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Antiviral Activities of Ribavirin, 5-Ethynyl-1-β-D-Ribofuranosylimidazole-4-Carboxamide, and 6′-(*R*)-6′-*C*-Methylneplanocin A against Several Ortho- and Paramyxoviruses

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5-Ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR) and 6′-(*R*)-6′-*C*-methylneplanocin A (TJ13025) are two novel antiviral agents which are targeted against IMP dehydrogenase and *S*-adenosylhomocysteine hydrolase, respectively. These compounds have been examined for their activities against various strains of orthomyxoviruses (influenza virus) and paramyxoviruses (parainfluenza virus, mumps virus, measles virus, and respiratory syncytial virus) in vitro. EICAR was 10- to 59-fold more active than ribavirin and TJ13025 was 32- to 330-fold more active than ribavirin against parainfluenza virus (types 2 and 3), mumps virus, and measles virus. EICAR was also more active than ribavirin against respiratory syncytial virus and influenza virus, whereas TJ13025 was virtually inactive against these viruses. The 50% virus-inhibitory concentrations of EICAR and TJ13025 were generally within the 0.1- to 1-μg/ml range. Although the compounds did not prove cytotoxic to stationary host cells (HeLa, Vero, MDCK, and LLCMK2) at a concentration of 200 μg/ml, concentrations of 4 to 13 μg/ml inhibited the growth of dividing cells. EICAR and TJ13025 should be further pursued as candidate drugs for the treatment of ortho- and paramyxovirus infections.

Ribavirin has been licensed for clinical use (as an aerosol) in the treatment of respiratory syncytial virus (RSV) infections. Inhalation of aerosolized ribavirin by infants with RSV pneumonia or college students with influenza A leads to an improvement of the clinical symptoms in these patients (7, 19, 20). The mechanism of action of ribavirin and its metabolism by cells have been investigated (5, 6, 17, 18, 21); the main targets for the antiviral action of ribavirin and its phosphorylated form appear to be IMP dehydrogenase, viral mRNA cap formation, and viral RNA transcriptase or polymerase.

Recently, we have reported two novel nucleoside analogs, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR) and 6′-(*R*)-6′-*C*-methylneplanocin A (TJ13025), as broad-spectrum antiviral agents active against a wide variety of pox-, toga-, reo-, myxo-, rhabdo-, and arenaviruses in vitro (3, 16). In this study, we have evaluated the inhibitory effects of EICAR and TJ13025 in comparison with those of ribavirin on the in vitro replication of several ortho- and paramyxoviruses.

MATERIALS AND METHODS

Compounds. The sources of the three compounds used in this study were as follows: ribavirin, ICN Pharmaceuticals, Costa Mesa, Calif.; EICAR, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan (10); and TJ13025, Toyo Jozo Research Center, Ohito, Shizuoka, Japan (16).

Viruses. The origins of the viruses and preparations of the virus stocks have been reported previously (8, 9, 15). Subtypes, strains, abbreviations, and passage histories of the viruses were as follows. Influenza viruses (FLUV) A/Bangkok/10/83/H1N1, A/Ishikawa/7/82/H3N2, and B/Singapore/222/79 (13) and parainfluenza virus (PFLUV) type 1 (Sendai virus, Fushimi strain) were gifts from N. Ishida, Tohoku University, Sendai, Japan; they were passaged more than 10 times in fertile hen's eggs and 3 times in MDCK cells or LLCMK2 cells respectively. PFLUV type 2 (CA, Greer strain) and type 3 (HA-1, C243 strain) (11) and mumps viruses (MPSV) ECXH-3-II, WV-3, and BMV-0320 (vaccine strain; from M. Hishiyama, National Institute of Health, Japan) were passaged more than six times in Vero cells. MPSV F-1213 was isolated from a patient with mumps by K. Honzumi and passaged twice in Vero cells. Measles viruses (MLSV) Sugiyama (8), Yamagata-1 (isolated from a patient with subacute sclerosing panencephalopathy [SSPE]) (8), Edmonston, and Toyoshima (from T. Kohama, National Institute of Health, Japan) were passaged more than five times in Vero cells. RSV type A (strains Long, FM-58-8, and NS-100) and RSV type B (strain SM-6148) were passaged in HEp-2 cells. Strains FM-58-8, NS-100, and SM-6148 were fresh isolates from patients and were used for experiments within three passages.

