Inhibition of Rhinovirus RNA Synthesis in Diploid Cells by a Substituted Guanidine Compound having a different Mode of Action from Guanidine

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SUMMARY

The compound ICI 65,709 is a substituted guanidine which has antiviral activity in human embryo lung cells (Bucknall et al. 1973). We have found that ICI 65,709 reversibly inhibits the synthesis of RNA by rhinovirus type 2. The three species of virus RNA were equally affected. Comparison of the effect with that of guanidine, a well known inhibitor of picornavirus RNA synthesis, showed that ICI 65,709 and guanidine mediated their antiviral effects in different ways. This conclusion was supported by the failure of ICI 65,709 to inhibit multiplication of a bovine enterovirus which was inhibited by guanidine. ICI 65,709 also inhibited the multiplication of poliovirus type 1.

The antiviral activity of a substituted guanidine compound, designated ICI 65,709 (Fig. 1) has recently been described (Bucknall et al. 1973). The compound was most active in inhibiting the multiplication of members of the picornavirus group, rhinoviruses, Coxsackie viruses and echoviruses, but was also active against vaccinia virus. It had slight activity against herpes simplex, Semliki Forest and Sindbis viruses but was inactive against equine rhinovirus, paramyxovirus type 1, coronavirus 229 E, respiratory syncytial virus and type A myxoviruses. The antiviral effect of ICI 65,709 was greatest in cultures of human embryo lung cells. In HeLa, KB or monkey kidney cells, the antiviral activity was much reduced or absent. ICI 65,709 appeared to inhibit a replicative or maturation process since it neither inactivated virus particles nor prevented their penetration into the host cell; it did not appear to bind to cellular components nor cause irreversible changes to the cell since antiviral activity was absent after removing the drug from the culture medium.

The antiviral activity of guanidine itself is well known (Sergiescu, Horodniceanu & Aubert-Combiescu, 1972). Like ICI 65,709, guanidine is most active in inhibiting the multiplication of picornaviruses and its effects are readily reversed. However, guanidine is active in a wide variety of cells. While it is known that guanidine inhibits virus RNA replication, its mode of action is not understood. It has been proposed that primarily guanidine (a) prevents the formation of active virus RNA polymerase (Lwoff, 1965; Caliguri & Tamm, 1968) or (b) prevents the release of newly synthesized RNA from the replication complex (Baltimore, 1969).

We have found that ICI 65,709 inhibited the replication of rhinovirus RNA in HEL cells. Because of the similarity of structure and spectrum of antiviral activity of ICI 65,709 and guanidine we have compared their action in the rhinovirus-HEL cell system, and have concluded that ICI 65,709 and guanidine do not have the same mode of action. In these studies we have used the gluconate salt of ICI 65,709. Its toxicity and antiviral activity were comparable with ICI 65,709 hydrochloride, used by Bucknall et al. (1973) (R. A. Bucknall, unpublished data).

Rhinovirus type 2, poliovirus type 1 (LSc 2ab) and bovine enterovirus (VG-5-27) were
grown and titrated as described by Koliais & Dimmock (1973). Monolayers of diploid human embryo lung (HEL) cells of the MRC 5 line were used except where indicated. Details of their preparation and culture have been previously described (Koliais & Dimmock, 1973). Cells were infected with virus at an input multiplicity of 5 to 10 for 1 h at 33 °C before washing and further incubation at 33 °C. Time after infection was measured from the time of virus addition. Radioactive virus RNA was isolated as described by Koliais & Dimmock (1973). Actinomycin D was used to inhibit cellular RNA synthesis and was usually added 4 h before radioactive labelling at a concentration of 1 μg/ml. Extracted RNA was analysed by polyacrylamide gel electrophoresis. The radioactivity in peaks of RNA from different gels was compared only after the counts above the background level of the gel had been summed and normalized with respect to internal O.D. markers of non-radioactive ribosomal RNA which were extracted from the same infected cells (Koliais & Dimmock, 1973).

