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Use of a Flavivirus RNA-dependent RNA polymerase assay to investigate the antiviral activity of selected compounds

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Abstract

We have developed an assay using flavivirus RNA-dependent RNA polymerase to test the inhibitory activity of potential antiviral agents. The effects of antiviral agents on RNA synthesis were examined in this assay using extracts of Vero cells infected with dengue virus type 2 or Kunjin virus. Several different classes of known polymerase inhibitors were tested. The synthesis of double-stranded replicative form RNA was inhibited in a dose-dependent fashion in the presence of the polyoxometalate HPA-23 $[(NH_4)_{18}(NaW_{21}Sb_9O_{86})_{17}].14\ H_2O$ and several structurally related compounds.

Key words: HPA-23; Flavivirus; RNA-dependent RNA polymerase

1. Introduction

Viruses which are members of the family *Flaviviridae*, contain a plus-sense RNA genome of approximately 11 kb. Infected cells contain genomic length RNA (44S) as well as a double-stranded replicative form (RF) and a partially double-stranded

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replicative intermediate (RI). Previous work has shown that the replicative form can be utilised as a template for RNA synthesis in vitro (Bartholomeusz and Wright, 1993). It has been proposed that genomic RNA is preferentially made from the RF template (Chu and Westaway, 1985). Thus, the RF RNA is a potential target for antiviral agents.

The family *Flaviviridae* contains many human pathogens including yellow fever virus, tick-borne encephalitis virus, dengue virus, Japanese encephalitis virus, Saint Louis encephalitis virus, Murray Valley encephalitis virus and West Nile virus. Another probable member of the family is hepatitis C virus (HCV). Dengue virus and Kunjin virus were used in this work as representatives of the family. Dengue virus epidemics occur in tropical and subtropical areas of the world (Halstead, 1992), and are associated with a relatively benign disease, dengue fever, as well as the life-threatening illnesses of dengue shock syndrome and dengue haemorrhagic fever. Kunjin virus is in the same serogroup as the last four viruses listed above. There is a high degree of similarity between Kunjin virus and these other viruses in the genomic nucleotide sequence and the deduced amino acid sequence (Coia et al., 1988).

Few reports of antiviral drugs effective against flaviviruses have been published (Koff et al., 1981; Koff et al., 1982; Huggins et al., 1984; Yamamoto et al., 1990; Nair and Ussery, 1992). One of the most investigated compounds is ribavirin. It was not effective in protecting rhesus monkeys against infection with dengue virus (Malinoski et al., 1990), but in a pilot study of ribavirin therapy for HCV infection, a decrease in serum levels of viral RNA has been reported (Di-Bisaglie et al., 1992). Previous in vitro studies have not usually examined the specific mechanisms of action of the drugs being tested. However, we have developed an antiviral testing procedure which uses the RNA-dependent RNA polymerase (RDRP) from either dengue or Kunjin virus-infected cells (Chu and Westaway, 1985; Bartholomeusz and Wright, 1993). Several antiviral agents with known activity against RNA viruses were tested in this assay.

Representative drugs from five major classes of inhibitors of RNA replication and viral RNA polymerase were evaluated. (1) Enzyme-binding compounds: HPA-23, suramin and rifampicin (Wu and Gallo, 1974; Ono et al., 1984; Balzarini et al., 1986). (2) Template/primer-binding compounds: ethidium bromide (Waring, 1968). (3) Substrate analogues: phosphonoformate (Eriksson et al., 1980; Oberg, 1989). (4) Modified nucleosides: tubercidin, tubercidin monophosphate, ara-A and ddC (De Clercq, 1993; Secrist et al., 1993). (5) Enzyme-binding or template-binding compounds: catechin (a flavonoid) (Spedding et al., 1989). (6) Unknown mechanism of action: benzimidazoles derivatives (Ennis et al., 1967).

Of the compounds tested, the enzyme-binding compound HPA-23 was found to inhibit the synthesis of the replicative form (RF) of dengue virus and Kunjin virus in the RDRP assay. Five derivatives of HPA-23 inhibited Kunjin virus RNA synthesis in the RDRP assay. HPA-23 and one derivative $[NH_4]_{17}[CaSb_9W_{21}O_{86}]\cdot_{14}H_2O$ also inhibited Kunjin virus replication in a cell-culture based assay.

