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Use of the yellow fever virus vaccine strain 17D for the study of strategies for the treatment of yellow fever virus infections

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Abstract

We have employed the attenuated vaccine strain 17D of yellow fever virus (YFV) to evaluate the inhibitory effect of a selected series of compounds on YFV in Vero cells. Use of the vaccine strain does not require high-level microbiological containment facilities and should allow extensive screening. In addition, YFV may serve as a model for other flaviviruses including hepatitis C virus (HCV), and thus strategies for the treatment of YFV infections may apply to flavivirus infections in general. In the present study, several compounds belonging to different classes of nucleoside analogues and polyanions were evaluated for their inhibitory effect on the replication of YFV. Compounds that are targeted at: (i) IMP dehydrogenase (ribavirin, EICAR, tiazofurin, selenazofurin and mycophenolic acid), (ii) OMP decarboxylase (pyrazofurin and 6-azauridine), (iii) CTP synthetase (carbodine and cyclopentenyl cytosine), (iv) dihydrofolate reductase (methotrexate) and the (v) sulfated polymers (dextran sulfate and PAVAS) proved inhibitory to the replication of YFV. Mycophenolic acid (EC_{50}: 0.08 μg/ml), EICAR (EC_{50}: 0.8 μg/ml) and methotrexate (EC_{50}: 0.07 μg/ml) were the most effective. The finding that EICAR and mycophenolic acid, despite their potent anti-YFV activity, had little or no effect on the replication of the bunyavirus Punta Toro or herpes simplex virus in Vero cells, indicates that their anti-YFV activity is rather specific and does not merely result from cytotoxicity. Inhibitors of *S*-adenosylhomocysteine hydrolase (SAH hydrolase) and thymidylate synthase were found to be devoid of anti-YFV activity.

Keywords: EICAR; Flaviviruses; Hepatitis C virus; Mycophenolic acid; Ribavirin; Yellow fever virus

1. Introduction

Despite the proven safety and efficacy of the yellow fever virus (YFV) vaccine prepared from the attenuated 17D strain, large population groups have remained unimmunized. Yellow fever is still the most important cause of hemorrhagic fever in man today (Monath, 1987). Between 1988 and 1990 a worldwide total of 13 027 cases and 2924 deaths were reported to the WHO, the highest reported incidence since 1948. These figures are believed to be underestimations, the

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WHO estimates that YFV strikes some 200 000 persons worldwide each year, causing some 30 000 deaths (WHO Notes and News, 1993).

Treatment of yellow fever is mostly supportive, although most patients with yellow fever are unable to benefit from modern intensive care units. A number of compounds with antiviral activity in vitro have been described, including ribavirin (Huggins et al., 1984). Trials with ribavirin in experimentally-infected monkeys have yielded conflicting results (Monath et al., 1990). Interferon treatment of monkeys resulted in the delayed onset of viremia and illness without any effect on survival (Arroyo et al., 1988). It is therefore important to have at hand a molecule that efficiently inhibits the replication of YFV. To be able to elaborate a convenient screening for anti-YFV activity, it would be useful to have a virus preparation that does not require high-level microbiological containment facilities for handling. Besides the importance of discovering antivirals for the treatment of YFV infections, this virus may also serve as a model for other flaviviruses, such as the hepatitis C virus (HCV), for which antiviral drug testing is not (yet) feasible because of the lack of appropriate cell culture assay systems. For these reasons we employed the vaccine strain 17D to evaluate the potential anti-YFV activity of a selected series of substances.

2. Materials and methods

2.1. Viruses and cells

Green monkey kidney (Vero) cells (ATCC CCL81) were grown in minimum essential medium (MEM, Gibco, Paisley, Scotland) supplemented with 10% inactivated fetal calf serum (FCS, Gibco), 1% L-glutamine and 0.3% bicarbonate. The vaccine strain 17D (Stamaril®, Pasteur Merieux) was used to infect 75 cm^2 bottles of Vero cells. After a 5 day incubation period at 37°C, extensive cytopathic effect was observed; the cultures were freeze-thawed, cell debris removed by centrifugation and the supernatant aliquoted and stored at −70°C. The virus was titrated on Vero cells grown to confluency in 96-well microtiter plates. Only the primary virus stock was used for experimental purposes, and a new stock was always prepared from the original vaccine.