Rhinovirus was adapted to grow in the presence of guanidine hydrochloride (Sigma Chemical Co.) by titrating the virus in HEL monolayers in rolled tubes containing 20, 60 or 100 μg/ml of inhibitor (Acornley et al. 1968). Virus growth was detected by c.p.e. and the highest dilution of virus which grew at the highest concentration of guanidine was passaged again. Complete resistance to the highest concentration used was achieved by the fifth passage and stocks were prepared from virus of the seventh passage. Plaque titration in HeLa cells showed that the resistant mutant was not significantly inhibited by 100 or even 200 μg/ml guanidine.

The effect of ICI 65,709 (kindly provided by Dr R. A. Bucknall, I.C.I. Pharmaceuticals Division, Alderley Edge, Cheshire) on the synthesis of cellular DNA, RNA and protein was determined by incubating HEL cells in the presence of 0 to 5 μg/ml for 9 h at 37 °C and then by adding [3H]-uridine or a mixture of [14C]-thymidine and [3H]-valine for a further 2 h. Fig. 2 shows the effect of ICI 65,709 on the incorporation into acid-insoluble radioactivity. Cellular RNA synthesis was not inhibited at any of the concentrations used, but along with DNA and protein synthesis showed an increased activity at 1-0 μg/ml, which was not further investigated. DNA synthesis fell by 15% and protein synthesis by 36% at a concentration of 2 μg/ml, a dose which produced a 90% inhibition of rhinovirus multiplication and which was used in subsequent experiments.

When 2 μg/ml ICI 65,709 was added immediately after rhinovirus infection, the cells pulsed with [3H]-uridine for 2 h from 9 to 11 h post-infection and the RNA extracted and analysed by polyacrylamide electrophoresis (Koliais & Dimmock, 1973) it was found that all three species of virus RNA, replicative intermediate (RI), replicative form (RF) and single-stranded RNA (SS), were inhibited to a similar extent (about 70% inhibition). Virus RNA synthesis was also inhibited without preference by the addition of ICI 65,709 well after it could be detected in the cell. When the compound was added at 7 h post-infection and the cells pulsed from 9 to 11 h the overall inhibition was about 60%. ICI 65,709 added at 1 h
Fig. 2. The effect of ICI 65,709 on RNA, DNA and protein synthesis in uninfected HEL cells. Cells were incubated with ICI 65,709 for 9 h and then for 2 h with radioactive precursors. ••••, [3H]-uridine; O——O, [14C]-thymidine; ▲——▲, [3H]-valine.

also inhibited the virus c.p.e. which extended to over 80% of the cells by 11 h after infection.

The reversibility of the inhibitory effect of ICI 65,709 on rhinovirus RNA synthesis was investigated next. Cells were treated with 2 µg/ml of ICI 65,709 from 7 to 8 h post-infection and then washed twice, re-incubated in fresh medium and pulsed with [3H]-uridine from 9 to 11 h. Total virus RNA synthesis was inhibited by 27%, which was half the value obtained when ICI 65,709 was not removed at 8 h. A similar recovery of RNA synthesis was also demonstrated when the compound was present from 0 to 3.5 h and cells pulsed from 5 to 7 h.

Since ICI 65,709 is a substituted guanidine it was of interest to establish whether or not ICI 65,709 and guanidine had identical modes of action. The first experiments examined some parameters of guanidine inhibition of the multiplication of rhinovirus type 2 in HEL cells. Rhinovirus was grown in the presence of increasing concentrations of guanidine for 21 h, and the combined yield of extra- and intra-cellular virus measured. Significant inhibition was observed at a concentration of 30 µg/ml and inhibition increased to over 99.9% at 100 µg/ml (Fig. 3). The latter concentration was used in the experiments on RNA synthesis described below to ensure maximum inhibition and is known not to affect macromolecular synthesis in HEL cells (Bablanian, Eggers & Tamm, 1965).

The extent of inhibition by guanidine added at different times and for varying periods was investigated by pulsing with [3H]-uridine from 9 to 11 h after infection and analysing the RNA by polyacrylamide electrophoresis. When guanidine was present from 0 to 11 h RNA synthesis was inhibited completely and when present from 7 to 11 h it was inhibited by 95%.

