Inhibition of an Early Stage of Rhinovirus Replication by Dichloroflavan (BW683C)

By MARGARET TISDALE* AND J. W. T. SELWAY
Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS, U.K.

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SUMMARY

The mechanism of action of a new anti-rhinovirus compound, dichloroflavan, was examined using a variety of techniques. Dichloroflavan was shown to bind to the highly sensitive rhinovirus type 1B (50% inhibitory concentration 0.007 μM) and at a much lower level to the insensitive rhinovirus type 4 (50% inhibitory concentration > 25 μM). Binding of the compound to rhinovirus 1B was accompanied by a reduction (about 0.5 log₁₀ units) in virus infectivity which could be restored by treatment with chloroform. Maximum inhibition of virus yield (about 2 log₁₀ units) occurred only when compound and virus were added together, but some inhibition (about 0.7 log₁₀ units) occurred even when the compound was added near the end of a single cycle of replication. This late reduction in infectivity could be abolished by treating with chloroform, and was therefore caused by the compound binding to progeny virus. The compound did not interfere with adsorption of virus to cells, nor with uncoating of the viral RNA. However, little or no viral RNA and protein synthesis occurred in the presence of dichloroflavan provided that the compound was added with the virus. Addition of the compound after virus adsorption had no effect on either viral RNA or protein synthesis. These results indicate that dichloroflavan binds directly to rhinovirus 1B and appears to prevent virus replication at a point immediately after uncoating of the viral RNA.

INTRODUCTION

4',6-Dichloroflavan (BW683C) is a new anti-rhinovirus compound (Bauer et al., 1981). The spectrum of activity appears to be limited to members of the rhinovirus group, but even within this group there is a wide variation in the sensitivity of the different serotypes, with some showing complete resistance. This variation in sensitivity indicates that the compound possesses specific antiviral activity, and this is supported by its low toxicity in vitro. The compound had no effect on cells at a concentration of 3-6 μM, which is several hundred times the 50% inhibitory concentrations (IC₅₀) for the more sensitive serotypes.

The most sensitive serotypes, of which rhinovirus 1B has been selected for study, have IC₅₀ values by plaque reduction down to 0.007 μM. The resistant serotypes are unaffected by dichloroflavan at concentrations up to 25 μM; they include rhinovirus 4 which has been selected for comparative studies with rhinovirus 1B. In the present study we have attempted to elucidate the mechanism of inhibition of rhinovirus 1B by dichloroflavan and to explain the difference between sensitive and resistant serotypes.

METHODS

Cells. M. HeLa cells, rhinovirus-sensitive HeLa cells (Fiala & Kenny, 1966) were obtained from Glaxo Laboratories, Greenford, Middlesex, U.K. and grown in minimal essential medium containing 10% foetal calf serum with 50 units/ml penicillin and 50 μg/ml streptomycin. Monolayer cultures were used throughout.

Viruses. Rhinovirus type 1B was obtained from Glaxo Laboratories and rhinovirus type 4 from the Clinical Research Centre, Harrow, Middlesex, U.K. The passage history of these viruses is not known.

Growth of normal and radioactive virus. Virus stocks were prepared in M. HeLa cells which were infected at 0.5 to 1 p.f.u./cell and incubated at 33 °C for approximately 24 h. The virus was harvested when the cells showed an extensive cytopathic effect.
To prepare radioactive virus, 0.05 mCi/ml of either [3H]uridine or [35S]methionine was added to cell cultures (2 x 10⁶ cells/culture) 3 h after addition of the virus. For [3H]uridine label, cells were pretreated for 2 h with 1 µg/ml actinomycin D and the virus grown in serum-free medium plus actinomycin D. For [35S]methionine label, the virus was grown in methine-free medium without serum.