2.2. Compounds

The test compounds and their sources were as follows: xylotubercidin, M.J. Robins (Chemistry Department, Brigham Young University, Provo, Utah); neplanocin A, Toyo Jozo Co. (Tagata-Gun, Shizuoka-Ken, Japan); C-c^3Ado (carbocyclic 3-deazaadenosine), J.A. Montgomery (Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Alabama); (*S*)-DHPA [(*S*)-9-(2,3-dihydroxypropyl)adenine], A. Holy (Institute of Organic Chemistry and Biochemistry, Academy of Science of the Czech Republic, Prague, Czech Republic); 3-deazaneplanocin A, cyclopentenyl cytosine, V.E. Marquez (Laboratory of Pharmacology and Experimental Therapeutics, National Cancer Institute, Bethesda, Maryland); DHCA [9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)adenine], R.T. Borchardt (Department of Biochemistry and Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas); carbodine (carbocyclic cytidine, cyclopentyl cytosine), J.A. Montgomery; ribavirin [1-(β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide], ICN Nutritional Biochemicals (Cleveland, Ohio); pyrazofurin [3-(β-D-ribofuranosyl)-4-hydroxypyrazole-5-carboxamide], Calbiochem Behring Corporation (Lucerne, Switzerland). Mycophenolic acid and tiazofurin were kindly provided by Dr. R. Cooney and Dr. D.G. Johns (National Cancer Institute, NIH, Bethesda, Maryland, USA). EICAR (5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide) was synthesized by Dr. A. Matsuda (Hokkaido University, Sapporo, Japan). BVDU [(*E*)-5-(2-bromovinyl)-2′-deoxyuridine] was synthesized following a modification of the method described by Jones et al. (1979). FdU (5-fluoro-2′-deoxyuridine) was obtained from Aldrich, FU (5-fluorouracil) was from Calbiochem, TFT (trifluorothymidine) was from Sigma, IDU (5-iodo-2′-

deoxyuridine) was from Sigma, and methotrexate (methylamino-pterin) was purchased from Lederle. Dextran sulfate (M_r 10 000) was obtained from Pfeifer and Langen (Dormagen, Germany). PAVAS (a co-polymer of acyclic acid with vinyl alcohol sulfate) was obtained from Prof. S. Görög and was synthesized as reported before (Schols et al., 1990). Uridine and guanosine were purchased from Sigma.

2.3. Inhibition of virus-induced cytopathogenicity

Serial dilutions of the test compounds (in medium supplemented with 2% FCS) were added to confluent Vero cell cultures in microtiter trays after which the cells were infected with 10 $CCID_{50}$ (cell culture infective dose, 50%) of virus. Cultures were further incubated at 37°C. Viral cytopathogenicity was recorded 7–8 days postinfection. Cultures were fixed with 70% ethanol, stained with Giemsa solution (50-fold dilution; 2 h staining), washed and air-dried. The antiviral activity of the compounds is expressed as the effective concentration required to inhibit the viral cytopathic effect (CPE) by 50% (EC_{50}).

2.4. Cytotoxicity

Cytotoxicity measurements were based on two parameters: (i) alteration of normal cell morphology and (ii) inhibition of uninfected host cell growth. To evaluate cell morphology, confluent cell cultures which had not been infected but treated with various concentrations of the test compounds were incubated in parallel with the virus-infected cell cultures and examined microscopically at the same time as viral cytopathogenicity was recorded for the virus-infected cell cultures. A disruption of the cell monolayer, e.g. rounding up or detachment of the cells, was considered as evidence for cytotoxicity. Inhibition of cell growth was assessed as follows: the cells were seeded at a rate of 3×10^3 cells per well in a volume of 0.1 ml into 96-well microtiter plates and allowed to proliferate for 24 h in MEM containing 20% FCS, 1% L-glutamine and 0.3% sodium bicarbonate. Twenty-four hours later, 0.1 ml MEM (with 2% FCS, 1% L-glutamine, and 0.3% sodium bicarbonate) containing different concentrations (in duplicate) of the test compounds were added to each well. After 3 days of incubation at 37°C in 5% CO_2, the cell number was determined with a Coulter counter. The minimum cytotoxic dose was expressed as the CC_{50}, or concentration required to reduce cell growth by 50%.

3. Results

Several compounds belonging to different classes of nucleoside analogues and sulfated polymers were evaluated for their inhibitory effects on the replication of YFV, by means of a cytopathic effect reduction assay. The EC_{50} values of the different compounds required for inhibition of YFV-induced cytopathic effect are given in Table 1.

