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A newly developed immunofluorescent assay for determining the Pichinde virus-inhibitory effects of selected nucleoside analogues

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Summary

An immunofluorescent assay (IFA) for Pichinde virus (PCV), a member of the family Arenaviridae, was developed for antiviral drug assays against the virus. The assay was performed by adding fluorescein-labeled anti-PCV monoclonal antibody to virus-infected cells at 24 h after the initial infection and counting the infected cells with an epifluorescence microscope. The average 50% effective dose (ED_{50}) for a series of nucleoside analogues tested against PCV using this IFA was: 2-β-D-ribofuranosylselenazole-4-carboxamide (selenazofurin), < 1.0 μg/ml; 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin), 6.0 μg/ml; ammonium 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5′-phosphate hydrate (ribavirin-5′-monophosphate), 15.8 μg/ml; ammonium 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5′-hemisuccinate (ribavirin-5′-hemisuccinate), 14.7 μg/ml; ammonium 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5′-(2,3-dimethyl)hemisuccinate [ribavirin-5′-(2,3-dimethyl)hemisuccinate], 213.5 μg/ml; 4-hydroxy-1-β-D-ribofuranosyl-2-pyridone (3-deazauridine), 5.2 μg/ml; and (*S*)-9-(2,3-dihydroxypropyl)adenine, ([*S*]-DHPA), 471.0 μg/ml. In comparison, the ED_{50} of ribavirin using inhibition of marginal PCV-induced cytopathogenic effect after 12 days was 6.0 μg/ml and using plaque reduction after 5 days was 2.5 μg/ml, indicating that this IFA was of comparable sensitivity to these other tests.

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Immunofluorescence antiviral assay; Pichinde virus; Ribavirin derivative; 3-Deazauridine; (*S*)-9-(2,3-dihydroxypropyl)adenine

Introduction

The Arenaviridae family contains a group of viruses that have proved to be serious human health threats, manifesting themselves in the form of hemorrhagic fevers. Several members of this family of viruses, such as Junin, Machupo, and Lassa (the etiological agents of Argentine hemorrhagic fever, Bolivian hemorrhagic fever, and Lassa fever, respectively), are endemic in some areas of Africa and South America. Pichinde virus (PCV) is an Arenavirus from South America that is much less pathogenic to humans, but causes a very severe hemorrhagic disease in guinea pigs (Jahrling et al., 1981) and hamsters (Murphy et al., 1977). Ribavirin has previously been shown to have in vitro and in vivo antiviral activity against several of the arenaviruses (McCormick et al., 1986; Stephen et al., 1980).

We have had an interest in developing a simple arenavirus antiviral assay. Standard antiviral assays that depend upon plaque reduction (PR) or inhibition of viral cytopathogenic effect (CPE) are not suitable for working with PCV because this virus does not cause consistently quantifiable CPE (Oldstone, 1987). This report describes an immunofluorescence assay for use in evaluating potential antiviral compounds against PCV. The assay is based on enumeration of virus infected cells utilizing fluorescein-labeled anti-PCV monoclonal antibody 24 h after exposure of cells to the virus. Utilization of this assay as an antiviral test procedure appears reproducible and provides the means to rapidly screen many compounds while reducing both the sample size and assay time.

Materials and Methods

Cells

The Vero 76 cells used in this study were obtained from the American Type Culture Collection (Rockville, MD). Cells were passaged in minimum essential medium (MEM, Gibco, Grand Island, NY) supplemented with 9% fetal bovine serum (FBS, HyClone Labs, Logan, UT) and 50 μg gentamicin/ml. For antiviral assays, cells were seeded at 4×10^4 cells/well in 96-well polystyrene cell culture plates (Corning, Corning, NY) and incubated at 37°C in humidified 5% CO_2 in air. The plates were used 20–24 h after seeding.

Virus

PCV strain An 4763 was provided by Dr. Joseph D. Gangemi, University of South Carolina School of Medicine, Columbia, SC. Virus stocks were prepared in Vero 76 cells from twice plaque-purified PCV. The virus was grown at 37°C and was harvested 2–3 days post-virus exposure, aliquoted into ampules, and stored at