**Purification of virus.** To purify the virus, the culture medium was clarified by centrifugation at 700 g for 15 min at 4 °C, and then the virus was sedimented at 60000 g for 1 h. After resuspension in phosphate-buffered saline (PBS), the pellets were either treated with 1% Nonidet P40 (BDH) or they were shaken for 5 min with an equal volume of Arcton 113 (ICI) and the aqueous fraction separated by low-speed centrifugation. The virus suspension was then layered onto a 15 to 45% linear sucrose gradient and centrifuged at 100000 g for 3 h on a MSE 3 x 20 ml rotor. Fractions (1 ml) were collected and assayed either for infectivity as described below or for radioactivity. For radioactivity assays, 50 µl samples were added to 1 ml vol. of 0.1 M-sodium hydroxide which in turn were added to 10 ml vol. of P.C.S. scintillation fluid (Hopkin and Williams) for counting.

**Infectivity assays.** Infectivity was determined by plaque assay in M. HeLa cells as described by Fiala & Kenny (1966). Assays for infectious RNA were carried out in M. HeLa cells using 1 mg/ml DEAE-dextran in the diluent, as described by Fiala & Saltzman (1969).

**Extraction of viral RNA.** This was carried out as described by Fiala & Saltzman (1969) with virus suspended in PBS and using two extractions with water-saturated phenol at 60 °C.

**Assays for radioactive RNA.** For experiments on virus adsorption and uncoating the methods were based on those of Lonberg-Holm & Korant (1972). Briefly, samples of cell lysate were treated with 25 µg/ml ribonuclease A (Sigma) for 30 min at 37 °C and then with 10% trichloroacetic acid (TCA) for 16 h at 4 °C. The samples were centrifuged at 400 g for 10 min at 4 °C, and the supernatant fluids added to 10 ml vol. of P.C.S. for scintillation counting. The precipitates were washed with 5% TCA, resedimented, dissolved in 1 ml vol. of 0.15 M-sodium hydroxide and added to 10 ml vol. of P.C.S. for scintillation counting.

For experiments on RNA synthesis, viral RNA was labelled during the growth cycle by incubating cells (0.7 x 10⁶ cells/culture), with 7 µCi [3H]uridine for 30 min at 33 °C and the cells then treated with 10% TCA at 4 °C for 16 h. The cells were scraped from the plastic, sedimented, washed and radioactivity determined as described above.

**Polyacrylamide gel electrophoresis.** [35S]Methionine-labelled samples were examined by discontinuous electrophoresis based on the method of Laemmli (1970) using 7.5 to 15% linear gradient gels. Electrophoresis was carried out at 50 V for approximately 18 h. The gels were fixed in 20% methanol, 9% acetic acid for 30 min, dried, and autoradiograms prepared on Cronex 4 X-ray film (Dupont). ¹⁴C-labelled molecular weight markers (Amersham International) were included in each run.

**Chemicals.** Dichloroflavan was synthesized by Dr J. Batchelor (Wellcome Research Laboratories, Beckenham, U.K.). [3H]Dichloroflavan (sp. act. 2.5 Ci/mmol) was labelled at positions 3 and 4 (Dr J. Batchelor). [5,6-³H]Uridine (sp. act. 40 to 60 Ci/mmol) and L-[³⁵S]methionine (sp. act. > 600 Ci/mmol) were obtained from Amersham International.

### RESULTS

#### Direct effect of dichloroflavan on virus

The first experiments on the mechanism of action of dichloroflavan were to determine whether the compound had any direct effect on the virus. Virus was incubated with compound or with diluent for increasing lengths of time at 37 °C. With rhinovirus 4, a 10 µM final concentration of compound was used; with rhinovirus 1B, a lower concentration, 1 µM, was used to ensure that the compound was diluted below its inhibitory level in the titrations.