Inhibition was reversible since it was found that when guanidine was present from 7 to 8 h, RNA synthesis, measured at 9 to 11 h, had risen to 49% of the control. Provided that sufficient time was allowed following reversal of inhibition, complete recovery of RNA synthesis was observed even when guanidine was added at the time of infection.

We then compared the effects of guanidine and ICI 65,709 on the multiplication of several picornaviruses. Bucknall et al. (1973) have shown that ICI 65,709 inhibited the multiplication of a wide range of viruses in HEL cells, although the greatest activity was against rhinoviruses. We have investigated the extent of inhibition of two other picornaviruses, bovine enterovirus and poliovirus by ICI 65,709 or guanidine.

We found that 100 µg/ml guanidine added to HEL cells at 0 h inhibited the rate of bovine
enterovirus multiplication and final yield of virus (Fig. 4), RNA synthesis and the appearance of c.p.e. However, 2 μg/ml ICI 65,709 added at 0 h had no effect on these properties. In contrast, both guanidine and ICI 65,709 inhibited the multiplication of poliovirus, and, as expected, inhibited synthesis of poliovirus RNA (data not shown).

Finally, we investigated the effect of ICI 65,709 on RNA synthesis by a guanidine-resistant

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Fig. 3. Inhibition of rhinovirus multiplication by guanidine. The yield of combined intra- and extra-cellular virus is presented. Cytopathic effect at the time of harvest (24 h) is indicated by + (50% of cells affected) or ++ (over 80% of cells affected).

Fig. 4. Effect of ICI 65,709 (2 μg/ml) and guanidine (100 μg/ml) on the multiplication of bovine enterovirus in HEL cells. Both compounds were present continuously from 0 h.

Fig. 5. Effect of guanidine and ICI 65,709 on the RNA synthesis by a guanidine-resistant rhinovirus mutant. Guanidine (100 μg/ml), or ICI 65,709 (2 μg/ml), were present from 0 h and cells were pulsed with [3H]-uridine from 9 to 11 h. The replicative form (RF) and single-stranded virus (SS)RNA are marked. The replicative intermediate which normally enters the top 5 fractions was not apparent in this experiment. The continuous line is the E_{70S} indicating the position of a peak of DNA and 28 S ribosomal RNA extracted from the infected culture.
The resistance of bovine enterovirus to ICI 65,709 but not to guanidine indicated that the modes of action of the two compounds were not identical. However, since rhinovirus replication was inhibited by both compounds we selected a mutant which was resistant to guanidine to see if resistance to ICI 65,709 was acquired simultaneously.

RNA synthesis by the guanidine-resistant rhinovirus was measured in the presence of 100 µg/ml guanidine or 2 µg/ml ICI 65,709 (Fig. 5). The result was unequivocal: guanidine had no effect while ICI 65,709 inhibited RNA synthesis. We concluded that the modes of action of guanidine and ICI 65,709 in inhibiting RNA synthesis of rhinovirus were completely different. This difference is corroborated by the failure of ICI 65,709 to inhibit bovine enterovirus RNA synthesis and virus particle production under conditions where guanidine was inhibitory. The action of ICI 65,709 also differs from that of guanidine since it did not give rise to drug-resistance and it is not reversed by the 'anti-guanidine' group of compounds (Bucknall et al. 1973).

The maximum inhibition of virus RNA synthesis by ICI 65,709 had a mean value over a large number of experiments of 85%. By comparison 30 µg/ml guanidine were required to inhibit virus particle formation to the same extent as 2 µg/ml ICI 65,709. The latter was thus 15-fold more effective on a weight basis. On a molar basis ICI 65,709 gluconate (mol. wt. 571.5) was 90-fold more effective than guanidine hydrochloride (mol. wt. 95.5). Increasing the concentration of ICI 65,709 above 2 µg/ml did not significantly increase inhibition of virus RNA synthesis whereas increasing the concentration of guanidine to 100 µg/ml resulted in the absence of detectable virus RNA synthesis and the reduction of virion yield by over 99.9%.

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REFERENCES

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