Cell culture media. HeLa cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% newborn calf serum, 1.6% glucose (a concentration 16 times

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higher than that in ordinary MEM), 100 U of penicillin G per ml, and 100 μg of streptomycin per ml. For RSV infection of HeLa cells, we used a maintenance medium (MM) consisting of MEM with 2% heat-inactivated fetal calf serum, a fourfold-increased concentration of L-glutamine, 1.6% glucose, and antibiotics as cited above. Vero, MDCK, and LLCMK2 cells were cultured in MEM plus 10% newborn calf serum and antibiotics. For infection of Vero cells with PFLUV (type 2 or 3), MPSV, or MLSV, MM consisting of MEM with 2% heat-inactivated fetal calf serum and antibiotics was used. For the infection of LLCMK2 cells with PFLUV type 1 and of MDCK cells with FLUV, MM consisting of MEM with 0.2% bovine serum albumin, 2.5 μg of crystallized trypsin per ml (Sigma Chemical Co., St. Louis, Mo.), and antibiotics was used.

Antiviral assays. All cell cultures were seeded in 24-well tissue culture plates (Falcon 3407; Becton Dickinson Co., Oxnard, Calif.) at 1.5×10^5 cells per well and incubated at 37°C. After 1 to 2 days of incubation, when the cell cultures became confluent, the growth medium was withdrawn and the cell monolayers were washed once with MM. To each well was added 20 to 40 virus PFU in 0.1 ml of MM. Samples (0.2 ml) of several fourfold dilutions of compounds in MM and 0.7 ml of 1% methylcellulose (Methocel A-4M Premium; Dow Chemical Co., Midland, Mich.) were added to the wells simultaneously with the virus inocula. The infected cell cultures were incubated at 35°C in a CO_2 incubator. After 3 to 4 days of incubation, the overlay medium was withdrawn and the cell sheets were stained with a 0.1% neutral red solution in MM for 2 h at 37°C. After the staining, the cell sheets were fixed with 5% formalin in phosphate-buffered saline (pH 7.2) and the plaque numbers were counted under the microscope (magnification, ×40). For FLUV and PFLUV type 1, focus formation was monitored by hemadsorption of guinea pig erythrocytes to infected cells at 4°C for 1 h before fixation of the cell sheets. The minimal concentration of compound required to inhibit by 50% the number of plaques or hemadsorbing foci was estimated as the 50% effective concentration (EC_{50}).

Cytotoxicity tests. HeLa, Vero, and MDCK cells in growth medium were seeded at 5×10^4 cells per well in 24-well tissue culture plates (Falcon 3702; Becton Dickinson Co.) and allowed to adhere to the plates for a period of 15 or 36 h at 37°C. The medium was then replaced by fresh medium containing different concentrations of the test compounds. Two sets of experiments were carried out. In the first set, medium was replaced by MM containing compound after 36 h of seeding (when a confluent monolayer had formed) and the cells were further incubated at 35°C. In the second set, the medium was changed with growth medium containing compound after 15 h of seeding (when the monolayer was not yet confluent) and the cells were further incubated at 37°C. Each dilution of compound was added to three wells per cell culture. After 3 days of incubation at 35 or 37°C in 5% CO_2, the cytotoxicity of each compound was monitored by counting the viable cell numbers with a hemocytometer. The numbers of viable cells were determined by trypan blue exclusion. Cell viability was also examined by the ability of the cells to incorporate 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Cells were seeded in 96-well tissue culture trays (Falcon 3402) at 5×10^3 cells per well and allowed to adhere to the plate as in the cell-counting experiment. The precise MTT procedure has been described elsewhere (12). The cytotoxicity of each compound was expressed as the 50% inhibitory concentration (IC_{50}), which is the minimal concentration required to reduce the number of viable cells to 50% of the control. The selectivity index of each compound was determined as the ratio of the IC_{50} for cell viability to the EC_{50} for virus replication.