The results in Fig. 1 show that with rhinovirus 4 there was no difference in infectivity between the test and the control mixtures; but with rhinovirus 1B, virus titres in the test mixtures were consistently slightly lower than those in the control mixtures, on average by 0.5 log₁₀ units. Therefore, dichloroflavan has a direct effect on sensitive virus but this differs from conventional direct inactivation in that the fall in titre does not increase with time. Dichloroflavan is a non-polar compound and very soluble in chloroform. Virus infectivity could be restored by shaking samples for 5 min with an equal volume of chloroform, before separating the two phases by low-speed centrifugation. These results suggest that reversible binding of the compound to the virus had occurred.
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Fig. 1. Effect on infectivity of incubating mixtures of virus and dichloroflavan for increasing lengths of time at 37°C. (a) Rhinovirus 4 + 10 μM-dichloroflavan; (b) rhinovirus 1B + 1 μM-dichloroflavan. ●, Virus control; ■, virus + dichloroflavan.

Fig. 2. Effect of time of addition of dichloroflavan to rhinovirus 1B-infected cultures. Cells (1 × 10⁶ per culture) were infected at 10 p.f.u./cell for 1 h. Dichloroflavan (0.5 μM) was added at the times indicated during the virus growth cycle. At 8 h, the cells plus medium of all cultures were freeze-thawed three times. Virus contents were titrated by plaque assay before (●) and after (■) treatment with chloroform, which removed dichloroflavan and prevented interference with subsequent titrations.

Effect of time of addition of dichloroflavan on virus inhibition

Further experiments were carried out to determine the point of action of the compound in a single cycle of virus growth. The results in Fig. 2 show that the compound had its greatest effect when added with the virus, inhibiting yield by 1-9 log₁₀ units. However, there was some reduction in virus yield even when the compound was added at the end of the growth cycle. The apparent inhibition produced by adding compound at any time after viral adsorption was completely reversed by treating the virus samples with chloroform. This indicated that the low infectivities were the result of binding of compound to progeny virus which was present in normal amounts. Dichloroflavan therefore acts very early in the virus growth cycle. Shortening
Table 1. Effect of dichloroflavan on release of virus after single cycle replication

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Concentration of dichloroflavan (μM)</th>
<th>Infectivity of cells plus medium (p.f.u./ml)†</th>
<th>Infectivity of medium (p.f.u./ml)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus control</td>
<td>0</td>
<td>1.3 \times 10^7</td>
<td>3.2 \times 10^5</td>
</tr>
<tr>
<td>Compound added after viral adsorption</td>
<td>0.5</td>
<td>1.2 \times 10^7</td>
<td>5.8 \times 10^5</td>
</tr>
</tbody>
</table>

* Cells were infected at 10 p.f.u./cell for 1 h, maintenance medium plus or minus compound added and the cultures incubated at 33 °C for 8 h.
† Virus was treated with chloroform to remove dichloroflavan before plaque titrations.

Table 2. Effect of pretreatment of cells with dichloroflavan on inhibition of rhinovirus 1B replication

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration of dichloroflavan (μM)</th>
<th>Virus yield† (p.f.u./ml)</th>
<th>log_{10} Reduction in virus titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus control</td>
<td>0</td>
<td>7.6 \times 10^6</td>
<td>--</td>
</tr>
<tr>
<td>Pretreatment* before infection</td>
<td>0.5</td>
<td>3.8 \times 10^5</td>
<td>1.30</td>
</tr>
<tr>
<td>Compound added with virus</td>
<td>0.5</td>
<td>4.4 \times 10^5</td>
<td>1.24</td>
</tr>
</tbody>
</table>

* Cells were incubated with dichloroflavan in maintenance medium for 30 min at 37 °C. The cells were washed three times with PBS before infection at 10 p.f.u./cell.
† Virus cultures, after 8 h incubation at 33 °C, were freeze-thawed three times and treated with chloroform.

Evidence that the compound binds to virus

The results of previous experiments suggested that the compound became bound to infectious virus, and experiments were therefore designed to verify this. Arcton-treated purified rhinovirus types 1B and 4 were mixed with an equal volume of 25 μM tritiated dichloroflavan and incubated at 33 °C for 30 min. The mixtures were layered onto 15 to 45% sucrose gradients and centrifuged at 100000 g for 3 h at 4 °C. Fractions were collected, radioactivity counted and infectivity determined as described earlier (Fig. 3).

