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Influence of twenty potentially antiviral substances on in vitro multiplication of hepatitis A virus

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Summary

A multiwell tissue culture system was developed to study the influence of various substances on hepatitis A virus (HAV) propagation. A panel of 20 substances of different structure types, each with known effect against at least some viruses, was studied at a concentration of 100 μM. Three substances showed reproducible inhibition. The strongest inhibitor, arabinosylcytosine, also produced cytotoxic changes in cells down to a concentration of 1 μM, and its effect was considered as nonspecific. Amantadine and ribavirin showed a moderate effect at 100 μM. A stronger inhibition was seen at 250 and 500 μM, doses that are toxic and impractical for clinical use. Although no promising candidates for antiviral treatment of hepatitis A have emerged from the present study, the assay model described here would seem useful in the screening of substances with inhibitory effects on HAV.

hepatitis A; arabinosylcytosine; amantadine; ribavirin

Introduction

Antiviral therapy has so far only been accepted clinically in a limited number of viral diseases, mainly those caused by viruses of the herpes group and influenza A [3,6–8]. However, the search for new substances and new application forms of established drugs continues.

Although hepatitis A infection during childhood tends to be mild or subclinical, the same infection in adults can be severe and of several weeks duration. The mortality rate

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is low but not negligible (0.1–0.2%) [4]. The changing epidemiology of hepatitis A in many Western countries [2] in the last decades has resulted in a lack of naturally acquired immunity to hepatitis A in many adults. In such populations, epidemical outbreaks with several icteric cases can occur if the virus is re-introduced [14].

The relative severity of hepatitis A together with increasing susceptibility in many populations prompted the search for substances that would be active against hepatitis A virus replication. Propagation of hepatitis A virus (HAV) in cell culture, first described by Provost and Hillemann [10], provides a new way to screen such substances. Two substances with known effect against other picornaviruses, namely guanidine and 2-(α-hydroxybenzyl)-benzimidazole, have already been shown to lack activity against HAV in vitro [12]. In the present study, 20 substances of different structural type were screened for inhibitory effect against HAV.

Materials and Methods

Cells

A continuous cell line (Frhk-4), originating from fetal rhesus monkey kidney, was a kind gift from Dr. Bertram Flehmig, Hygiene Institut, Tübingen, F.R.G.

Virus

Hepatitis A virus (H 141 strain) was isolated in our laboratory from the stools of a patient with hepatitis A, two days after onset of jaundice [15]. The H 141 strain was serially passaged, and infectious material at higher passages (12–21) was used in the present study.

Media

Eagle's minimum essential medium with Hank's balanced salts solution (Gibco, Paisley, U.K.) was used with 3% fetal calf serum (Flow Laboratories, Irvine, U.K.). Penicillin (250 000 IU/l) and streptomycin (100 mg/l) were added.

Trypsin solution (0.25%) (Flow Laboratories, Irvine, U.K.) with 0.02% EDTA (Titriplex III, Merck, Darmstadt, F.R.G.) was prepared in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline.

Culture vessels

Sterile, disposable 24-well plates with lids (Nunclon Delta, Roskilde, Denmark) were used. The wells were seeded with approximately 40 000 cells per well in 1.5 ml of medium containing 10% of fetal calf serum. The plates were kept in a humidified incubator (Hereus B5060, Hanau, F.R.G.) with 5% CO_2 at 37°C. Cell morphology was observed via an inverse microscope (Labovert, Leitz, Wetzlar, F.R.G.) equipped with a camera (OM-2, Olympus, Tokyo, Japan).

Detection of HAV antigen

A solid phase radioimmunoassay following a method described by Purcell et al. [11] was used. Briefly, test samples were incubated in the wells of a microtitre plate

previously coated with human anti-HAV. After washing, ^{125}I-labelled anti-HAV-IgG was added and the bound radioactivity was measured. The mean of negative controls (N) was calculated and HAV antigen reactivity was expressed as the ratio between the individual sample (S) and N. S/N ratios above 2.1 were regarded as positive.