RESULTS

Inhibitory activities of ribavirin, EICAR, and TJ13025 against several ortho- and paramyxovirus strains. Ribavirin, EICAR, and TJ13025 were examined for their activities against four strains of RSV, three strains each of PFLUV and FLUV, and four strains each of MPSV and MLSV (Table 1). Ribavirin inhibited the replication of RSV and FLUV at EC_{50}s of 1.38 to 5.3 μg/ml and the replication of PFLUV, MPSV, and MLSV at EC_{50}s of 8.6 to 67 μg/ml. EICAR exhibited a potent inhibitory activity against all viruses (EC_{50}s, 0.06 to 2.3 μg/ml). TJ13025 also showed very low EC_{50}s against the paramyxoviruses (0.12 to 0.62 μg/ml), except for PFLUV type 1 and RSV, but was inactive against the orthomyxoviruses (EC_{50}, >100 μg/ml).

From the ratios of the EC_{50} of ribavirin to the EC_{50} of EICAR or TJ13025, it appears that EICAR is about 10- to 59-fold more potent than ribavirin against paramyxoviruses and about 2- to 4-fold more potent than ribavirin against FLUV. TJ13025 also showed a 32- to 330-fold-greater potency than ribavirin against all paramyxovirus strains, except for RSV and PFLUV type 1 (Sendai). TJ13025 is at least 20- to 50-fold less active than ribavirin against FLUV.

Cytotoxicities of ribavirin, EICAR, and TJ13025 for HeLa, Vero, MDCK, and LLCMK2 cells. None of the compounds showed cytotoxicity at a concentration of 200 μg/ml for any of the cell lines in the stationary phase, whether the viable-cell number was assessed by the MTT method or hemocytometer (Table 2; the data obtained by the MTT method are not shown). When cytotoxicity measurements were done with replicating cells, EICAR and TJ13025 showed much lower IC_{50}s (based on the reduction in cell counts) of 4.1 to 13.0 and 5.6 to 12.0 μg/ml, respectively. Ribavirin also proved cytotoxic to replicating cells, with IC_{50}s ranging from 14 to 69 μg/ml. The corresponding IC_{50}s obtained by the MTT method varied from 6.6 to 80 μg/ml (data not shown).

Selectivity indices of ribavirin, EICAR, and TJ13025 for selected strains of ortho- and paramyxoviruses. The selectivity indices of EICAR and TJ13025 (Table 3) against paramyxoviruses (except for RSV) were much higher than those of ribavirin, whether the cells were incubated while stationary or growing, in the cytotoxicity test. However, the differences were much lower when the cells were growing. EICAR showed selectivity indices three to four times higher than those of ribavirin against FLUV against both stationary and growing cells. TJ13025 did not show any positive (>1) selectivity indices against RSV and FLUV.

DISCUSSION

In previous papers (3, 16), we have reported that EICAR and TJ13025 have broad-spectrum antiviral activities against a variety of DNA and RNA viruses. In this study, we demonstrated that both compounds are particularly active against representative paramyxoviruses and that EICAR is also active against orthomyxoviruses. EICAR was 10- to 59-fold more active than ribavirin and TJ13025 was 32- to 330-fold more active than ribavirin against paramyxoviruses.