2. Materials and methods

2.1. Virus and cell culture

Vero cell monolayers were infected with either dengue virus type 2 (DEN-2; New Guinea C strain), or Kunjin virus (KUN; strain MRM61C; Boulton and Westaway, 1972). During infection cells were maintained in Eagle's Minimum Essential Medium (MEM).

2.2. RNA-dependent RNA polymerase assay for antiviral activity

Vero cells were infected with either KUN or DEN-2 at a mutiplicity of infection of 7. At 24 or 36 h pi cytoplasmic cell extracts were prepared from KUN or DEN-2-infected cells, respectively, as previously described (Chu and Westaway, 1985; Bartholomeusz and Wright, 1993). Test compounds ranging in concentration from 0.5 to 100 μM were incubated for 10 min with cell extracts containing 0.5 units/ml of RNasin (an inhibitor of RNases), then the other components of the RDRP assay were added. The final RDRP assay (total volume of 50 μl) contained the mixture of cell extract and inhibitor, 50 mM Tris-HCl (pH 8.0), 10 mM magnesium acetate, 7.5 mM potassium acetate, 10 mM 2-mercaptoethanol, 6 μg actinomycin D, 5 mM phosphoenolpyruvate, 3 units/μl pyruvate kinase, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 25 μM GTP and 5 μCi [α-^{32}P]GTP (Amersham, specific activity 410 Ci/mmol). The assay was incubated for 60 min at 37°C before the reaction was stopped by the addition of EDTA to a final concentration of 10 mM. An equal volume of buffer containing 50 mM Tris-acetate (pH 7.6), 0.1 M sodium acetate, 1 mM EDTA and 2% SDS was added to disrupt membranes and RNA was then extracted with phenol and precipitated with ethanol.

The RNA samples were mixed with an equal volume of TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA) containing 7 M urea and 0.5% bromophenol blue, and electrophoresed through 3% polyacrylamide gels containing 7 M urea in TBE. The gels were dried, and radiolabeled bands detected by autoradiography.

2.3. 3-(4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) (MTT) assay

The MTT assay was a modification of the procedure established by Mosmann (1983). Briefly, maintenance medium was removed from infected and uninfected Vero cell monolayers grown in 24-well [1.9 cm^2] plates and the cells incubated at 37°C for 1 h in the presence of MTT (2 mg/ml in PBS, 200 μl/well). The MTT was then removed and the cells solubilized in DMSO (400 μl/well). The solubilized cells were dispensed from the 24 well trays into 96 well trays and the absorbance in each well measured at 570 nm using a Microplate Reader (Bio-Rad model 450). The 50% cytotoxic concentration (CC_{50}) of drug is that which reduces metabolic activity of uninfected cells by 50%. The IC_{50} is the concentration of drug which reduces the metabolic activity of the infected cells by 50% compared to the untreated controls.

2.4. Antiviral agents

HPA-23 and analogous compounds were synthesised using methods previously described (Jasmin et al., 1974; Michelon et al., 1980). These compounds and rifampicin (Sigma), phosphonoformate (Fluka), suramin (May and Baker), ethidium bromide (Sigma), the benzimidazoles, 2-(2-imidazolin-2-yl)-benzimidazole (as hydrochloride salt), 2-(2-imidazolin-2-yl)-5-benzimidazole sulfonic acid (as sodium salt) (Ennis et al., 1967), tubercidin (Sigma), tubercidin monophosphate (Sigma), ara-A (Sigma), ddC (Sigma) and catechin (Sigma) were tested at concentrations from 0.5 to 100 μM in the in vitro RDRP assay. All compounds were dissolved in water except for rifampicin and catechin, which were dissolved in methanol and ethanol, respectively. Methanol and ethanol at the concentrations used had no effect in the RDRP assay (results not shown).

2.5. Preparation of cell extracts from dengue-infected cells treated with HPA-23

Vero cells (infected and uninfected) were incubated in the presence of HPA-23 at a range of concentrations up to 10 μM. Significant cytotoxicity was not observed by light microscopy in uninfected cells at concentrations < 10 μM. Cell extracts were prepared at 36 h pi, the time of maximum polymerase activity in the infected cells in the absence of the drug (Bartholomeusz and Wright, 1993). To remove HPA-23 from these cells, detached cells floating in the medium were washed three times with PBS and combined with similarly washed cells of the monolayer. Cytoplasmic cell extracts were prepared as previously described (Bartholomeusz and Wright, 1993).

