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Comparative efficacy of broad-spectrum antiviral agents as inhibitors of rotavirus replication in vitro

Setsuko Kitaoka[1], Tasuke Konno[1] and Erik De Clercq[2]

[1]*Department of Pediatrics, Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai 980, Japan, and* [2]*Department of Human Biology, Division of Microbiology, Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium*

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Summary

Several nucleoside analogues which have previously been established as broad-spectrum antiviral agents, i.e. ribavirin, vidarabine, pyrazofurin, tubercidin, carbodine, (*S*)-9-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA], carbocyclic 3-deazaadenosine (C-c^{3}Ado), (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid [(*RS*)-AHPA] isobutyl ester and neplanocin A were compared for their potency and selectivity as inhibitors of human rotavirus (strains Wa, KUN and MO) replication in vitro. As the most efficacious inhibitors emerged (*S*)-DHPA, C-c^{3}Ado, (*RS*)-AHPA isobutyl ester and neplanocin A, with a minimum inhibitory concentration of 60, 1.4, 1.2 and 0.2 μg/ml, and a selectivity index of >3, 70, 80 and >20, respectively. As has been postulated for their antiviral action in general, these adenosine analogues probably owe their anti-rotavirus activity to inhibition of *S*-adenosylhomocysteine hydrolase, a key enzyme in regulating methylations including those that are required for the maturation of viral mRNA.

adenosine analogues; ribavirin; vidarabine; pyrazofurin; tubercidin, carbodine; (*S*)-DHPA; C-c^{3}Ado; (*RS*)-AHPA; neplanocin A; human rotavirus; *S*-adenosylhomocysteine hydrolase

Introduction

Acute diarrheal diseases are among the leading causes of morbidity and mortality in infants and young children, particularly in developing countries. Rotaviruses have been recognized as the single most important pathogen causing diarrhea worldwide

[12,15]. Rotavirus diarrhea not only appears to be common, but tends also to be quite severe in the first 2 years of life. The annual death rate due to rotavirus diarrhea in developing countries has been estimated at one million or more [26].

Various strategies have been pursued to produce a rotavirus vaccine: live attenuated human rotavirus strains, heterologous (i.e. bovine) rotavirus strains, reassortant human-animal strains and viral polypeptides obtained through recombinant DNA technology. In particular, the live oral attenuated bovine rotavirus vaccine (derived from Nebraska calf diarrhea virus) seems to be promising [26], although its efficacy, safety and usefulness remain to be assessed by further studies.

An important supportive measure in the treatment of rotavirus diarrhea is oral or parenteral rehydration [17], but the ideal therapeutic approach should obviously be directed against the virus itself. The development of potential anti-rotavirus compounds has been hampered by difficulties in propagating human rotavirus in cell culture and, hence, the lack of adequate assay systems to monitor inhibition of rotavirus replication in vitro. Recently, however, successful in vitro cultivation methods for human rotavirus have been described [14,16,25], and, using these methods, we have now compared several nucleoside analogues for their potency and selectivity as inhibitors of rotavirus replication in vitro.

All compounds that were selected for evaluation of their anti-rotavirus activity had previously been established as broad-spectrum antiviral agents; thus, (i) ribavirin, which has the broadest antiviral spectrum ever reported for a synthetic material that does not act through interferon induction [22]; (ii) vidarabine, which has been licensed as an antiherpetic drug, but is also inhibitory to RNA and DNA viruses other than herpesviruses, i.e. vaccinia virus, rabies virus, vesicular stomatitis virus and murine leukemia virus [11,21]; (iii) pyrazofurin, which is, as compared to ribavirin, much more potent as an antiviral agent in vitro [10]; (iv) tubercidin (7-deazaadenosine), which is, like pyrazofurin, a very potent antiviral agent, but also very toxic for the host cells [1]; (v) carbodine (carbocyclic cytidine), which has significant antiviral activity in vitro against several RNA viruses, including influenza virus [20] and reovirus (E. de Clercq, R. Bernaerts, Y.F. Shealy and J.A. Montgomery, unpublished data, 1984); (vi) (*S*)-9-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA], the first acyclic adenosine analogue to be accredited with broad-spectrum antiviral activity [6,7]; (vii) carbocyclic 3-deazaadenosine (C-c^{3}Ado), the first among the adenosine analogues targeted at *S*-adenosylhomocysteine (SAH) hydrolase [19] that was found selective in its antiviral action [9]; (viii) (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid (AHPA) alkyl (i.e. isobutyl) esters [8]; and (ix) neplanocin A [2,3], which are, like (*S*)-DHPA and C-c^{3}Ado, assumed to acquire their antiviral activity through inhibition of SAH hydrolase [4].

