Inhibition of the Replication of Influenza A and B Viruses by a Nucleoside Analogue (Ribavirin)

(Accepted 20 May 1975)

SUMMARY

A synthetic nucleoside analogue 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin or RTCA) inhibits the replication in tissue culture of influenza B virus and also a wide range of influenza A viruses of human, animal and avian origin. The synthesis of influenza virus-induced antigens and also structural and non-structural polypeptides is inhibited by RTCA as detected by immunofluorescence and by pulse labelling experiments with [35S]-methionine. The inhibitory effects of RTCA on influenza A virus replication in tissue culture is reversed by a molar excess of guanosine or xanthosine which suggests that the compound acts at an early stage of virus RNA synthesis prior to the utilisation of the latter nucleosides. A possible inhibitory effect of RTCA on cellular DNA replication is not excluded.

A synthetic nucleoside analogue 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin or RTCA) has been shown to inhibit the multiplication of both DNA- and RNA-containing viruses including influenza viruses (Sidwell et al. 1972; Streeter et al. 1973; Suganuma & Ishida, 1973). We describe here the inhibition of influenza type A and B virus replication in tissue culture cells by RTCA and show that the compound acts at an early stage in the influenza-infected cell, possibly by inhibiting the production of essential nucleotides and hence RNA synthesis. The compound, as a reversible inhibitor, is of interest in the analysis of the early steps in influenza virus replication and as a potential compound for the chemoprophylactic control of influenza.

Influenza strains were from the collection of the World Influenza Centre, National Institute for Medical Research, Mill Hill, and were grown in embryonated hen’s eggs by standard techniques. Calf kidney and chick embryo fibroblast (CEF) cells were grown as described previously (Oxford & Schild, 1975).

For radioactive labelling experiments cells (5 x 10^6 cells/60 mm plastic Petri dish) were infected at a multiplicity of approx. 10 p.f.u. of influenza virus/cell for 30 min at 37 °C in Geys balanced salt solution, washed to remove excess unadsorbed virus and re-incubated at 37 °C in Geys balanced salt solution. Cells were pulse labelled for 20 min at varying times after infection by removing the medium and replacing with 0.4 ml of Geys balanced salt solution containing 10 μCi/ml of [35S]-methionine (10 Ci/mmol, Radiochemical Centre, Amersham). The monolayers were then washed with cold Geys salt solution to remove excess isotope and solubilised by adding 0.5 ml per Petri dish of a mixture of 8 M-urea, 2 % β-mercaptoethanol and 1 % SDS in 0.05 M sodium phosphate buffer at pH 7.2.

Cell extracts or virus samples in the above solubilisation mixture were heated at 100 °C for 2 min and electrophoresis carried out in a slab gel apparatus with 8 channels containing 7.5 % acrylamide with a current of 80 mA for 17 h and using the neutral pH urea-SDS phosphate buffered system described previously (Oxford, 1973). The gel slabs were stained in 0.3 % Coomassie blue, for 3 h, destained in a mixture of 10 % (v/v) acetic acid:50 % methanol for 2 days, laid on a porous filter covered with cellophane and dried under
vacuum for 16 h. The dried and flattened slab was removed and developed by autoradiography using standard techniques. Purified influenza A/Hong Kong/1/68 virus was co-electrophoresed in one channel (100 μg of virus protein in 20 μl saline) and the migration position of the virus structural polypeptides used to tentatively identify isotopically labelled polypeptides in extracts of virus infected cells.

The inhibitory effect of RTCA on the replication of a number of different influenza A and B viruses was measured using the egg piece system (Fazekas de St Groth & White, 1958). In these experiments RTCA was added to egg pieces in WHO plates at a final concentration of 0.04 mM at the time of virus infection and the virus was titrated in RTCA treated and in control untreated plates and the plates incubated for 3 days at 37 °C. The nucleoside analogue (0.04 mM) caused a very significant inhibition (> 4.0 log ID₅₀/ml) of the replication of a range of influenza A strains of human, equine, avian and swine origin including A/Duck/England/62 (Hav3Nav1), A/Swine/Cambridge/39 (Hswine1N1), A/Equine/-Miami/63 (Heq2Neq2) and A/Port Chalmers/1/73 (H3N2) viruses. In addition, influenza B/Hong Kong/8/73 virus was inhibited to a similar degree to the influenza A viruses.