−90°C until used. The virus stock used in these experiments had a titer of 4×10^5 plaque-forming units (PFU)/ml. The virus was also grown in the following cell lines: KB, human oral epidermoid carcinoma, HeLa markers, ATCC CCL 17; BHK, BHK-21, kidney cells derived from one-day-old Syrian hamsters, ATCC CCL 10; MDBK, Madin-Darby bovine kidney cells, ATCC CCL 22; L, L929 cells, in growth medium containing 10% fetal bovine serum rather than 10% horse serum; L929, NCTC clone 929, mouse connective tissue cell line grown in media supplemented with 10% horse serum, ATCC CCL 1; Vero, Vero 76, African green monkey kidney, ATCC CRL 1587; MDCK, Madin-Darby canine kidney cells, ATCC CCL 34; MRC-5, fetal male human lung diploid cells, ATCC CCL 171; WI-38, fetal female human lung diploid cells, ATCC CCL 75; LLC-MK_2D, rhesus monkey kidney, ATCC 7.1; HaK, adult Syrian hamster kidney cells, ATCC CCL 15; MA-104, embryonic African green monkey kidney cells, passage 52 from Dr. Mary Estes, Baylor University, Houston, TX. This was done in an attempt to find a cell line in which PCV would produce consistently quantifiable CPE.

Antiviral compounds

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) and 3-deazauridine (4-hydroxy-1-β-D-ribofuranosyl-2-pyridone) were provided by ICN Pharmaceuticals (Costa Mesa, CA). Selenazofurin (2-β-D-ribofuranosylselenazole-4-carboxamide) was provided by Dr. P.D. Cook, Warner Lambert/Parke Davis, Inc. (Ann Arbor, MI). Ribavirin-5′-monophosphate (ammonium 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5′-phosphate monohydrate), ribavirin-5′-hemisuccinate (ammonium 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5′-hemisuccinate), and ribavirin-5′-(2,3-dimethyl)hemisuccinate (ammonium 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5′-[2,3-dimethyl]hemisuccinate) were synthesized in one of our laboratories (M.D.). The (*S*)-DHPA [(*S*)-9-(2,3-dihydroxypropyl)adenine] was provided by Dr. Erik De Clercq, Rega Institute for Medical Research, Leuven, Belgium. All compounds were stored in sealed vials at room temperature, in the presence of dessicant. For antiviral testing, the compounds were solubilized in MEM and then stored at 4°C until used.

Monoclonal antibodies

The hybridoma cell lines PC4.9A6 and PC4.9D3 were derived through the fusion of FOX-NY myeloma cells with the spleen cells from RBF/Dn mice that had been hyperimmunized with PCV. The adenosine phosphoribosyltransferase procedure as described by Taggart and Samloff (1983) was used to select for hybridoma cell lines. The PC4.9A6 and PC4.9D3 hybridoma cells lines secreted anti-PCV monoclonal antibodies of the IgG2A isotype. The two monoclonal antibodies bind to two different epitopes on a Pichinde virus glycoprotein expressed on the surface of PCV-infected cells. The hybridoma lines had been cloned twice and the cells were in the exponential growth phase when injected into recipient (BALB/c × RBF/Dn) F_1 mice that had been primed by intraperitoneal injection with 1.0 ml of 2,6,10,14-tetramethylpentadecane (pristane). The resulting ascites fluids contained anti-PCV monoclonal antibodies at concentrations ranging from 5–15 mg/ml.

Preparation of fluorescein-labeled monoclonal antibodies

Immunoglobulins, predominantly monoclonal antibodies, were isolated from ascites fluids by precipitation with ammonium sulphate and further purified by affinity chromatography on protein A using the Bio-Rad Affi-Gel Protein A MAPS II Kit (Bio-Rad Laboratories, Richmond, CA). Immunoglobulin samples were dialyzed against 0.1 M Na_2HPO_4, pH 9.0. Following dialysis, precipitates were removed by centrifugation for 15 min at 550 × *g*. Sufficient fluorescein isothiocyanate (FITC, Isomer 1 from Sigma Chemical Co., St. Louis, MO) was added to result in a protein to FITC wt/wt ratio of 50:1 in the conjugation reaction mixture. The FITC was added as a solution containing 1.0 mg/ml of FITC dissolved in 0.2 M Na_2HPO_4, pH 9.0; this FITC solution was prepared just before addition to the immunoglobulin. The conjugation of fluorescein to protein was then accomplished by bringing the pH of the immunoglobulin-FITC mixture to 9.5 by dropwise addition of 0.2 M Na_3PO_4 and allowing the reaction to proceed at 25°C for 2.5 h. The conjugation conditions were selected to yield a fluorescein to protein ratio in the final conjugate of approximately 10 fluorescein molecules per molecule of immunoglobulin (Hebert et al., 1972). The unconjugated FITC was removed by dialysis against Dulbecco's phosphate-buffered saline: 0.5 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4 and 140 mM NaCl, pH 7.2 (PBS), containing 0.1% sodium azide. The fluorescein-labeled monoclonal antibody preparations were then further purified by gel exclusion chromatography on Sephadex G-75 (Pharmacia Fine Chemicals, Piscataway, NJ). The resulting fluorescein labeled monoclonal antibody preparations were titered in a direct immunofluorescent cell assay using PCV-infected Vero 76 cells. There was only the slightest nonspecific staining at dilutions of 1:100 or greater, while the specific staining was very clear and intense out to dilutions of at least 1:1600.