EICAR can be considered an analog of ribavirin, in which the N in position 2 of the triazole ring has been replaced by a C—C≡CH group. Accordingly, EICAR has an antiviral-activity spectrum quite similar to that of ribavirin. TJ13025

TABLE 1. Inhibitory effects of ribavirin, EICAR, and TJ13025 on replication of ortho- and paramyxoviruses in vitro

Virus type and strain	EC_{50} (μg/ml)[a]		
	Ribavirin	EICAR	TJ13025
RSV			
A, Long	1.9 (1.1–2.5)	0.13 (0.09–0.2)	25 (12.5–50)
A, FM-58-8	3.5 (2.8–4.0)	0.09 (0.06–0.12)	25 (25–25)
A, NS-100	1.6 (0.2–2.5)	0.06 (0.03–0.1)	13.4 (3.0–25)
B, SM-6148	1.6 (0.3–2.5)	0.06 (0.04–0.1)	14.0 (4.0–20)
PFLUV			
1, Sendai Fushimi	30.0 (19–50)	0.97 (0.32–2.4)	15.0 (10.0–20.0)
2, CA Greer	25.8 (6.4–52)	0.63 (0.13–1.5)	0.47 (0.09–0.69)
3, HA-1 C243	27.2 (8.9–43)	0.58 (0.44–0.66)	0.16 (0.08–0.2)
MPSV			
ECXH-3-II	23.7 (13.6–32)	1.26 (0.51–1.56)	0.62 (0.06–0.98)
WV-3	13.9 (9.1–20)	0.46 (0.13–0.7)	0.43 (0.06–0.98)
F-1213	28.7 (14.0–52)	0.49 (0.29–0.85)	0.2 (0.08–0.32)
BMV-0320	32.8 (29–36.5)	0.57 (0.37–0.76)	0.44 (0.02–0.86)
MLSV			
Sugiyama	21.9 (9.7–39)	0.69 (0.2–1.8)	0.15 (0.05–0.2)
Toyoshima	9.1 (4.5–19.7)	0.9 (0.06–0.11)	0.25 (0.07–0.39)
Edmonston	66.7 (29–100)	1.51 (0.36–2.6)	0.2 (0.07–0.39)
SSPE Yamagata-1	8.6 (3.9–16.0)	0.32 (0.22–0.39)	0.12 (0.08–0.17)
FLUV			
A, Bangkok/10/83/H1N1	1.5 (0.3–3.0)	0.40 (0.1–0.75)	>100
A, Ishikawa/7/82/H3N2	5.3 (5.1–5.6)	2.3 (2.1–2.5)	>100
B, Singapore/222/79	1.38 (1.2–1.6)	0.44 (0.22–0.7)	>100

[a] Average values (with ranges in parentheses) for three to four independent experiments.

can be considered a 6′-(*R*)-methyl-substituted derivative of neplanocin A, which itself corresponds to cyclopentenyladenine. EICAR and ribavirin are assumed to exert their antiviral actions primarily through depletion of the intracellular GTP pool levels, following inhibition of IMP dehydrogenase activity (3, 18). On the other hand, neplanocin A is known as a potent inhibitor of *S*-adenosylhomocysteine (SAH) hydrolase (1). Neplanocin A has broad-spectrum activity against a variety of DNA and RNA viruses but is also fairly toxic to the host cells (2). Also, TJ13025 is a potent inhibitor of SAH hydrolase (16) and, in addition, has marked activity against a broad range of DNA and RNA viruses (16). SAH hydrolase inhibitors are assumed to inhibit the methylation reactions (with *S*-adenosylmethionine as the methyl donor) that are required for maturation of the viral mRNA (2, 4).

TJ13025 did not prove inhibitory to FLUV replication at concentrations of up to 100 μg/ml. Other SAH hydrolase inhibitors, i.e., carbocyclic 3-deazaadenosine, apparently fail to block the replication of orthomyxoviruses, despite unequivocal evidence of their activities against paramyxoviruses both in vitro and in vivo (4, 16, 22). The differential behaviors of TJ13025 and other SAH hydrolase inhibitors against orthomyxoviruses and paramyxoviruses may be related to differences in methylation requirements of the ortho- and paramyxoviral mRNAs. In fact, it has been well estab-

TABLE 2. Cytotoxicities of ribavirin, EICAR, and TJ13025 for different cell cultures