Test substances

Twenty substances, synthesized or received from the Department of Antiviral Chemotherapy, Astra Läkemedel, most of them with known in vitro effect against RNA or DNA viruses were tested [3,6–8]. Most of the substances were diluted in distilled water at concentrations of 2–10 mM and stored at –70°C until final dilutions in medium were made. Some substances (marked with *) could only be dissolved when final dilutions of 110 μM were made. The test substances belonged to three different groups: Group 1 (acids): trisodium phosphonoformate, disodium phosphonoacetate, 2-phosphonoundecanoic acid, aurintricarboxylic acid and caffeic acid*. Group 2 (amines): amantadine, rhodanine, *N*-(1,3,4-thiodiazol-2-yl)-thiourea*, 6-diazo-5-oxo-L-norleucin. Group 3 (nucleoside analogs): arabinosyladenine (ara-A), arabinosylthymine* (ara-T), arabinosylcytosine (ara-C), acycloguanosine, 9-(4-hydroxybutyl)-guanine, *S*-9-(3,4-dihydroxybutyl)guanine, *R*-9-(3,4-dihydroxybutyl)guanine, 9-[3-(hydroxymethyl)-4-hydroxybutyl]guanine, 5-iodo-5′-amino-2′,5′-dideoxyuridine*, 3′-deoxyguanosine*, and ribavirin.

Assay model

At the time they were inoculated with virus, the cells had been confluent for at least four days.

The old medium was removed and 1.35 ml of fresh medium was added per well. The medium except for the virus control wells contained the test substances at an initial concentration of 110 μM. Each substance was present in a row of six wells. Within 15–20 min, 0.150 ml of virus inoculum diluted in medium was added per well. In this way, the concentration of the test substances was reduced to 100 μM and the virus inoculum was further diluted 10-fold. All substances were tested in duplicate wells and with two different inoculation doses, one at 3–30 ID_{50} (50% infectious dose) and the other at 300–3000 ID_{50}. The remaining two uninfected wells with each substance were used as cytotoxicity controls and for calculation of negative RIA levels. Duplicate wells, containing infected cells, served as virus controls and were also used for infectivity titration included in each test (inocula diluted 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and control).

For some substances (see below) further concentrations of both test substances and inocula were investigated, using the same principles of duplicate wells and infectivity controls.

After inoculation, the plates were kept in a humidified CO_2-incubator for seven days. On days three and seven, cell morphology was evaluated and photographs were taken from unstained living cells incubated in the presence of the test substances. On day seven, the medium was removed by aspiration using Pasteur pipettes, and 0.5 ml of trypsin EDTA was added to each well. After the cells had been detached, the plates were freeze-thawed three times and 50-μl portions of the homogenates were thereafter

tested by RIA. An arithmetic mean (N) was calculated for all uninfected wells. Arithmetic means of duplicate wells were calculated for each substance and each inoculation dose. Results are expressed as S/N ratios at each inoculation dose and were compared with S/N ratios obtained from wells to which no substance had been added.

Results

Variations in the assay model

To test the natural variation of HAV-RIA results in the assay, performed over 7 days, three 24-well plates with Frhk-4 cells were infected with three different virus concentrations without any test substance added. Each inoculum was added to altogether 18 wells, evenly distributed over the plates. Wells were infected with 3 ID_{50}, 30 ID_{50} and 300 ID_{50}, and 18 wells were kept uninfected as negative controls. The mean S/N ratio of uninfected wells was 1.00 ± 0.18, and the mean ratio of wells infected with 3 ID_{50} was 4.75 ± 0.91. Inoculation with 30 ID_{50} resulted in a mean S/N ratio of 11.0 ± 1.81 and 300 ID_{50} resulted in a mean S/N of 17.3 ± 1.97. The differences between uninfected and 3 ID_{50}, between 3 and 30 ID_{50}, and between 30 and 300 ID_{50}, were each highly significant ($P < 0.001$, Student's *t*-test). Only rarely, in two out of 72 wells, did RIA results slightly overlap between two inoculation doses.

Screening test

The 20 substances were studied in parallel for their influence on HAV replication and cell morphology. The infectivity titration performed along with the screening test, when no substance was added, showed that the inoculum contained $10^{4.5}$ ID_{50}/1.5 ml. Since inoculum dilutions of 10^{-2} and 10^{-4} were used, the test wells were challenged with 3 and 300 ID_{50} per well. When no test substance was added, 3 ID_{50} after one week gave a mean S/N ratio of 4.04 in the HAV antigen RIA, whereas 300 ID_{50} gave a mean S/N ratio of 13.4. The results of inoculation with 3 ID_{50} and 300 ID_{50} in the presence of each of the 20 substances are shown in Table 1.

As shown, most substances did not affect HAV replication but four substances amantadine, ribavirin, 6-diazo-5-oxo-L-norleucin and arabinosylcytosine (ara-C) gave an S/N ratio below 2.1 at 3 ID_{50} and a greater than 50% reduction of S/N at 300 ID_{50}. These substances were further tested. For ara-C that caused the most pronounced depression of HAV replication, there were also signs of direct cell toxicity, whereas the other three substances did not affect cell morphology. The four substances were further evaluated in separate tests.

