, TRZABN, NPABN, and CPABN against RSV were 10, 25, 7.5, and 13.5. These SI values were comperable to that of ribavirin against RSV. The selectivity indexes of these compounds against PFLUV-3 and measles in Vero cells were up to 30-82 and obviously exceed that of rivabirin against PFLUV-3 and measles. Similar EC_{50} were obtained when the compounds were added together with the virus or after the virus adsorption period, which suggest that they do not interfere with virus adsorption. On the other hand, the compounds inhibited syncytium formation at a concentration that was significantly lower than the concentration required to inhibit viral antigen production. This may be interpreted to mean that the compounds would interfere with fusion between RSV, PFLUV-3-, and measles-infected cells and uninfected cells.

The mechanism by which these compounds might inhibit syncytium formation remains to be elucidated. For fusion of virus-infected and uninfected cells, F-glycoproteins of RSV, PFLUV-3, or measles have to be cleaved to F1 and F2 by cellular protease. Dubovi et al. (7) reported that some bis-amidinobenzimidazoles inhibit both cellular protease activity and RSV-induced syncytium formation. In this experiments, these compounds did not inhibited the activity of trypsin or clotting facter X (data not shown). An endoprotease homologous to this clotting factor would be responsible for the cleavage of the F-glycoproteins (8). Inhibitory of syncytium formation by these compounds may prevent virus from the cells to cell spread in vivo lesion. On the other hand, failure to inhibit the viral antigen production by these compounds may induce the stimulation of the immune system in vivo and protect the body from the progress of infection. Aminomethylenebenzimidazole derivatives should be further pursued as candidate drugs for the treatment of ortho and para-myxovirus infection.

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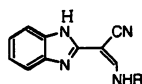
R = ethoxycarbonyl (ECABN)
R = tetrazolyl (TEZABN)
R = 1,3,4-triazolyl (TRZABN)
R = m-nitrophenyl (NPABN)
R = m-chlorophenyl (CPABN)

Table 1 Inhibitory effects of aminomethylenebenzimidazoles on the replication of viruses

Virus	EC_{50}(μg/ml)					CC_{50}(μg/ml)				
	ECABN	TEZABN	TRZABN	NPABN	CPABN	ECABN	TEZABN	TRZABN	NPABN	CPABN
RSV	4.4	20	12.5	15	27	200	200	50	200	200
PFLUV-3	15	9.4	200	23	>50	50	>50	>50	200	200
measles	24	41	200	>50	>50	50	>50	>50	200	200

Table 2 Selectivity index of aminomethylenebenzimidazoles

$$S.I. = \frac{EC_{50}}{CC_{50}} \times 100$$

Virus	S.I.				
	ECABN	TEZABN	TRZABN	NPABN	CPABN
RSV	2.2	10	25	7.5	13.5
PFLUV-3	30	18.8	--	46	--
measles	48	80	--	--	--

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