A definite peak of compound coincided with the peak of rhinovirus 1B, but only a very small peak was associated with rhinovirus 4, showing that both viruses bind the compound, but that binding to rhinovirus 1B is much greater than to the insensitive rhinovirus 4. The particle : p.f.u. ratios were determined by measuring the adsorption of purified virus suspension at 260 nm and the number of molecules of compound bound per virion were calculated (Korant et al., 1972). Under the conditions stated, rhinovirus 1B bound approximately 24 molecules per virion and rhinovirus 4 approximately 2 molecules per virion. If the binding of dichloroflavan to virus is reversible by dilution, then the levels of binding may be higher than these results indicate.

Effect of dichloroflavan on free viral RNA

Since dichloroflavan appeared to bind to sensitive virus, it was of interest to determine whether the compound would affect the infectivity of free viral RNA under similar conditions.

Extracted viral RNA (3 \times 10^4 p.f.u./ml) was mixed with an equal volume of dichloroflavan
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Fig. 3. Binding of [3H]dichloroflavan to rhinovirus. Purified virus was mixed with an equal volume of 25 μM [3H]-labelled dichloroflavan and centrifuged on a 15 to 45% linear sucrose gradient. (a) Rhinovirus 4; (b) rhinovirus 1B. ■, [3H]-labelled dichloroflavan; ○, infectivity.

Fig. 4. Comparison of the rate of adsorption of [3H]uridine-labelled rhinovirus 1B, and the rate of uncoating of its RNA into M. HeLa cells, in the presence (■, □) or absence (○, ○) of dichloroflavan. Cells and virus (approx. 22000 ct/min/sample) were pretreated with 25 μM-dichloroflavan or with medium for 30 min before infection. The cultures (3 × 10⁶ cells/culture) were incubated with virus at 1 p.f.u./cell for increasing lengths of time at 33 °C. The virus inoculum was removed and the cells placed on ice and washed with cold PBS. The cells were frozen and thawed and treated with ribonuclease and TCA as described in Methods. The acid-soluble and -precipitable counts were measured. Adsorption is indicated by the total radioactivity associated with cells (■, ●), and uncoating by the acid-soluble radioactivity after treatment with 25 μg/ml ribonuclease (□, ○).

Fig. 5. Incorporation of [3H]uridine into newly synthesized RNA of rhinovirus 1B in the presence and absence of dichloroflavan (added with virus). Cellular RNA synthesis was inhibited by actinomycin D (5 μg/ml) added 2 h before infection and present throughout the growth cycle. Virus was incubated with either medium (controls) or with 5 μM-dichloroflavan for 30 min at 37 °C before infecting cells at 10 p.f.u./cell. Virus was adsorbed for 1 h at 33 °C and then the cells were washed with PBS, and maintenance medium with or without compound (5 μM) was added. Cultures were incubated for the times indicated and pulse-labelled with 7 μCi [3H]uridine for 30 min before precipitation with TCA. Cell controls were included for every reading and the cell background activity was deducted from each reading. ●, Virus control; ■, virus + dichloroflavan mixed before infection.

(0.01 or 1 μM final concentration). The mixtures were incubated at 37 °C for 30 min and then titrated in M. HeLa cells using DEAE-dextran as diluent. No difference in infectivity titres was observed between control and test mixtures, and therefore the compound does not appear to have any direct effect on the viral RNA.
Fig. 6. Effect of dichloroflavan on polypeptide synthesis of rhinovirus 1B-infected and uninfected cell cultures. Cultures (1 × 10⁷ cells/culture) were pretreated for 2 h before infection and throughout the cycle with maintenance medium plus 5 μg/ml actinomycin D and 150 mM-NaCl. Cultures were treated with 5 μM-dichloroflavan either before or after adsorption of virus (50 p.f.u./cell for 30 min) and polypeptide synthesis determined by labelling with [35S]methionine for 1 h, between 6 and 7 h after infection. Virus and cell controls, plus a compound control to determine the effect of dichloroflavan on cell protein synthesis, were included. Polyacrylamide gel electrophoresis was carried out as described in Methods. Lane 1, uninfected cells; lane 2, uninfected cells treated with dichloroflavan; lane 3, infected cells; lane 4, infected cells treated with dichloroflavan added with virus; lane 5, infected cells treated with dichloroflavan added after viral adsorption. Structural polypeptides were identified by comparison with labelled purified virus.