(*S*)-DHPA, C-c^{3}Ado, (*RS*)-AHPA isobutyl ester and neplanocin A are remarkably similar in their antiviral spectrum in that they are particularly active against poxviruses (vaccinia), (-)RNA viruses such as measles, parainfluenza and vesicular stomatitis virus, and reoviruses (reo-1) [3,4,8,9]. Because of their activity against reovirus, it appeared mandatory to explore further the inhibitory effects of these adenosine analogues on the replication of rotavirus. Of the nine compounds that were examined for inhibition of rotavirus replication, only ribavirin and (*S*)-DHPA have been the subject of previous studies on their inhibitory effect on rotaviruses [23,24]. Also

included in these studies were 3-deazaguanine and 3-deazauridine, and of the four antiviral agents evaluated, (*S*)-DHPA appeared to have the greatest potential for human use as an anti-rotavirus inhibitor [24]. However, these previous studies were carried out with bovine, porcine, simian and murine rotaviruses [22,23], whereas the present study was conducted with human rotavirus (strains Wa, KUN and MO).

Materials and Methods

Test compounds

The origin of the test compounds was as follows: ribavirin, ICN Nutritional Biochemicals, Cleveland, Ohio; vidarabine, Parke, Davis & Co., Detroit, Michigan; pyrazofurin, Calbiochem Behring Corp., Lucerne, Switzerland; tubercidin, Upjohn Co., Fine Chemicals Division, Kalamazoo, Michigan; carbodine and C-c^{3}Ado, Dr. Y.F. Shealy and Dr. J.A. Montgomery, Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Alabama; (*S*)-DHPA and (*RS*)-AHPA isobutyl ester, Dr. A. Holý, Czechoslovak Academy of Sciences, Prague, Czechoslovakia; neplanocin A, Toyo Jozo Co., Mifuku Ohito-Cho, Tagata-Gun, Shizuoka-Ken, Japan.

Viruses

Three different serotypes of human rotavirus, designated Wa, KUN and MO, were used in the present study. The KUN (subgroup 1, serotype 2) and MO (subgroup 2, serotype 3) strains were isolated and adapted to cell culture growth in our laboratory [16]. The Wa strain (subgroup 2, serotype 1) was supplied by Dr. R. Wyatt (National Institutes of Health, Bethesda, Maryland) [28].

Cells

MA 104 is an established cell line derived from embryonic rhesus monkey kidney. The cell line was originally supplied by Dr. S. Matsuno (National Institutes of Health, Japan). MA 104 cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS). Cells were incubated at 37°C throughout all experiments.

Cytotoxicity

The cells were seeded in culture tubes (10 × 110 mm) at 10^5 cells per ml growth medium per tube. After 1 day, the culture medium was replaced by fresh growth medium containing varying concentrations of the test compounds, and the cells were further cultured for 1, 2 or 3 days. The cells were then suspended in phosphate-buffered saline (PBS) containing 0.8 mg/ml trypsin and 0.4 mg/ml EDTA and enumerated by a trypan blue dye exclusion assay. Cell counts increased until the 3rd day, levelled off on the 4th and declined thereafter. Assessments for cytotoxicity were based on the reduction in cell number on the 1st, 2nd and 3rd day. The results obtained on the 3rd day are shown (Figs. 1–9).