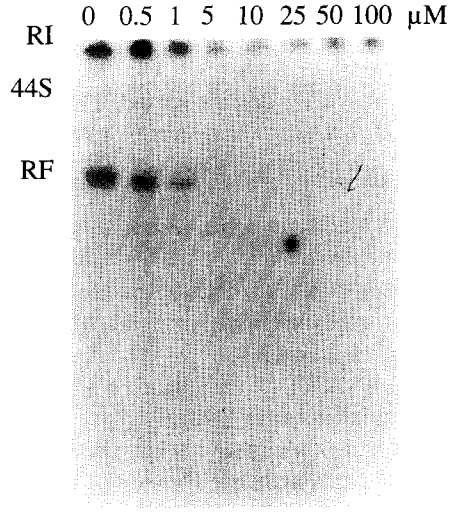

Fig. 1. Effect of HPA-23 (0.5 to 100 μM) on DEN-2 RNA synthesis in the RDRP assay. RNA was labeled with [α-^{32}P]GTP for 60 min and the RNA products separated by electrophoresis through a urea polyacrylamide gel.

Analysis of nucleic acid contents of aliquots of the cell extracts showed little variability following the above procedure (results not shown). The cell extracts were radiolabeled for 60 min in the standard in vitro RDRP assay.

3. Results

3.1. Testing of antiviral agents in the RDRP assay

The inhibition of DEN-2 RDRP activity by HPA-23 is shown in Fig. 1 and a summary of the results of all the compounds tested using the RDRP assay is shown in Tables 1 and 2. Neither the nucleosides ara-A, ddC, tubercidin and tubercidin monophosphate, nor catechin, the flavonoid compound, and the benzimidazole derivatives had any affect on RNA synthesis at the concentrations tested. The inhibitory activity of HPA-23 analogues against RDRP from KUN-infected cell extract is shown in Table 2.

Of the enzyme-binding compounds HPA-23, suramin and rifampicin, only HPA-23 significantly inhibited viral RNA synthesis (Fig. 1 and Table 1). The total incorporation of radiolabel into the RNA was reduced in the presence of HPA-23 and there was almost complete inhibition of RF RNA synthesis at a concentration of 5 μM (Fig. 1). The total incorporation of radiolabel into RNA remained approximately the same in RDRP assays containing suramin. While this drug partially inhibited RF RNA synthesis at 50 μM, the level of inhibition remained the same from 50 to 500 μM (results not shown), indicating that the inhibition was not dose-dependent. Rifampicin did not inhibit RNA synthesis at any of the concentrations tested.

Ethidium bromide inhibited the incorporation of radiolabel into all RNA species, with dose-dependent inhibition between 10 and 100 μM. Phosphonoformate at 100 μM did not inhibit RNA synthesis in the RDRP assay. This drug inhibited the incorporation of radiolabel into RNA only when 500 μM was added to the RDRP

Table 1
Antiviral activity of compounds tested in the DEN-2 RDRP assay

Class	Antiviral compound	Concentration (μM)					
		0.5	1	5	10	50	100
Enzyme-binding	HPA-23	0[a]	73	96	98	98	99
	Suramin	0	0	0	15	47	49
Template/primer-binding	Ethidium bromide	0	0	69	74	98	100

[a]Percentage inhibition of RF RNA synthesis relative to the drug-free control (estimated from X-ray films using a laser densitometer).

[b]The following compounds had no significant inhibitory effect in the RDRP assay at the concentrations tested; rifampicin, phosphonoformate, tubercidin, tubercidin monophosphate, ara-A and ddC, catechin, 2-(2-imidazolin-2-yl)benzimidazole and 2-(2-imidazolin-2-yl)-5-benzimidazole sulfonic acid.