The multiplication of fowl plague virus A/FPV/Dutch/27 (HavlNeq1) in CEF cells was inhibited by RTCA as determined by plaque inhibition tests. The results indicated that 0.02 mM-RTCA in the overlay medium inhibited virus plaque formation by approx. 50 % (Fig. 1). In addition, the plaques produced in the presence of the nucleoside analogue were significantly smaller than in the control untreated plates.
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Table I. Effect of RTCA on the production of influenza A/HK/1/68 virus structural antigens in infected cells

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<tr>
<th>RTCA concentration (mM)</th>
<th>% cells fluorescing after treatment with antisera to influenza structural antigens</th>
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<tbody>
<tr>
<td></td>
<td>HA</td>
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<tr>
<td>0</td>
<td>98.0</td>
</tr>
<tr>
<td>0.1</td>
<td>23.1</td>
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<tr>
<td>0.2</td>
<td>8.8</td>
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Separate studies of the effect of RTCA on cell metabolism of normal CEF, calf kidney cells and also of egg pieces indicated that 0.04 mM concentrations of RTCA had no effect on the uptake and incorporation of [75Se]-methionine or [3H]-uridine into acid precipitable product after prior incubation with the compound for 72 h. [75Se]-methionine was used for these experiments since TCA-precipitable counts could be estimated in a whole egg piece sample and avoided the technical problem of solubilisation of egg piece fragments. Incubation of egg pieces with relatively high concentration (1.2 mM) of RTCA for 72 h was required to produce a 50% decrease in [3H]-uridine incorporation although this concentration had no effect on [75Se]-methionine incorporation into acid precipitable product. For the short incubation times of 3 to 8 h used in later experiments to determine the effect of the compound on virus polypeptide or antigen synthesis, experiments established that incubation of CEF cells with 8 mM-RTCA resulted in a 50% decrease in [3H]-uridine and a 20% decrease in [75Se]-methionine incorporation into acid precipitable product. For the short incubation times of 3 to 8 h used in later experiments to determine the effect of the compound on virus polypeptide or antigen synthesis, experiments established that incubation of CEF cells with 8 mM-RTCA resulted in a 50% decrease in [3H]-uridine and a 20% decrease in [75Se]-methionine incorporation into acid precipitable product. For the short incubation times of 3 to 8 h used in later experiments to determine the effect of the compound on virus polypeptide or antigen synthesis, experiments established that incubation of CEF cells with 8 mM-RTCA resulted in a 50% decrease in [3H]-uridine and a 20% decrease in [75Se]-methionine incorporation into acid precipitable product. For the short incubation times of 3 to 8 h used in later experiments to determine the effect of the compound on virus polypeptide or antigen synthesis, experiments established that incubation of CEF cells with 8 mM-RTCA resulted in a 50% decrease in [3H]-uridine and a 20% decrease in [75Se]-methionine incorporation into acid precipitable product. For the short incubation times of 3 to 8 h used in later experiments to determine the effect of the compound on virus polypeptide or antigen synthesis, experiments established that incubation of CEF cells with 8 mM-RTCA resulted in a 50% decrease in [3H]-uridine and a 20% decrease in [75Se]-methionine incorporation into acid precipitable product. For the short incubation times of 3 to 8 h used in later experiments to determine the effect of the compound on virus polypeptide or antigen synthesis, experiments established that incubation of CEF cells with 8 mM-RTCA resulted in a 50% decrease in [3H]-uridine and a 20% decrease in [75Se]-methionine incorporation into acid precipitable product.

To examine the effect of RTCA on influenza virus antigen synthesis in infected cells, control or RTCA (0.2 mM)-treated calf kidney cells were infected with influenza A/Hong Kong/1/68 (H3N2) virus at a multiplicity of 10 p.f.u./cell and incubated for 8 h at 37 °C. The cells were then fixed in acetone and examined for the presence of influenza A virus structural antigens using monospecific antisera and the indirect immunofluorescence test as described previously (Oxford & Schild, 1968; Oxford & Schild, 1975). The results (Table I) indicated that the nucleoside analogue had no selective inhibitory effect on the synthesis of any individual virus structural antigen. The production of all virus-induced structural antigens investigated (NP, MP, HA and NA) was inhibited to a similar degree. Whereas approx. 90% of cells were infected and showed the presence of all the different virus structural antigens by immunofluorescence in the control infected monolayer, only 5 to 8% of cells were fluorescing in RTCA (0.2 mM)-treated infected cells. In the low percentage of cells showing the presence of fluorescent antigen in the RTCA treated monolayers, the fluorescence showed identical intensity and distribution intracellularly when compared with the control infected cells.

Experiments were designed to provide additional data on the effect of RTCA on influenza virus protein synthesis in infected cells and particularly the effect of the compound on the synthesis of the non-structural protein (NS1) and the structural polypeptide P2 which are detected relatively early after infection of cells with influenza A virus (Skehel, 1972). The corresponding antigens could not be detected by immunofluorescence because at present monospecific antisera are not available. Influenza virus infected (10 p.f.u./cell) control or
RTCA (4 mM)-treated calf kidney monolayers were pulsed with \[^{35}S\]-methionine for 20 min periods at 3 h after infection. The cells were lysed and solubilized as described above and total cell extracts electrophoresed on slab polyacrylamide gels. Examination of the densitometer tracings of the autoradiographs indicated that the nucleoside analogue at a concentration of 4 mM had no effect on the polypeptide pattern in treated uninfected cells. However, the compound completely inhibited the production of the early influenza virus-induced structural polypeptides P2 and NP and the non-structural polypeptide (NS1). Identical results were obtained with A/FPV, A/HK/1/68 and A/England/42/72 viruses.