Inhibition of virus replication

The cells were seeded in culture tubes at 10^5 cells per ml growth medium per tube. After 2 days, the culture medium was replaced by fresh medium (MEM) without FCS. One day later, the cell monolayers were inoculated with rotavirus (strain Wa, KUN or MO) at a multiplicity of 0.1 and further incubated in MEM containing varying concentrations of the test compounds. After 24 h, the cells were harvested and frozen at -80°C. After thawing and another cycle of freeze–thawing the cell lysates were centrifuged at 1000 rpm for 5 min. Serial dilutions of the supernatant were then inoculated on MA 104 cell monolayers grown on multichamber glass slides (Lab-Tek Products). After 20-h incubation, the cells were fixed with cold acetone and evaluated by an indirect immunofluorescent assay using rabbit antiserum against MO strain and fluorescein isothiocyanate-conjugated anti-rabbit goat immunoglobulin (Behringwerke). Virus titers are expressed as fluorescent cell focus (forming) units (fcfu).

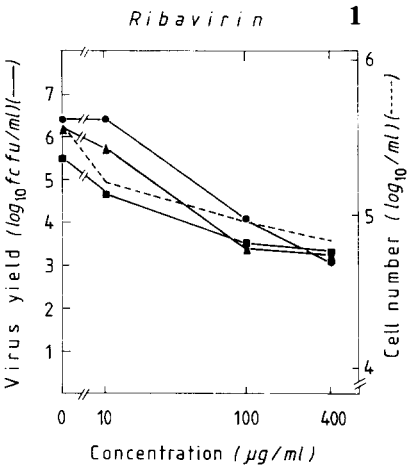
Results

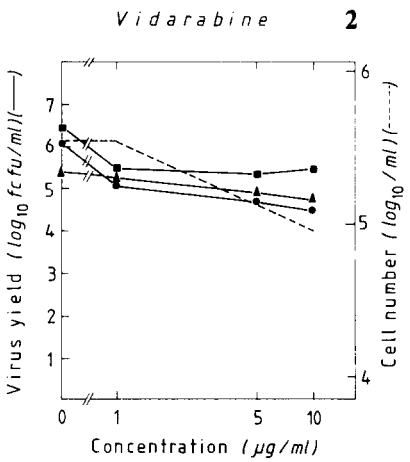
In preliminary experiments (carried out with the KUN strain) it was found that virus yield reached its peak value at 24 h after infection and levelled off thereafter. In these preliminary experiments it was also assessed that the dose-dependent reductions in virus yield achieved by the test compounds [ribavirin, (*S*)-DHPA, C-c^{3}Ado, (*RS*)-AHPA isobutyl ester] were similar, whether the virus yields were measured at 24, 48 or 72 h after infection (data not shown). In all further tests aimed at comparing the virus yield reductions effected by the nine test compounds with the three rotavirus strains, virus yields were uniformly measured at 24 h after infection.

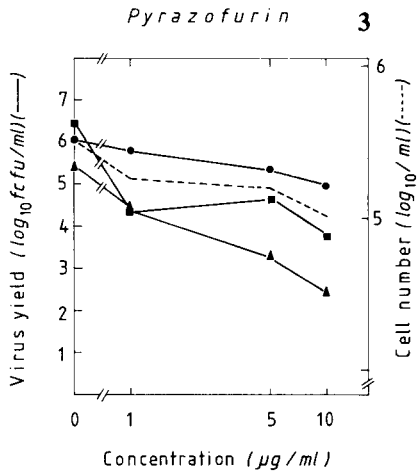
Ribavirin caused a dose-dependent inhibition of the multiplication of all three rotavirus strains, but, as shown in Fig. 1, concomitantly with the virus titer reductions, ribavirin also effected a reduction in cell growth. Vidarabine showed little effect on virus yield, even at a concentration (10 μg/ml) at which there was a significant reduction in cell growth (Fig. 2). Pyrazofurin achieved a slightly greater reduction in virus yield, particularly of the MO strain, than vidarabine, but, again, concomitantly with a decrease in host cell growth (Fig. 3). Tubercidin was highly cytotoxic: at concentrations $\geqslant$0.4 μg/ml it suppressed the proliferation of the host cells, and at subtoxic concentrations ($\leqslant$0.1 μg/ml) it did not markedly affect virus replication (Fig. 4). Carbodine clearly suppressed growth of the host cells within the concentration range (1–10 μg/ml) which proved barely inhibitory to virus replication (Fig. 5).