Table 2
Antiviral activity of HPA-23 and five derivatives in KUN-infected Vero cells

Compound	Inhibitory conc. (μM) RDRP assay	50% Cytotoxic conc. (μM) (CC_{50})	50% Antiviral conc. (μM) (IC_{50})	Antiviral index (CC_{50}/IC_{50})
$[NH_4]_{18}[NaSb_9W_{21}O_{86}] \cdot {}_{14}H_2O$	5[a]	20	10	2
$[NH_4]_{17}[CaSb_9W_{21}O_{86}] \cdot nH_2O$	2–5	>25	4.5	>5
$Na_{18}[NaSb_9W_{21}O_{86}] \cdot nH_2O$	10–50	10	N.A.[b]	N.A.
$K_{18}[KSb_9W_{21}O_{86}] \cdot nH_2O$	5–10	7.5	N.A.	N.A.
$[NH_4]_{18}[RbSb_9W_{21}O_{86}] \cdot nH_2O$	10	15	N.A.	N.A.
$[NH_4]_{18}[NH_4Sb_9W_{21}O_{86}] \cdot nH_2O$	10	15	N.A.	N.A.

[a]Concentration at which RF RNA synthesis is inhibited more than 75% in the KUN RDRP assay.
[b]N.A. Not applicable as metabolic activity of virus-infected cells did not reach the level which was 50% of uninfected cells.

assay (results not shown), and at this concentration RF synthesis was only partially inhibited.

3.2. The effect of HPA-23 on dengue virus-infected Vero cells

The antiviral activity of HPA-23 was examined by analysing DEN-2 RDRP activity in infected cell monolayers maintained in the presence of 1.0 to 10 μM

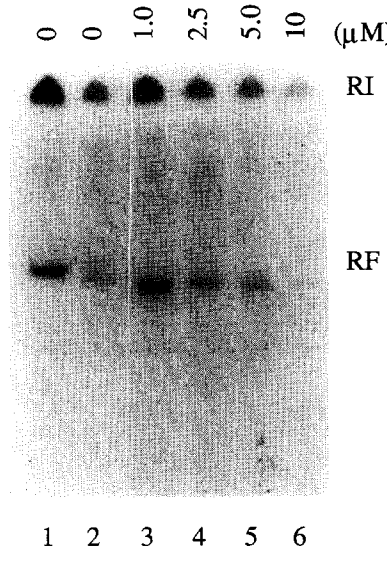

Fig. 2. RDRP activity in extracts of DEN-2-infected cells that were incubated in medium containing various concentrations of HPA-23 for 36 h post-infection. The products of the RDRP assay were separated by electrophoresis as outlined in Section 2. Lane 2 contains half the amount of sample used in the other lanes.

HPA-23. RNA synthesis in the RDRP assay decreased when extracts were prepared from cells incubated in the presence of 2.5 to 10 μM (Fig. 2, lanes 4 to 6). These results were consistent with those obtained when HPA-23 was added directly to the RDRP assay (Fig. 1). The lower levels of RDRP activity in cell extracts from HPA-23-treated cells most likely indicates a reduced number of polymerase molecules, or template RNA, or both.

3.3. The antiviral activity of HPA-23 and five polyoxometalates in Kunjin-infected Vero cells

HPA-23 and five derivatives were tested in the RDRP assay using KUN infected cell extracts and all were found to inhibit RNA synthesis (Table 2). However, when allowance was made for the cytotoxicity of these compounds as measured in the MTT assay, only two (HPA-23 and $[NH_4]_{17}[CaSbW_{21}O_{86}].nH_2O$) had an antiviral index suggestive of specific activity.

4. Discussion

We report here an assay capable of detecting inhibitory activity against RDRP, a virus-specific enzyme which is an important target for antiviral drugs directed against RNA viruses. RNA-dependent RNA polymerases of animal and plant RNA viruses share common sequence motifs (Poch et al., 1989). These polymerases are unique to RNA viruses. We used the RDRP assay to investigate the activity of several potential inhibitors of the enzyme from cells infected with DEN-2 or KUN. Primary screening of the inhibitors utilised the RDRP assay rather than the more conventional methods of testing the drugs on infected cell monolayers. There are several advantages of using the RDRP assay to evaluate compounds targeted to inhibit RNA synthesis. (1) Only flavivirus RNA is radiolabeled in the assay and the inhibition of all three RNA forms can be assayed, (2) the same cell extract can be used to test a number of compounds, thus the eliminating variabilities of testing compounds in tissue culture, and (3) the assay is relatively quick.