Testing of ara-C

The influence of ara-C on HAV replication and cell morphology was studied at decreasing ara-C concentrations (100, 50, 25, 12.5, 6.25 and 1 μM) with three different doses of virus inoculation. The ID_{50} titer of the virus in this test was $10^{-5.5}$ and the virus inoculum/well was 30, 300 and 3000 ID_{50}. As previously, all testing was performed in duplicate. Cell morphology was studied more in detail. As can be seen in Table 2, there

TABLE 1

Effect on HAV propagation of 20 substances, each evaluated at 100 μM concentration

		S/N ratios (challenge dose)	
		300 ID_{50}	3 ID_{50}
	No substance added	13.4	4.04
Group 1: acids	Trisodium phosphonoformate	18.7	3.73
	Disodium phosphonoacetate	15.7	4.23
	2-Phosphonoundecanoic acid	10.7	2.33
	Aurintricarboxylic acid	16.0	2.00
	Caffeic acid	15.5	4.67
Group 2: amines	Amantadine[a]	5.23	1.32
	Rhodanine	13.7	4.60
	N-(1,3,4-Thiodiazol-2-yl)thiourea	19.0	4.61
	6-Diazo-5-oxo-L-norleucin[a]	3.74	1.19
Group 3: nucleoside analogs	Arabinosyladenine (ara-A)	10.3	3.52
	Arabinosylthymine (ara-T)	12.7	3.28
	Arabinosylcytosine (ara-C)[a]	2.02	0.95
	Acycloguanosine	12.3	3.16
	9-(4-Hydroxybutyl)guanine	8.44	2.99
	S-9-(3,4-Dihydroxybutyl)guanine	16.3	3.25
	R-9-(3,4-Dihydroxybutyl)guanine	12.4	2.94
	9-[3-(Hydroxymethyl)-4-hydroxybutyl]guanine	7.22	2.62
	5-Iodo-5'-amino-2',5'-dideoxyuridine	13.2	3.16
	3'-Deoxyguanosine	14.6	5.18
	Ribavirin[a]	2.67	1.09

[a] These substances were selected for further testing.

was a pronounced influence on HAV antigen expression at levels down to 12.5 μM of ara-C, and even at 1 μM some influence was observed. This paralleled the influence by ara-C on cell morphology, which was clearly seen down to 6.25 μM and still discernible at 1 μM (Fig. 1). Cell changes consisted mainly of vacuolization and reduction of cell density. Detachment of cells was not observed; neither were pH changes observed during the 7-day incubation period.

To investigate whether HAV viability had become reduced after virus had grown in the presence of ara-C, cell homogenates (medium-free) from selected wells were also tested for infectivity. These infectivity titrations were limited to four pairs of wells, namely those infected with 30 and 3000 ID_{50} and kept in ara-C free or ara-C containing (50 μM) medium. HAV antigen expression by RIA in these wells varied greatly (S/N 34.0–1.17) and two almost identical values, 7.46 and 7.56 were noted. These values were obtained with 30 ID_{50} without ara-C and with 3000 ID_{50} plus 50 μM ara-C.

Results of the infectivity titrations, presented in brackets in Table 2, show that identical infectivity titres ($10^{-4.5}$) were obtained when original S/N ratios were the same (about 7.5), irrespective of the influence of ara-C on virus growth. Furthermore, at the

TABLE 2

Effect of different concentrations of ara-C on HAV propagation

	S/N ratios (challenge dose)		
	3000 ID_{50}	300 ID_{50}	30 ID_{50}
No substance added	34.0 *i* ($10^{-5.5}$)	17.4	7.46 *i* ($10^{-4.5}$)
Ara-C 100 μM	11.1	4.63	2.00
50 μM	7.56 *i* ($10^{-4.5}$)	3.88	1.17 *i* ($10^{-3.5}$)
25 μM	10.3	4.26	1.67
12.5 μM	11.3	4.15	2.60
6.25 μM	16.2	9.05	3.90
1.0 μM	22.4	11.0	3.16

Material marked with *i* underwent subsequent infectivity titrations; ID_{50} titres are given in parentheses.