Immunofluorescence assay for PCV-infected cells

The IFA for PCV-infected cells used a cocktail of fluorescein-conjugated murine monoclonal antibodies towards PCV designated PC4.9A6 and PC4.9D3. These were diluted to approximately 0.01 to 0.03 mg protein/ml and then combined in equal volumes in PBS prior to use. Unused antibody was kept frozen at −20°C until thawed immediately prior to use. At 20–24 h postinfection (p.i.), the medium was removed from the wells of the 96-well plates, and the cells were allowed to dry thoroughly at room temperature. If the cell sheets were not allowed to dry thoroughly prior to adding the 80% acetone fixative, many of the cells would detach from the well during the fixation and staining procedures. The cells were then fixed in 80% acetone by adding cold distilled water (50 μl/well) followed by immediate addition of cold (−15°C) acetone (200 μl/well) to the water in each well. If the acetone and water were mixed prior to addition to the wells, or if the acetone was added first, the plastic was etched, thus spoiling the specimens for microscopic examination. The fixation was allowed to proceed for 5 min and then the fixative was poured from the plate and the cell sheets were allowed to dry thoroughly. Cells were either immunostained immediately after drying or stored at −15°C.

Fluorescein labeled monoclonal antibody towards Pichinde virus was used at a dilution of 1:500 to provide intense specific staining with only minimal nonspecific background. For immunostaining, 50 μl of the fluorescein labeled antibody was added to each well and immunostaining was allowed to proceed at 37°C for 1–2 h before the immunostain was simply poured from the plate. The plate was inverted on absorbent paper and allowed to drain, a drop of mounting medium (Heimer and Taylor, 1974) was added, and immunostained cells were viewed through the bottom of the plate using a 16 × objective and 10 × eyepieces on a Zeiss epifluorescence microscope. The number of fluorescent cells in one strip across the center of each well was counted and the total fluorescent cells per well determined as described by Barnett et al. (1975) and Tu et al. (1974). The multiplication factor calculated for the one strip counts in this study was 6.36.

Immunofluorescence antiviral assay (IFA) and toxicity evaluation

Growth medium was aspirated from confluent monolayers of Vero 76 cells growing in 96-well culture plates and 100 μl of PCV diluted in MEM with 2% FBS was added to the appropriate wells. The virus inoculum was such that there were approximately 10 fluorescing cells per microscope field in virus control wells, the multiplicity of infection (MOI) was approximately 0.002. The virus was allowed to adsorb for 30 min at room temperature after which time 100 μl of test compound diluted in MEM was added without removing the virus inoculum. Seven concentrations of test compound were used: 1000, 320, 100, 32, 10, 3.2 and 1 μg/ml. The plates were then sealed with plastic wrap (Saran Wrap™, Dow Chemical) and incubated for 20–24 h at 37°C. The cells were fixed and immunostained to detect PCV-infected cells as decribed above. The number of fluorescing cells in treated wells was determined and compared to the number of fluorescing cells in wells without drug. Each assay included toxicity controls, as well as virus and normal cell controls similar to a virus reduction assay described previously (Sidwell and Huffman, 1971). Three virus-containing wells were used for each dose, with one additional well being used for toxicity controls (cells + sterile virus diluent + drug). Six wells on each plate were used for normal cell controls (cells + sterile virus diluent + drug diluent). Six wells on each plate were used for virus controls (cells + virus + drug diluent). The antiviral activity was expressed as the 50% effective dose (ED_{50}), which was the concentration of drug required to reduce the number of fluorescing infected cells by 50%. The ED_{50} was determined by plotting the percent inhibition versus compound concentration on a semilogarithmic chart.

Drug toxicity was evaluated by examining the toxicity control cells microscopically for morphological changes, when compared with normal cell controls. The 50% cytotoxic dose (CD_{50}) was defined as that concentration which caused approximately a 50% change in cellular morphology of toxicity control cells. This toxicity determination, while subjective and approximate, is based on graded morphological changes observed at increasing levels of the test compounds, with 0% representing no morphological change and 100% representing total cell destruction. The therapeutic index (TI) is defined as the CD_{50}/ED_{50}.