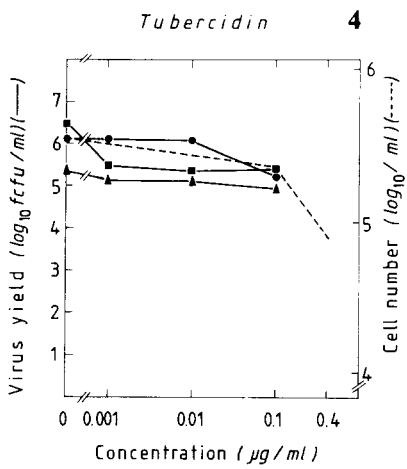
(*S*)-DHPA effected a 1–2 $\log_{10}$ reduction in virus yield at a concentration of 100–200 μg/ml; at these concentrations it did not impair host cell growth (Fig. 6). C-c^{3}Ado

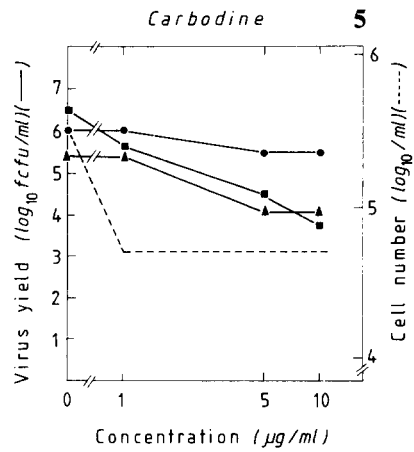
Figs. 1–9. Inhibitory effects of antiviral compounds on the replication of 3 human rotavirus strains, Wa (●), KUN (■) and MO (▲), and the proliferation of uninfected host (MA 104) cells. Virus yield was measured 24 h after infection by a fluorescent cell focus assay (see Materials and Methods). Cell counts were determined 3 days after incubation of the cells in the presence of the test compounds: ribavirin (Fig. 1), vidarabine (Fig. 2), pyrazofurin (Fig. 3), tubercidin (Fig. 4), carbodine (Fig. 5), (*S*)-DHPA (Fig. 6), C-c^{3}Ado (Fig. 7), (*RS*)-AHPA isobutyl ester (Fig. 8), and neplanocin A (Fig. 9).

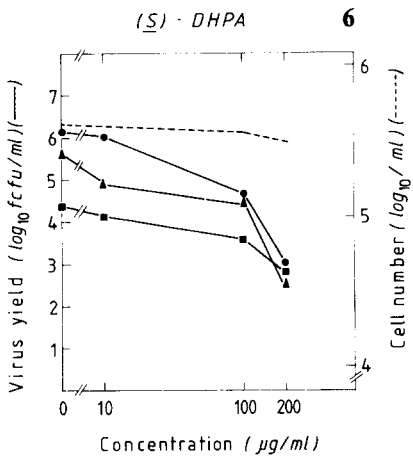

Ribavirin
1
Virus yield (log10 fcfu/ml)(——)
Cell number (log10/ml)(------)
Concentration (µg/ml)
0
10
100
400