The sulfated polymers dextran sulfate and PAVAS prevented the infection of the cells with YFV at concentrations that had no effect on cell morphology or cell growth. The compounds had to be present at the time of virus adsorption to exert their antiviral effect; when added after virus adsorption was allowed to occur, the compounds lost virtually all activity (data not shown). PAVAS proved at least 3–4 fold more effective than dextran sulfate (M_r 10 000).

Next, a series of compounds that have been well recognized as inhibitors of *S*-adenosylhomocysteine hydrolase (SAH hydrolase) were evaluated. None of these compounds (i.e. C-c^{3}Ado, (*S*)-DHPA, DHCA, neplanocin A, 3-deazaneplanocin A) had a significant effect on YFV replication.

Five different compounds that inhibit IMP-dehydrogenase activity (i.e. ribavirin, EICAR, tiazofurin, selenazofurin and mycophenolic acid) were evaluated. From this class of compounds ribavirin was found to elicit the weakest antiviral effect. In contrast, EICAR and mycophenolic acid efficiently blocked virus replication. As reported before (Huggins et al., 1984; Kirsi et al., 1983), tiazofurin and selenazofurin proved to be more potent inhibitors of YFV replication than rib-

Table 1
Anti-YFV activity of a selected series of compounds

Compound	EC_{50} (μg/ml)[a]	MTC (μg/ml)[b]	CC_{50} (μg/ml)[c]
Adenosine analogues			
Xylotubercidin	≥20	100	28
C-c^{3}Ado	100	>400	5.8
(*S*)-DHPA	>100	>400	>200
DHCA	100	>400	0.9
Neplanocin A	3.5 ± 3.8	4	0.4
3-Deazaneplanocin A	100	>100	0.1
Inhibitors of OMP decarboxylase			
Pyrazofurin	0.37 ± 0.18	>400	25
6-Azauridine	0.41 ± 0.07	>100	8 ± 9
Inhibitors of IMP dehydrogenase			
Ribavirin	28 ± 18	>100	63
EICAR	0.8 ± 0.6	>100	1.7 ± 0.4
Tiazofurin	6.2 ± 5.0	>100	2.6 ± 0.9
Selenazofurin	2.2 ± 1.5	>100	3.0 ± 2.8
Mycophenolic acid	0.08 ± 0.05	>100	0.3 ± 0.2
Inhibitors of CTP synthetase			
Carbodine	0.3 ± 0.3	>100	0.5 ± 0.2
Cyclopentenyl cytosine	0.5	≥ 10	0.4
Inhibitors of thymidylate synthase and dihydrofolate reductase			
Trifluorothymidine	>4	20	1.1 ± 0.7
5-Fluoro-deoxyuridine	>20	100	<0.03
5-Fluorouridine	>4	20	—
5-Iodo-deoxyuridine	>100	>100	—
(*E*)-5-(2-bromovinyl)-deoxyuridine	>100	>100	>100
5-Formyl-deoxyuridine	40	>100	30
Methotrexate	0.07	20	0.9 ± 1.2
Polyanions			
Dextran sulfate (M_r 10 000)	27 ± 3	>100	>100
PAVAS	8.7 ± 4.6	>100	>100

[a]Concentration required to inhibit virus-induced CPE formation by 50% (± S.D.).
[b]Minimal concentration required to alter normal cell morphology.
[c]Concentration required to inhibit the growth of non-infected Vero cells by 50%. (± S.D.).
Data are mean values for 2–4 separate experiments.

avirin. None of the IMP dehydrogenase inhibitors affected normal cell morphology at the concentrations tested, although they had a marked inhibitory effect on the growth of uninfected cells. The anti-YFV activity of all five IMP-dehydrogenase inhibitors could be reversed upon addition of guanosine (Table 2).

The OMP decarboxylase inhibitors that were evaluated (i.e. pyrazofurin and 6-azauridine) inhibited YFV replication at EC_{50} values of about 2 μg/ml. Exogeneously added uridine reversed the antiviral activity of these molecules (Table 2). Also, carbodine and cyclopentenyl cytosine, two compounds that are targeted at CTP synthetase, had a pronounced anti-YFV activity at concentrations that did not alter normal cell morphology of quiescent cell cultures. Finally, several compounds that are targeted at thymidylate synthase (TS)

Table 2
Anti-YFV virus activity of IMP dehydrogenase and OMP decarboxylase inhibitors in the presence or absence of guanosine or uridine