Table 3. Effect of time of addition of dichloroflavan on RNA synthesis of rhinovirus 1B

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration of dichloroflavan (μM)</th>
<th>Incorporation* of [3H]uridine (ct/min × 10⁻⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus control</td>
<td>0</td>
<td>7.411</td>
</tr>
<tr>
<td>Compound added with virus</td>
<td>5</td>
<td>0.015</td>
</tr>
<tr>
<td>Compound added after virus adsorption</td>
<td>5</td>
<td>7.561</td>
</tr>
</tbody>
</table>

* Cells were treated with actinomycin D and infected as described in Fig. 5. Cultures were pulse-labelled with 7 μCi [3H]uridine for the 30 min between 5.5 and 6 h after infection and samples precipitated with TCA as described in Methods. Results given are averaged from duplicate cultures after adjustment for cell background.
Effect of dichloroflavan on adsorption and uncoating of virus

Maximum inhibition by dichloroflavan occurred very early in the replication cycle and therefore it appeared possible that entry of virus into the cell was affected. To test this, cultures were infected with purified rhinovirus 1B labelled with $[^3H]uridine$.

From the distribution of radioactive label the percentage of whole virus adsorbed to the cells and the amount of RNA that had become uncoated and susceptible to ribonuclease were calculated (Fig. 4). Neither adsorption of virus nor uncoating of viral RNA was inhibited by dichloroflavan. However, adsorption was slightly higher at 4h in the presence of dichloroflavan. The reason for this difference is not known.

Effect of dichloroflavan on viral RNA synthesis

Since viral RNA was shown to be uncoated in the presence of the compound, the next step was to determine whether RNA and protein synthesis could occur in the presence of the compound.

In untreated cultures viral RNA synthesis reached a peak at about 6h after infection (Fig. 5). In the presence of the compound no viral RNA synthesis occurred, provided that the compound was added with the virus. If the addition of the compound was delayed until after the period of viral adsorption, no detectable inhibition of viral RNA synthesis occurred (Table 3). These results therefore indicate that the compound has no detectable effect on viral RNA synthesis but blocks a stage in replication before initiation of RNA synthesis. The compound had no effect on cellular RNA synthesis at the concentration tested.

Effect of dichloroflavan on viral and cellular protein synthesis

Protein synthesis was examined by polyacrylamide gel electrophoresis of extracts of cultures pulse-labelled with $[^35S]methionine$. The results are shown in the autoradiogram in Fig. 6. From a comparison of lanes 1 and 2, it may be seen that dichloroflavan did not affect cellular protein synthesis. From lanes 3, 4 and 5 it may be seen that when dichloroflavan was added with virus, complete inhibition of viral protein synthesis occurred, with no switch-off of cellular proteins; however, when dichloroflavan was added after adsorption, all viral proteins were produced in similar quantities to the virus controls. Therefore, dichloroflavan had no direct effect on viral protein synthesis. These results agree with the results obtained with the inhibition of viral RNA synthesis, and both indicate that dichloroflavan acts very early in the growth cycle, before viral replication has commenced.

Discussion

Dichloroflavan has been shown to bind to the sensitive rhinovirus 1B serotype, and to bind about 12-fold less efficiently to the insensitive rhinovirus 4 serotype. This difference between the two serotypes indicates that binding is probably important to the inhibitory action of the compound, but that some of this binding is non-specific. The difference in sensitivity between serotypes implies that viral proteins rather than viral RNA are involved in the binding reaction. This was supported by the observation that the infectivity of free RNA was not affected by incubation with the compound. Dichloroflavan is a highly lipophilic compound and may bind to hydrophobic regions on some of the viral structural proteins.