The effect of delayed addition of RTCA to virus infected cells was investigated. CEF cells were infected with 10 p.f.u./cell of FPV and RTCA (4 mM) was added at varying times after infection. At 6 h post-infection the monolayers were pulse labelled with \[^{35}S\]-methionine for 20 min, solubilized and examined by electrophoresis in slabs of polyacrylamide for inhibitory effect of the compound on influenza virus induced polypeptide synthesis. The RTCA sensitive step occurred within 1 h of infection of cells at this high multiplicity. In comparison, the influenza A virus inhibitor amantadine, which inhibits influenza virus replication at the stage of uncoating (Kato & Eggers, 1969) or virus penetration (Hoffmann et al., 1965), had to be added within 30 min of infection to obtain any inhibitory effect in the same experiment. The nucleoside analogue did not exert its inhibitory activity via an effect on virus adsorption, since in experiments in which RTCA was present only during the virus adsorption period no inhibitory effects of the compound were detected. Also addition of the compound immediately after FPV virus adsorption to CEF cells inhibited plaque formation to an equivalent degree to cultures where RTCA was present during the entire adsorption period. Thus, the compound appeared to act after the stages of virus adsorption, penetration and uncoating, but before the synthesis of virus polypeptides. We therefore attempted to reverse the inhibitory effect of RTCA by a variety of nucleosides to determine if the compound was exerting its inhibitory effects on the nucleotide pathways to RNA. The inhibitory effect of RTCA (0.2 mM) on plaque formation in CEF cultures infected with 250 p.f.u. of A/FPV was reversed by a 0.6 mM concentration of the nucleoside, guanosine (197 plaques) and by xanthosine (155 plaques) incorporated together with the nucleoside analogue in the agar overlay. In contrast, inosine (no plaques) and adenosine (37 plaques) at the same concentration were not able to reverse the virus inhibitory effects of the compound. In these experiments plaques were counted from 2 replicate dishes after 3 days incubation at 37 °C. In infected plates with RTCA alone in the overlay no plaques were detectable.

To investigate the possible reversibility of the inhibitory effect of RTCA by simply removing the compound, CEF cells were infected with 10 p.f.u./cell of A/FPV in the presence of 4 mM-RTCA. After 4 h incubation at 37 °C the nucleoside analogue was removed by washing the cells twice in normal medium, cells were reincubated in normal maintenance medium and at varying times after this the monolayers were pulsed with \[^{35}S\]-methionine and cell extracts examined for virus polypeptide synthesis. After removal of RTCA, virus polypeptide synthesis was not detectable over a period of 5 h. After this time period virus-induced P2, NP and NS1 polypeptides were detected but comparison of autoradiographs of virus infected cells treated with RTCA with autoradiographs of virus infected cells alone indicated continued reduction of the synthesis of each polypeptide in RTCA treated cultures. At the high input multiplicity (10 p.f.u./cell) used in the experiments it might be expected that surviving virus could re-initiate infection after removal of RTCA and the apparent decreased synthesis of virus induced polypeptides could result from cells infected with a lower multiplicity of virus. Polypeptide synthesis in control uninfected cells was inhibited by incubation
of cells for 5 h with a high concentration of RTCA (8 mM) but the effect was reversible after removal of the compound.

X-ray structural analysis has indicated that the crystal form of RTCA is very similar to that of guanosine (Prusiner & Sundaralingham, 1973). 1-β-D-ribofuranosyl-1,2,4-triazole 3-carboxamide 5’ phosphate is a potent competitive inhibitor of inosine 5’ phosphate dehydrogenase isolated from Escherichia coli and Ehrlich ascites tumour cells, suggesting that the antiviral activity of RTCA may be mediated by inhibition of GMP biosynthesis at the stage of conversion of IMP to xanthosine 5’ phosphate (Streeter et al. 1973). The reversal results of the present study would be consistent with this hypothesis although the possibility is not yet excluded that the phosphorylated derivative 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide 5’ phosphate has an inhibitory effect on influenza RNA transcriptase. Biologically the compound is a potent inhibitor of the replication in tissue culture of a wide range of influenza A viruses of human, animal and avian origin, but of particular interest is the inhibition of influenza B virus replication by RTCA. In contrast 1-amino adamantane hydrochloride, a well investigated inhibitor of influenza replication (Oxford, 1975) has a more restricted inhibitory effect against certain influenza A subtypes and has no effect against influenza B viruses. The plaquing experiments with A/FPV demonstrated that even in the presence of high concentrations of RTCA (0.2 mM) small plaques were occasionally detected. Whether this indicates the selection of virus particles resistant to the inhibitory effects of RTCA requires further investigation. The nucleoside analogue with its ease of reversibility may be of use in the study of early replicative events in influenza virus infected cells.

The technical assistance of Miss Sue O'Connor is gratefully acknowledged. The nucleoside analogue 1-β-D ribofuranosyl-1,2,4-triazole-3-carboxamide, formerly known as virazole, was kindly supplied by Dr R. W. Sidwell, ICN Nucleic Acid Research Institute, Irvine, California.

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(Received 4 February 1975)