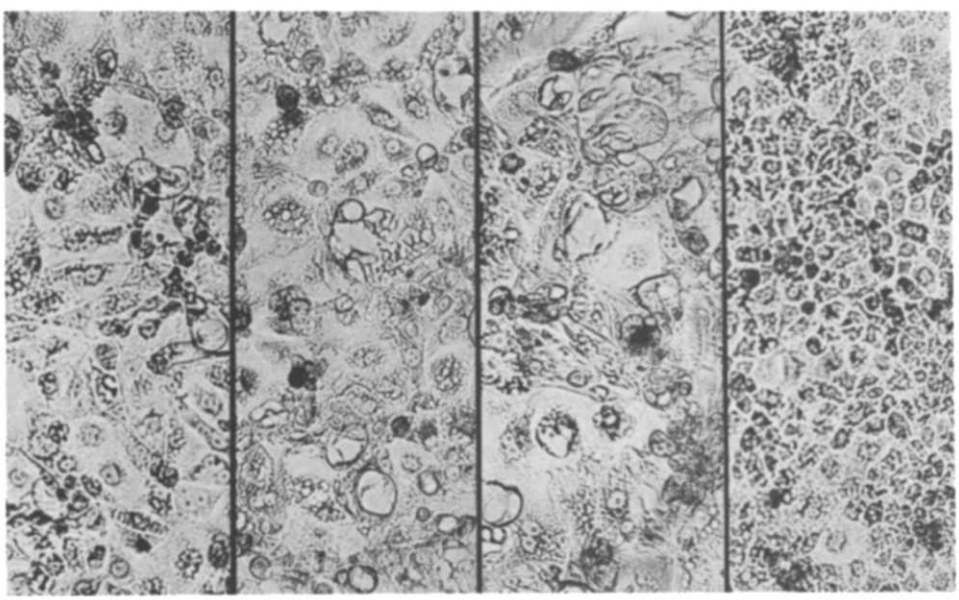

Fig. 1. Cell morphology 7 days post-inoculation with HAV. Control virus-infected cells (left) are shown together with infected cells incubated in the presence of three different levels of ara-C (100 μM, 6.25 μM and 1 μM).

concentration (50 μM) where ara-C lowered HAV antigen ratios from 34.0 to 7.56 and from 7.46 to 1.17, there was only a 10-fold fall in infectivity titre ($10^{-5.5}$ to $10^{-4.5}$ and $10^{-4.5}$ to $10^{-3.5}$). Inoculation of 30 ID_{50} in ara-C (50 μM) that made detection of HAV antigen by RIA negative (1.17), nonetheless allowed a virus propagation to an infectivity titre of $10^{-3.5}$.

Testing of remaining substances

One substance, 6-diazo-5-oxo-L-norleucin had a minor effect on HAV replication in a follow-up test but no effect in other tests (data not shown) and was excluded from further studies. The remaining two substances, amantadine and ribavirin, which in repeated tests showed inhibition on HAV replication, were tested at different concentrations (500, 250, 100, 50, 25 and 12.5 μM). In this experiment, the wells were infected with 3 and 30 ID_{50}. A combination with 50 μM of amantadine and 50 μM of ribavirin was also included and the results are presented in Table 3. Concentrations of both 250 and 500 μM of amantadine and ribavirin led to an inhibition of HAV expression. With ribavirin there was a strong influence on cell morphology at these levels whereas amantadine was less cytotoxic (Fig. 2). At 100 μM both substances caused some inhibition though less pronounced than in the screening tests. At lower concentrations, the inhibitory effects of both compounds disappeared. There was no sign of a synergistic or additive effect when ribavirin and amantadine were combined at a concentration of 50 μM.

TABLE 3

Effect of amantadine and ribavirin on HAV replication

		S/N ratios (challenge dose)	
		30 ID_{50}	3 ID_{50}
No substance added		11.6	3.95
Amantadine:	500 μM	1.74	1.30
	250 μM	2.99	1.33
	100 μM	7.88	2.13
	50 μM	7.69	3.53
	25 μM	10.2	2.67
	12.5 μM	12.4	4.07
Ribavirin:	500 μM	1.58	0.98
	250 μM	3.02	0.84
	100 μM	7.32	2.06
	50 μM	8.15	2.68
	25 μM	11.5	3.28
	12.5 μM	12.6	4.00
Amantadine 50 μM Ribavirin 50 μM	combined	8.60	3.82