Inhibition of cytopathogenic effect and plaque reduction assays

Ribavirin was run in side by side assays in order to compare the sensitivity of the immunofluorescence antiviral assay to the more standard antiviral assays such as inhibition of viral CPE (Sidwell and Huffman, 1971) and the PR (Huffman et al., 1973) assays. In the CPE assay, the drug was added in triplicate to wells of a 96-well plate in one-half $\log_{10}$ dilutions. The final concentration of drug ranged from 1000 μg/ml to 1.0 μg/ml. The plates were scored when virus controls reached a maximum level of CPE. In these studies using PCV at an MOI of 0.001, this did not occur until approximately 12 days after virus infection, and the maximum CPE obtained was 1+ on a scale of 0 to 4+. In the PR assay the candidate compound was incorporated into the overlay media and was then placed onto infected cell monolayers in 6-well plates. The monolayers in this assay were infected at an MOI of 0.0001. After 5 days of incubation, the plaques in the treated wells were counted and compared with those of the virus control wells.

Results

Attempts to find a cell line in which PCV would produce consistent and quantifiable CPE

Several cell lines, noted in Materials and Methods, were used in an attempt to find a cell line that produced consistently quantifiable CPE when infected with PCV. This was done in order to use the inhibition of CPE assay (Sidwell et al., 1971)

TABLE 1

Effect of ribavirin against Pichinde virus infection in Vero 76 cells as determined by the IFA procedure

Conc. (μg/ml)	Cytotoxicity[a] (μg/ml)	Fluorescent cells (No. of cells/well)	Mean fluorescent cells/well	Inhibition of fluorescent cells (%)
1000	50	0,0,0	0	100
320	25	0,0,0	0	100
100	0	0,0,0	0	100
32	0	0,0,0	0	100
10	0	0,0,0	0	100
3.2	0	133,89,57	93	43
1.0	0	127,146,147	140	14
0	0	171,235,178,114,173,102	162	0

ED_{50}[b]: 4.6 μg/ml
CD_{50}[c]: 1000 μg/ml
TI[d]: 217.4

[a] As determined by microscopic examination of toxicity control wells, expressed as enlarged, flattened cells. The percent indicated is the approximate degree of enlargement and flattening that occurred.
[b] ED_{50}: 50% effective dose, determined by plotting concentration of drug versus percent inhibition on a semilogarithmic chart.
[c] CD_{50}: 50% cytotoxic dose as determined by microscopic examination of toxicity control wells.
[d] Therapeutic index = CD_{50}/ED_{50}.

that is used as a standard antiviral screening test in our laboratory. Only one cell line, Vero 76, showed slight viral CPE, in this case appearing 12 days p.i. PCV did not produce adequate CPE in any of the other cell lines that were tested.

Antiviral activity of ribavirin, 3-deazauridine, (S)-DHPA, and ribavirin derivatives against PCV

An example of an antiviral test using the IFA procedure is seen in Table 1. Ribavirin was used as the test compound. Toxicity controls exhibited morphologic changes only at 1000 and 320 μg/ml dose levels; the CD_{50} was determined to be 1000 μg/ml. Exposure of the infected cells to ribavirin resulted in total inhibition of fluorescent cells as to through 1000 μg/ml dosage levels; a 43% inhibition of infected cells was seen at 3.2 μg/ml. The TI for ribavirin was calculated to be 217.4 in this test.

Using the IFA procedure, the compounds described earlier were evaluated against PCV. The virus was found to be most sensitive to selenazofurin (Table 2). The data also indicate that selenazofurin had the greatest TI of the compounds tested. Ribavirin-5′-hemisuccinate and, as described above, ribavirin, were very effective against PCV using this assay. In contrast, methyl groups incorporated at the 2- and 3- positions of the hemisuccinate side chain reduced the TI 21-fold. Ribavirin-5′-monophosphate and 3-deazauridine were also active against the virus. Very weak activity against PCV was seen with (*S*)-DHPA.

The infected cells were easily distinguished from uninfected cells by the bright apple green fluorescence of the immunostain. There was virtually no background staining of the uninfected cells. The stain appeared to bind to the membrane and cytoplasmic components of the fixed, infected cells. The nuclei of the infected cells did not appear to bind any immunostain. This pattern would be consistent with what is known concerning the replication and maturation of PCV particles.