	EC_{50} (μg/ml)[a]		
	No addition	Guanosine (10 μg/ml)	Guanosine (100 μg/ml)
Inhibitors of IMP-dehydrogenase			
Ribavirin	28 ± 18	30	>100
EICAR	0.8 ± 0.6	>50	>50
Tiazofurin	6.2 ± 5.0	100	100
Selenazofurin	2.2 ± 1.5	>100	>100
Mycophenolic acid	0.08 ± 0.05	30	>100
	No addition	Uridine (10 μg/ml)	Uridine (100 μg/ml)
Inhibitors of OMP decarboxylase			
Pyrazofurin	0.37 ± 0.18	>100	>100
6-Azauridine	0.41 ± 0.07	>100	>100

[a]Concentration required to inhibit virus-induced CPE formation by 50% (± S.D.).

were evaluated for their effect on YFV replication. None of these compounds had any effect. However, methotrexate, a molecule targeted at dihydrofolate reductase, emerged as a potent inhibitor of YFV replication.

4. Discussion

Clinically useful inhibitors of YFV replication would seem of importance, despite the availability of an effective vaccine. Since the evaluation of compounds for their potential inhibitory effect on the replication of YFV requires high level microbiological containment facilities, few laboratories have performed an intensive search for anti-YFV agents. We therefore employed the vaccine strain 17D of YFV [which was developed by passage of the wild-type YFV Asibi (Theiler and Smith, 1937)] and evaluated several substances for their inhibitory effect on YFV replication. Although the 50% effective concentrations (EC_{50}) obtained in the present study for the reference compounds ribavirin and tiazofurin are somewhat higher than those described by Kirsi et al. (1983) and Huggins et al. (1984), the relative activity of the compounds, as compared to each other, is comparable in both assay systems, which points to the usefulness of this method for initial screening programs. The difference in EC_{50} values obtained for the reference compounds by us and Huggins may result from differences in the sensitivity of both virus strains to the respective drugs.

Polyanionic substances, in particular sulfated polymers, have been well studied for their inhibitory effect on the virus adsorption process. As a rule, enveloped viruses are sensitive to the action of sulfated polymers. For HIV the site of attack of these polyanionic substances is the interaction between the viral envelope glycoprotein *gp*120 and the cellular CD4 receptor; their antiherpesvirus activity has been shown to be attributed to an inhibition of binding of virus particles to the host cell heparan sulfate (Wu-Dunn and Spear, 1989; Neyts et al., 1992). Use of the polyanionic substances could be helpful in further unraveling the initial interactions of flaviviruses with the host cell.

Ribavirin was one of the first compounds found to be effective in vitro against YFV, but was shown to have, however, no significant effect on viremia, mean time of death or mortality in monkeys experimentally infected with the virus (Monath, 1990). The primary target for the broad spectrum antiviral action of ribavirin has been originally identified as IMP-dehydrogenase, the enzyme that converts IMP to XMP (xanthylate), an intermediate metabolite in the synthesis of

GTP. As a consequence, ribavirin leads to a reduction of GMP, GDP and GTP pool levels and suppression of viral RNA synthesis. We have provided evidence that ribavirin indeed acts on YFV replication via depletion of the intracellular GTP pools since its antiviral effect is reversed upon addition of guanosine. Another general mechanism by which ribavirin may exert its antiviral activity includes phosphorylation to its 5′-triphosphate, in which form it may interfere directly with viral RNA synthesis, viral mRNA capping guanylylation, and primer generation and elongation during viral RNA transcription (Goswami et al., 1979; Wray et al., 1985).

In the present study we evaluated several other inhibitors of IMP dehydrogenase, i.e. the tiazole C nucleoside tiazofurin and its selenium analog selenazofurin (both of which have been reported to possess anti-YFV activity) (Kirsi et al., 1983; Huggins et al., 1984), mycophenolic acid and EICAR. EICAR (5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide) can be viewed as a close analog of ribavirin, whereby the N at position 2 of the triazole ring is replaced by an alkynyl-carbon moiety. The order of anti-YFV activity of these compounds ranked as follows: mycophenolic acid > EICAR > selenazofurin > tiazofurin > ribavirin. The order and potency of anti-YFV activity of these compounds correlates with the activity of the compounds against IMP dehydrogenase: mycophenolic acid > EICAR > selenazofurin > tiazofurin > ribavirin (Balzarini et al., 1993). This allows us to conclude that IMP dehydrogenase is an important target for the treatment of YFV infections.