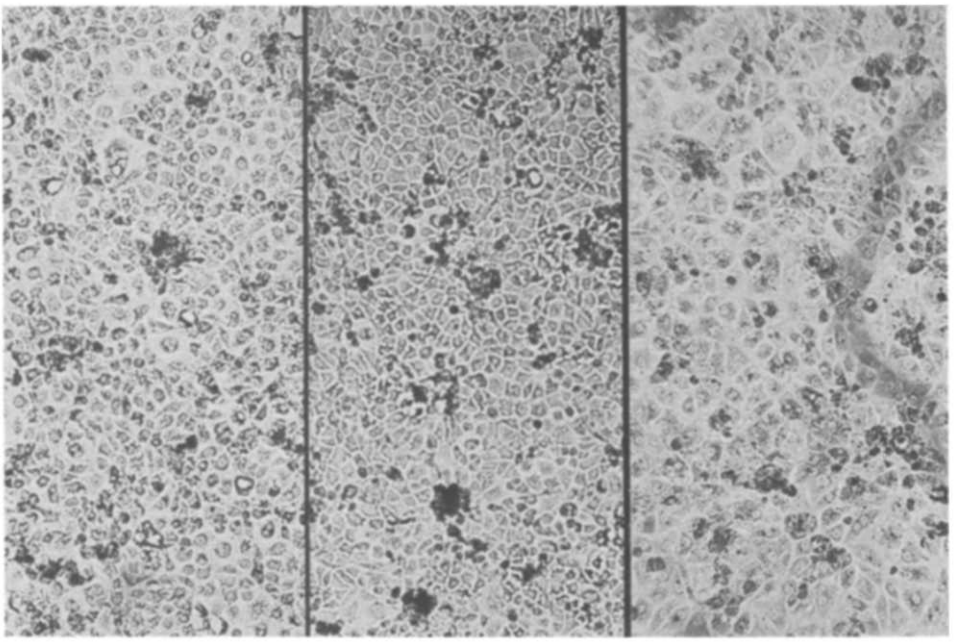

Fig. 2. Cell morphology 7 days post-inoculation with HAV. Control virus-infected cells (middle) are shown together with infected cells incubated in the presence of 500 μM amantadine or 500 μM ribavirin.

Discussion

The likely presence of a viral RNA polymerase [5], required for HAV replication, constitutes a target for antiviral drugs against disease caused by this virus. However, only one report has been published concerning the effect of inhibitors on HAV replication [12]; that there are no other reports is due to the difficulty of growing HAV in cell culture rather than the lack of interest in a therapy against HAV infections. The multiwell tissue culture assay used in the present study proved satisfactory and reproducible. The taxonomic position of HAV among the picornaviruses makes it natural first to investigate picornavirus inhibitors for activity against HAV but it might be useful at an early stage to screen compounds with a broader spectrum. The compounds used in this report display a wide spectrum both structurally and functionally.

The substance causing the strongest inhibition on HAV replication, ara-C, also caused disturbances in cell morphology even at low concentrations. It is known that levels even less than 1 μM inhibit the multiplication of human cells (Stenberg, K., personal communication). In our assay, very little influence on the cells was seen during the first 3 days post-inoculation whereafter there appeared gradually changes of a type shown in Fig. 1. In other tests the cytotoxic effects were somewhat less

pronounced but there was still a strong inhibition on HAV expression (data not shown). The infectivity titrations, performed on infected cells propagated in the presence or absence of ara-C, did not show any increased production of defective HAV under the influence of ara-C. Moreover, when 30 ID_{50} was used with 50 μM ara-C, and HAV antigen remained undetectable by RIA, virus still multiplied to a titre of $10^{-3.5}$. Thus, the inhibition of HAV replication by ara-C could be regarded as the consequence of its cytotoxic action.

Ribavirin showed inhibition on HAV replication especially at higher concentrations (250 and 500 μM), when cytotoxic effects were also observed. According to a placebo-controlled study [9] from India, oral ribavirin significantly shortened the duration of abnormal liver values in acute non-B hepatitis, presumably hepatitis A. Since these cases were not serologically confirmed, it is possible that the described hepatitis epidemic was of the recently described epidemic non-A, non-B type. The drug, 600 mg given daily (50 μmol/kg body weight), was well tolerated. Our in vitro study of ribavirin showed only moderate inhibition of HAV replication at non-cytotoxic levels, and it is doubtful whether this reflects any clinical usefulness. A 50% inhibition of cellular DNA synthesis has been reported at about 100 μM ribavirin [1].

Amantadine inhibited HAV replication to a degree similar to that caused by the same concentration of ribavirin, although cytotoxic effects were less pronounced. In clinical practice, the daily oral dose of 200 mg of amantadine against influenza A gives plasma drug levels of 2–5 μM [13]. These levels are below those at which any effect was noted in our assay. Amantadine inhibits the multiplication of human cells by 50% at 215 μM (Stenberg, K., personal communication). Thus, there are few indications that amantadine might be clinically useful against hepatitis A.

Although no promising candidates for treatment of hepatitis A emerged from the present study, the assay model provides a simple and practical way to evaluate potential candidate inhibitors of HAV. Should this procedure lead to the discovery of substances which inhibit HAV replication without afflicting normal cell morphology, extended cytotoxicity studies will have to be performed.

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