Previous work has shown that RF RNA can be utilised as a template for flavivirus RNA replication and may be the preferred template for the synthesis of the positive-sense genomic RNA (Chu and Westaway, 1987; Bartholomeusz and Wright, 1993). HPA-23 and the five related polyoxometalates were the most effective inhibitors tested in the RDRP assay producing a marked reduction in the synthesis of RF RNA (Fig. 1).

HPA-23 has previously been shown to inhibit a wide range of RNA and DNA viruses including murine leukaemia and sarcoma viruses (Jasmin et al., 1974), encephalomyocarditis virus (Werner et al., 1976), vesicular stomatitis virus (Werner et al., 1976), rabies (Pepin and Blancou, 1985) and various retroviruses (Dormont et al., 1985). Kinetic studies have shown that HPA-23 binds to the polymerase in a noncompetive manner with respect to the substrate and the template/primer (Ono et al., 1984). The concentration of HPA-23 reported to inhibit flavivirus RNA poly-

merase activity in the RDRP assay is similar to the concentration which inhibits the human immunodeficiency virus RNA-dependent DNA polymerase in a reverse transcriptase assay (i.e., 60–80% inhibition by 2 μM HPA-23; Ono et al., 1988).

Extracts prepared from DEN-2-infected cells treated with HPA-23 and then tested in the RDRP assay also showed a reduction in RNA synthesis as measured by the decreased incorporation of radiolabel into all three viral RNA species (Fig. 2). This is also consistent with the MTT assay results, which show that HPA-23 does have some antiviral activity in KUN-infected cells (Table 2). A consequence of the inhibition would be a reduction in the number of templates available for transcription and translation with obvious effects on virus replication.

HPA-23 has been administered to patients infected with HIV and with symptoms of AIDS, but was associated with significant adverse side-effects (Rozenbaum et al., 1985). The drug penetrates the blood-brain barrier and can also stimulate natural killer cells (Dormont et al., 1985). The use of HPA-23 as an antiviral agent against dengue or other flaviviruses has not been reported and may be precluded because of its toxicity. However, the inhibition of RF RNA synthesis by HPA-23 in the in vitro RDRP assay and the reduction of polymerase activity observed in DEN-2-infected cells is significant, and suggests that less toxic derivatives of the drug may have potential for the treatment of flavivirus infections, especially in case of acute life threatening encephalitic infections and chronic hepatitis C infection.

Most of the other compounds tested did not inhibit RNA synthesis in the RDRP assay. Rifampicin was used as a negative control in the RDRP assay and as expected did not effect viral RNA synthesis. The drug is active against RNA-dependent DNA polymerases but not against other RNA-dependent RNA polymerases (Sankar and Porter, 1991). Phosphonoformate marginally inhibited DEN-2 RNA synthesis at 500 μM. This is comparable to the degree of inhibition observed for other RNA-dependent RNA polymerases, including those of vesicular stomatitis virus and reovirus (Chandra and Banerjee, 1980). By contrast, influenza RNA polymerase was inhibited at lower concentrations (30–60 μM), similar to those which inhibit DNA polymerases and reverse transcriptase (Oberg, 1989). The modified nucleosides tested had no effect on RNA synthesis in the RDRP assay. Ribavirin which has been used in the treatment of patients infected with HCV was not tested in the RDRP assay as the drug inhibits IMP dehydrogenase which leads to a reduced intracellular pool of GMP, GDP and GTP. Since, in the RDRP assay the radiolabel [α-^{32}P]GTP is added, any inhibitory effect of ribavirin could not be detected using the assay.

We have used the RDRP assay to investigate antiviral agents known to inhibit RNA and DNA polymerase activity. Of the drugs tested, HPA-23 greatly inhibited flavivirus RNA synthesis. Our observations of inhibitory activity by HPA-23 in the RDRP assay suggest that there is potential for the treatment of flaviviral infections through the use of appropriately targeted, non-toxic compounds derived from HPA-23. These derivatives may be useful in the treatment of dengue infections in endemic areas where no effective vaccine is available. Since HPA-23 can cross the blood brain barrier (Dormont et al., 1985), they may also be useful in the treatment of encephalitic infection with flaviviruses such as Japanese encephalitis, Saint Louis

encephalitis, West Nile and Australian encephalitis viruses. They may also be effective in the treatment of chronic hepatitis caused by HCV. Further investigations into less toxic derivates of HPA-23 are currently in progress.

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