Although EICAR and mycophenolic acid were found to be highly active against YFV replication, these compounds did not show activity against the replication of the bunyavirus Punta Toro (data not shown). Furthermore, EICAR was shown to have no effect on the rhabdovirus vesicular stomatitis virus (VSV), the picornavirus poliovirus and the DNA viruses herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2) (De Clercq et al., 1991a). The differences in the sensitivity of different RNA viruses to EICAR and mycophenolic acid indicates that their inhibitory effect on viral replication is not strictly a cell-dependent (cytotoxic) event. If cytotoxicity was the main reason for the anti-YFV activity of the IMP dehydrogenase inhibitors, they should have been active against Punta Toro virus, VSV, polio, HSV-1 and HSV-2. It is thus of interest to unravel why some RNA viruses are sensitive to the action of IMP dehydrogenase inhibitors, whereas others are not. Interestingly, mycophenolic acid has recently been shown to inhibit HIV replication (Ichimura and Levy, 1995).

Recently the morpholinoethylester of mycophenolic acid (Mofetil) has entered clinical trials for use as an immunosuppressive agent (Taylor et al., 1994). Since (i) mycophenolic acid is in our hands about 350-fold more potent as an anti-YFV agent than ribavirin and (ii) toxicological and pharmacological data from human clinical trials with Mofetil will become available, mycophenolic acid (and/or its prodrug Mofetil) may be considered as candidate drug(s) for the treatment of YFV infections and, possibly, other Flavivirus infections as well. Although mycophenolic acid is cytostatic in cell cultures, Mofetil has shown low toxicity in man; side effects may include nausea, leukopenia, vomiting, diarrhea and dyspepsia (Taylor et al., 1994). A draw-back, however, could be an immunosuppressive effect (Eugui et al., 1991); although this effect may be expected to be moderate upon the short term usage required to combat flavivirus infections.

OMP decarboxylase converts OMP to UMP and is an essential enzyme in the pyrimidine metabolism. Pyrazofurin, an inhibitor of OMP decarboxylase, has potent activity against both (+)RNA and (−)RNA viruses (Descamps and De Clercq, 1978). Here we demonstrate that the compound is also endowed with potent anti-YFV activity. Also 6-azauridine (Janeway and Cha, 1977) resulted in an appreciable inhibitory effect on YFV replication. In addition, compounds that are targeted at CTP-synthetase (i.e. C-Cyd, carbodine) and cyclopentenyl cytosine (Ce-Cyd) (Glazer et al., 1985; Marquez et al., 1988; Kang et al., 1989) had a marked effect on YFV replication. C-Cyd has been described as being active against

(−)RNA viruses (orthomyxo, paramyxo and rhabdo), (+)RNA viruses (toga), and (±)RNA viruses (reo). Ce-Cyd is a more potent antiviral agent than its counterpart C-Cyd, and has, in addition, shown activity against picornaviruses and herpesviruses (De Clercq et al., 1991b).

Inhibitors of *S*-adenosylhomocysteine hydrolase activity were shown to posses potent activity against pox-, paramyxo-, rhabdo-, reo- and arenaviruses (De Clercq, 1987). However, SAH hydrolase inhibitors have not proven active against (+)RNA viruses (i.e. toga and picornaviruses). The lack of activity of this class of compounds against YFV is thus in agreement with their presumed spectrum of antiviral activity, although Tseng et al. (1989) described, in contrast to our results, anti-YFV activity for these compounds. Also inhibitors of TS did not show any effect on YFV replication. The observation that both inhibitors of SAH hydrolase and of TS have no effect on YFV replication (although the cytostatic potential of several of these compounds is comparable or even more pronounced than that of the IMP dehydrogenase and OMP decarboxylase inhibitors) again points to the specificity of the latter against YFV replication. Methotrexate (an inhibitor of dihydrofolate reductase) was found to inhibit YFV replication. It is at present not clear why methotrexate and TS inhibitors differ in their anti-YFV activity, since the antiviral activity of both groups of antimetabolites may be expected to result from a depletion of the dTTP pools.

In conclusion, we have employed the YFV vaccine strain to evaluate a series of compounds for their potential anti-YFV activity. The method presented here should allow a high output evaluation of compounds against YFV. In addition, it should be studied whether drug activity against YFV has predictive value for activity against other flaviviruses such as hepatitis C virus, dengue virus and Japanese encephalitis virus. In this context, it is interesting to note that ribavirin has demonstrated some activity against hepatitis C virus (Di-Bisaglie et al., 1992). The potent anti-YFV activity of mycophenolic acid and the fact that its prodrug Mofetil is used with little side effects in patients may suggest possible use of Mofetil for treatment of flavivirus infections.

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