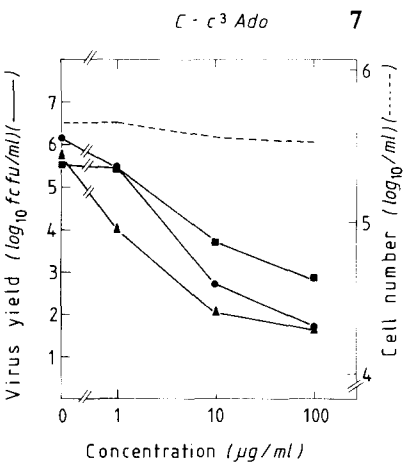

Vidarabine
2
Virus yield (log10 fcfu/ml)(——)
Cell number (log10/ml)(------)
Concentration (µg/ml)
0
1
5
10

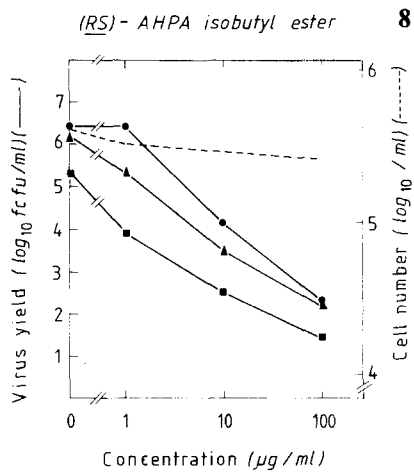

Pyrazofurin
3
Virus yield (log10 fcfu/ml)(——)
Cell number (log10/ml)(------)
Concentration (µg/ml)
0
1
5
10

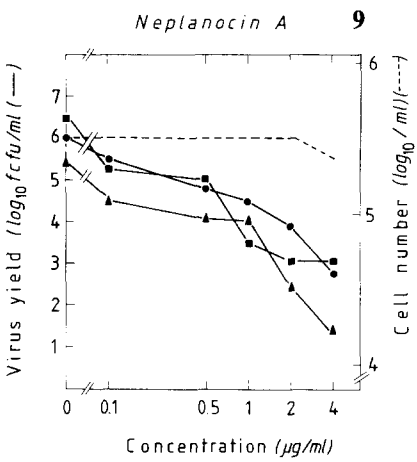

Tubercidin
4
Virus yield (log10 fcfu/ml)(——)
Cell number (log10/ml)(------)
Concentration (µg/ml)
0
0.001
0.01
0.1
0.4


Carbodine
5
Virus yield (log10 fcfu/ml)(——)
Cell number (log10/ml)(------)
Concentration (µg/ml)
0
1
5
10


(S) - DHPA
6
Virus yield (log10 fcfu/ml)(——)
Cell number (log10/ml)(------)
Concentration (µg/ml)
0
10
100
200







Figs. 7–9. For legend, see page 60.

brought about a 3 $\log_{10}$ reduction in virus yield at a concentration of 10–100 μg/ml, while not being toxic for the host cells within this concentration range (Fig. 7). Similarly, no significant inhibition of cell growth was noted with (*RS*)-AHPA isobutyl ester at a concentration of 100 μg/ml, which achieved an almost 4 $\log_{10}$ reduction in rotavirus replication (Fig. 8). A selective inhibition of virus yield, ranging from 1 $\log_{10}$ at 0.2 μg/ml to 3–4 $\log_{10}$ at 4 μg/ml, was also noted for neplanocin A; in this concentration range, neplanocin A did not interfere with normal host cell proliferation (Fig. 9).

If then the minimum cytotoxic concentrations and minimum antiviral concentrations of the compounds were calculated, and their selectivity index determined (Table 1), a clear distinction could be made between selective and non-selective inhibitors of human rotavirus replication. Ribavirin, vidarabine, pyrazofurin, tubercidin and carbodine emerged as non-selective inhibitors, whereas (*S*)-DHPA, C-c³Ado, (*RS*)-AHPA isobutyl ester and neplanocin A exhibited marked selectivity as anti-rotavirus agents.