Comparison of IFA with inhibition of viral CPE and PR antiviral assays

A comparison of the IFA to the inhibition of viral CPE and PR assays is shown

TABLE 2

Activity of nucleoside analogues against PCV as measured by the IFA procedure in Vero 76 cells

Drug	ED_{50}[a] (μg/ml)	CD_{50}[b] (μg/ml)	TI[c]
Selenazofurin	<1.0	320	>320.0
Ribavirin	6.0	1000	166.7
Ribavirin-5′-hemisuccinate	14.7	>1470	>100.0
Ribavirin-5′-monophosphate	15.8	>1000	>63.3
3-Deazauridine	5.2	320	61.5
Ribavirin-5′-(2,3-dimethyl)hemisuccinate	213.5	>1000	>4.7
(*S*)-DHPA	471.0	>1000	>2.1

[a] ED_{50}: 50% effective dose, determined by plotting concentration of drug versus percent inhibition on a semilogarithmic chart; each is an average of 1 to 4 experiments.

[b] CD_{50}: 50% cytotoxic dose.

[c] $TI = CD_{50}/ED_{50}$.

TABLE 3

Activity of ribavirin against PCV as measured by different antiviral assays in Vero 76 cells

Antiviral assay[a]	ED_{50} (μg/ml)[b]	Amount required/assay (mg)[c]	Time to endpoint (days)[d]
CPE	3.2–10	5	12
PR	1.0–3.2	50	5
IFA	3.2–10	5	1

[a] CPE = inhibition of cytopathogenic effect; PR = plaque reduction; IFA = immunofluorescence assay.
[b] ED_{50} values expressed as ranges, from multiple experiments.
[c] Amount of compound required to make initial stock of 2000 μg/ml.
[d] Time at which results could be determined.

in Table 3. The IFA sensitivity appeared comparable to that of the two other assays used, although the PR assay appears to be slightly more sensitive than the IFA or the inhibition of viral CPE assay. This may be, in part, due to the relative MOIs used in the different assays, with the PR assay being infected with 10–20-fold less virus than the inhibition of viral CPE or IFA assays, respectively. Also, the PR assay required larger amounts of the compound than either IFA or CPE procedure, and the time to endpoint was 4 days longer than that required in the IFA test.

Discussion

The IFA procedure described here appears to be an acceptable tool for antiviral drug testing. By comparing the results obtained from evaluating the antiviral efficacy of ribavirin using several different antiviral assays, the IFA demonstrates several important advantages: (1) The IFA is essentially as sensitive as either of the other assays used in this study; (2) it has the advantage of requiring much less compound than the PR assay; (3) the endpoint is much less ambiguous than that of the CPE assay, in which reproducible, readily discernible PCV-induced CPE was difficult to achieve; (4) the IFA assay can be performed in 24 h instead of the days required for PR testing or CPE inhibition. The procedure worked well for evaluation of all nucleoside analogues used in this study.

The relative costs of the three assays used in this study are comparable, if the production costs of the monoclonal antibodies are not taken into consideration. This procedure can be adapted for use as an indirect method using virus-specific polyclonal sera and FITC-labeled secondary antibody. The monoclonal antibodies to PCV produced in our laboratories that were used for this study are not available commercially.

The results reported here indicate that PCV is reasonably sensitive to selenazofurin, ribavirin, ribavirin-5′-hemisuccinate, ribavirin-5′-monophosphate and 3-deazauridine. Selenazofurin and ribavirin have previously been reported active against PCV using PR tests (Kirsi et al., 1983), and ribavirin has also exhibited in

vivo efficacy against this virus (Huggigs et al., 1984). 3-Deazauridine is known to inhibit a number of other RNA viruses, including rhino, influenza A and B, parainfluenza 1 and vesicular stomatitis viruses (Khare et al., 1972), as well as Gross and Rauscher murine leukemia viruses (Shannon, 1972). Ribavirin-5′-monophosphate exhibited both in vitro and in vivo efficacy against influenza A and B and murine hepatitis viruses (Allen et al., 1978). The aliphatic nucleoside (*S*)-DHPA has been reported to have a broad-spectrum antiviral activity (De Clercq et al., 1978) although prior to these experiments, it had never been tested against PCV. The results of testing (*S*)-DHPA against PCV indicate that the compound was active only at the highest concentrations. This is the first report of antiviral testing with the other ribavirin derivatives described here. The loss of activity associated with the incorporation of methyl groups at the 2- and 3- positions of the 5′-hemisuccinate of ribavirin indicates that steric hindrance may inhibit either the transport of the molecule into the infected cell or, more likely, the hydrolysis to free ribavirin.

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