The low levels of inhibition seen in the direct inactivation experiments suggest that the binding of the compound to the virus was largely reversible, most of the compound being removed during dilution of the virus. Treatment of the virus with organic solvents such as chloroform completely removed the compound from the virus and showed that no direct damage to the virus occurred during binding. However, uptake of the compound by cells was not readily reversible, presumably due to the highly lipophilic nature of the compound. This lipophilicity should mean that high concentrations of the compound are present in the cell membrane and thus in an ideal position to bind to and inhibit virus as it enters the cells. The concentration of the compound in the cell membrane, plus the reversible nature of the binding to the virus, should explain the differences seen between inhibition produced by direct inactivation and by yield studies under single cycle multiplication.
The results have shown that binding of compound to virus is followed by inhibition of a very early stage in viral replication. In single cycle experiments maximum inhibition did not occur unless the compound came into direct contact with either virus or cells before infection. The cut-off point was very sharp, and indicated that once the virus had begun to enter the cell, no inhibition could occur. These results suggested that the compound acts on entry of the virus into the cells. However, the experiments on adsorption and uncoating of virus indicated that neither of these events was inhibited. The latter results were supported by the finding that dichloroflavan did not stabilize virus against heat (50 °C) inactivation (M. Tisdale & J. W. T. Selway, unpublished data), in contrast to observations with other compounds which inhibit uncoating of picornaviruses (Caliguiri et al., 1980). It is possible that binding of the compound to one of the capsid proteins does not inhibit changes which occur during uncoating in the cell membrane, but prevents final release of the RNA from the capsid. Treatment of poliovirus with cell membrane extracts has revealed a particle which is sensitive to ribonuclease but where the RNA was still encapsidated (De Sena & Torian, 1980).

Experiments on viral RNA and protein synthesis confirmed the early effect of the compound, since addition of the compound with virus prevented synthesis of both viral RNA and viral protein. However, addition of the compound after adsorption indicated that dichloroflavan had no direct effect on viral RNA or protein synthesis. Thus, the compound could be inhibiting the initiation of viral RNA synthesis through binding to the virus. Binding to the viral protein VPg, which is covalently linked to the viral RNA, would be consistent with these observations. However, the lack of inhibition with infectious RNA, plus the variation in sensitivity seen with the different serotypes, suggests that VPg is not involved. Furthermore, it is possible that binding of dichloroflavan to one or more of the coat proteins could also prevent initiation of viral RNA replication. Thus, with guanidine, which is known to inhibit initiation of poliovirus RNA synthesis, capsid proteins have been implicated as determinants of guanidine resistance and sensitivity (Cooper et al., 1970).

Dichloroflavan is not the first compound which has been shown to bind to rhinovirus and cause inhibition of viral replication. Zinc ions bind to virus particles and also cause inhibition of cleavage of viral peptides (Korant & Butterworth, 1976). SDS binds to rhinovirus 2 and inhibits eclipse of the virus (Lonberg-Holm & Noble-Harvey, 1973). Several compounds have been shown to bind to poliovirus and other enteroviruses, including arildone (Caliguiri et al., 1980), rhodanine (Rosenwirth & Eggers, 1979), and the thiopyrimidine S-7 (Lonberg-Holm et al., 1975). Most of these latter compounds are flat lipophilic structures with little or no ionization, which probably bind to hydrophobic areas of the protein and inhibit uncoating of the virus (Cooper, 1977). Dichloroflavan shares many of the above properties of these compounds, but differs in that uncoating does not appear to be inhibited. Further studies with a dichloroflavan-resistant mutant are in progress to determine which component of the virus is involved in binding, and to help to elucidate the exact point of inhibition.

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