Cell line	Condition of cells[a]	Increase (fold) in cell number (± SD)[b]	IC_{50} (μg/ml)[c]		
			Ribavirin	EICAR	TJ13025
HeLa	Stationary	1.17 ± 0.3	>200	>200	>200
	Growing	5.10 ± 1.4	55 ± 26	13 ± 6.6	12 ± 6.0
Vero	Stationary	1.53 ± 0.21	>200	>200	>200
	Growing	4.30 ± 0.6	69 ± 11	4.1 ± 2.5	6.7 ± 2.6
LLCMK2	Stationary	1.67 ± 0.2	>200	>200	>200
	Growing	5.41 ± 1.3	65 ± 25	4.3 ± 2.4	5.6 ± 3.9
MDCK	Stationary	1.02 ± 0.11	>200	>200	>200
	Growing	3.40 ± 0.6	14 ± 10	5.6 ± 3.2	7.3 ± 2.2

[a] All stationary cultures were incubated at 35°C in MM. All growing cultures were incubated at 37°C in growth medium.
[b] From the time of medium replacement to the end of the experiment.
[c] IC_{50}s were estimated from counts of viable cells. Values are averages and standard deviations for four independent experiments.

TABLE 3. Selectivity indices of ribavirin, EICAR, and TJ13025 for selected strains of ortho- and paramyxoviruses

Virus type and strain	Cell line	Ribavirin		EICAR		TJ13025	
		Index A[a]	Index B[b]	Index A	Index B	Index A	Index B
RSV							
A, Long	HeLa	>105.3	28.9	>1,538	100	>8	0.48
B, SM-6148	HeLa	>125	34.4	>3,333	216	>14.8	0.86
PFLUV							
1, Sendai Fushimi	LLCMK2	>6.6	0.16	>206	4.4	>13.3	0.37
2, CA Greer	Vero	>7.8	2.7	>317	6.5	>425	14.2
3, HA-1 C243	Vero	>7.3	2.5	>344	7.1	>1,250	41.8
MPSV							
WV-3	Vero	>14.4	5.0	>435	8.9	>465	15.6
F-1213	Vero	>7.0	2.4	>408	8.4	>1,000	33.5
MLSV							
Edmonston	Vero	>3.0	1.0	>133	2.7	>1,000	33.5
SSPE Yamagata-1	Vero	>23.2	8.0	>625	12.8	>1,666	55.8
FLUV							
A, Bangkok/10/83/H1N1	MDCK	>133	9.3	>500	35	>2	<0.07
B, Singapore/222/79	MDCK	>145	10.1	>455	31.8	>2	<0.07

[a] Index A calculated as IC_{50} for stationary cells/EC_{50} for virus.
[b] Index B calculated as IC_{50} for growing cells/EC_{50} for virus.

lished that the 5′-terminal cap of FLUV mRNA is directly transferred from eukaryotic mRNA (13, 14). Thus, the formation of FLUV mRNA does not require de novo methylations, and this may well explain why SAH hydrolase inhibitors, although inhibitory to the replication of paramyxoviruses which apparently depend on these de novo methylations, do not affect the replication of orthomyxoviruses.

In the present study, RSV and PFLUV type 1 (Sendai) did not prove markedly susceptible to the inhibitory activity of TJ13025. This contrasts with the potent inhibitory effects of TJ13025 on the other paramyxoviruses. One of the reasons for this discrepancy may reside in the choice of cells (Vero cells for PFLUV types 2 and 3, MPSV, and MLSV and HeLa and LLCMK2 cells for RSV and PFLUV type 1). Methylation patterns and requirements may be different from one cell (or virus) type to another, and, consequently, the antiviral effects of SAH hydrolase inhibitors may also differ from one virus-cell system to another.

Finally, the potent inhibitory effects that have been noted for EICAR against ortho- and paramyxoviruses and for TJ13025 against paramyxoviruses make these compounds attractive candidate drugs for further study of ortho- and paramyxovirus infections.

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