TABLE 1

Comparative potencies and selectivities of broad-spectrum antiviral agents as inhibitors of human rotavirus replication

Compound	MTC[a] (μg/ml)	MIC[b] (μg/ml)	Selectivity index[c]
Ribavirin	10	20	0.5
Vidarabine	4	7	0.6
Pyrazofurin	1	1	1
Tubercidin	0.15	>0.1	<1.5
Carbodine	<1	4	<0.25
(*S*)-DHPA	>200	60	>3
C-c^{3}Ado	>100	1.4	70
(*RS*)-AHPA isobutyl ester	>100	1.2	80
Neplanocin A	>4	0.2	>20

[a] Minimum toxic concentration, required to effect a 50% (0.3 $\log_{10}$) reduction in cell number (see Figs. 1–9).
[b] Minimum inhibitory concentration, required to effect a 90% (1 $\log_{10}$) reduction in virus yield (average value for 3 rotavirus strains: Wa, KUN and MO; see Figs. 1–9).
[c] Ratio of MTC to MIC.

Discussion

All four compounds [(*S*)-DHPA, C-c^{3}Ado, (*RS*)-AHPA isobutyl ester and neplanocin A] that were found to be selective inhibitors of human rotavirus replication, have previously been shown to be potent inhibitors of SAH hydrolase. Thus, Votruba and Holý [27] noted a K_i of 3.5 μM of (*S*)-DHPA for rat liver SAH hydrolase and Merta et al. [18] found a K_i of 0.9 μM of (*S*)-DHPA for murine L1210 leukemia SAH hydrolase. For the *R* and *S* enantiomers of AHPA, Merta et al. [18] found a K_i of 0.04 and 0.12 μM, respectively. Montgomery et al. [19] and Houston et al. [13] reported for C-c^{3}Ado K_i values of 1 nM for hamster liver SAH hydrolase, and 4 nM for bovine liver SAH hydrolase. For neplanocin A Borchardt et al. [2] obtained a K_i of 8.39 nM for the beef liver enzyme.

When the four adenosine analogues were evaluated under the same experimental conditions for their inhibitory effects on bovine liver SAH hydrolase [5], their K_i values amounted to 1.4 μM for (*S*)-DHPA, 0.073 μM for (*RS*)-AHPA, 0.013 μM for C-c^{3}Ado and 2 nM for neplanocin A. When these K_i values were plotted in function of the minimum inhibitory concentrations of the compounds for the replication of vesicular stomatitis virus, a close correlation ($r = 0.986$) emerged [5], suggesting a causal relationship between the antiviral activities of the four adenosine analogues and their inhibitory effects on SAH hydrolase. The relative inhibitory potency of the compounds against human rotavirus is similar to that reported previously for vesicular stomatitis virus; thus, in order of increasing potency: (*S*)-DHPA < (*RS*)-AHPA (isobutyl ester) ≈ C-c^{3}Ado < neplanocin A. It is likely, therefore, that these compounds acquire their antiviral properties, and in particular their inhibitory effects on rotavirus replication, by an action targeted at SAH hydrolase.

The precise mechanism of action of the adenosine analogues remains to be explored. One may wonder how inhibition of SAH hydrolase can lead to a specific block of the virus replicative cycle without affecting normal cell metabolism. Indeed, SAH is a product inhibitor of *S*-adenosylmethionine (SAM)-dependent methylations, and such reactions are essential not only for viral mRNA maturation, i.e. 5'-terminal cap formation, but also for the normal metabolic integrity of the cell. Conceivably, SAM-dependent methylation reactions occurring in the virus-infected cell are quantitatively or qualitatively different from transmethylations in uninfected cells and therefore more vulnerable to SAH hydrolase inhibitors and the concomitant accumulation of SAH. Further studies should be directed at measuring SAH and SAM levels in virus-infected cells under the same conditions as used for determining the inhibitory effects of the compounds on virus multiplication.

The present findings support the rationale of designing selective rotavirus inhibitors based on the inhibition of SAH hydrolase. They also provide a series of compounds which themselves offer considerable potential as rotaviral inhibitors and which should therefore be pursued for their anti-rotavirus activity in animal models. Smee et al. [24] have recently described a convenient murine rotavirus model in which the compounds could be evaluated by the oral route.

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