Isolation and biological characterization of 3(2H)-isoflavene-resistant and -dependent poliovirus type 2 Sabin mutants

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Poliovirus type 2 Sabin mutants were selected for drug resistance and dependence by plating on HeLa cell monolayers in the presence of 3(2H)-isoflavene, a compound related to dichloroflavan, which prevents the shut-off of host translation and poliovirus RNA and protein synthesis. The drug-resistant mutants grew equally well in the presence and in the absence of the drug, while the drug-dependent mutants only grew in the presence of the compound. One dependent and one resistant mutant were characterized biologically in more detail. The resistant mutant did not exhibit thermolability. The mild thermolability exhibited by the dependent mutant was not affected by the addition of 3(2H)-isoflavene, indicating that the substance does not bind the poliovirus type 2 Sabin capsid. The translation of viral proteins and the shut-off of host protein translation during cell infection were not inhibited in either mutant. In the absence of the drug, the cleavage of the precursor VP0, a step in virus protein processing, was affected in the dependent mutant. The dependence of the mutant on the drug was due to the inability of 75S empty particles to reach maturation: our results strongly suggest that this phenomenon is strictly dependent on the reduction of RNA synthesis, confirming the existence of a dynamic equilibrium between RNA production and genome encapsidation during the poliovirus replication cycle.

Introduction

Enteroviruses form the largest genus of the family Picornaviridae. They include many human pathogens, which are aetiological agents of severe diseases of the heart, liver, eyes and pancreas and of well-known acute infections of the central nervous system. Polioviruses (the prototype of the enterovirus group) have a positive-sense single-stranded RNA genome with a long 5′ untranslated region containing several AUG codons upstream of the one used to initiate translation (Haller & Semler, 1995). The poliovirus genome is an effective mRNA although, in contrast to the majority of host mRNAs, it lacks a cap structure at the 5′ end.

The poliovirus genome is surrounded by an icosahedral capsid composed of 60 protomers, each made up of four capsid proteins, VP1, VP2, VP3 and VP4. Upon infection, the RNA is first translated into a polyprotein, which is then proteolytically cleaved into three precursors (P1, P2 and P3) by the virus-encoded proteases 2A and 3CD. The subsequent cleavage of these precursors by the 3CD protease gives rise to four structural and seven non-structural polypeptides (Hellen & Wimmer, 1995).

Replication occurs within the cytoplasm in virus-induced lipid-containing vesicles. These membranes, known as the replication complex (Caliguiri & Tamm, 1970; Bienz et al., 1987), are apparently necessary for the production of single-stranded RNA, both in vivo (Guinea & Carrasco, 1990) and in vitro (Dasgupta et al., 1980; Baron & Baltimore, 1982; Takedagami et al., 1983; Takeda et al., 1986). The viral proteins required for positive-sense RNA replication in vitro are unlikely to be supplied in trans (Bernstein et al., 1986; Giachetti et al., 1992); cis-acting elements seem to be necessary during this step of virus replication.

Different stages of virion assembly are associated with specific cleavages of capsid protein precursors. The formation of pentamer intermediates appears to require the cleavage of the precursor P1 to VP0, VP1 and VP3. At the final stage of virion assembly (conversion of the provirion to the mature virion), VP0 is cleaved to form the capsid proteins VP2 and VP4.
Over the last 10 years, there has been growing interest in antiviral chemotherapy of enterovirus infection. Indeed, a number of picornavirus inhibitors have been described and their mechanism of action studied by using drug-resistant mutants. Analysis of antiviral drug resistance is an extremely useful approach for investigating the function of picornavirus proteins (Carrasco, 1994). Among the antiviral agents, the natural compounds flavonoids have been demonstrated to be effective against picornavirus replication in vitro. In particular, 3-methylquercetin and Ro 09-0179 have been shown to inhibit poliovirus RNA synthesis selectively (Castrillo et al., 1986; González et al., 1990).

Another flavonoid, 4′,6-dichloroflavan, which has been reported to be a potent inhibitor of the replication of several rhinovirus serotypes, acts in a different way, by preventing virus uncoating (Bauer et al., 1981). Halogenated 3(2H)-isoflavenes and isoflavans, derivatives of 4′,6-dichloroflavan, have been synthesized and found to be effective not only against rhinovirus replication in vitro but also against a broader spectrum of picornaviruses. Studies on the mode of action demonstrated interference with some early steps of virus replication (Burali et al., 1987). Halogenated 3(2H)-isoflavenes and isoflavans, derivatives of 4′,6-dichloroflavan, have been synthesized and found to be effective not only against rhinovirus replication in vitro but also against a broader spectrum of picornaviruses. Studies on the mode of action demonstrated interference with some early steps of virus replication (Burali et al., 1987; Conti et al., 1988, 1990a; Superti et al., 1989; Genovese et al., 1995). We previously studied the mechanism of action of 3(2H)-isoflaven on poliovirus type 2 infection in vitro and our results led us to conclude that the compound interfered with the process of virus replication at a step between uncoating and translation. Indeed, the substance was neither virucidal nor able to protect virus infectivity from heat inactivation and, contrary to the mechanism of action on rhinovirus, had no effect on virus binding, penetration or uncoating. The presence of the drug at the beginning of infection or during the adsorption period prevented the virus-induced shut-off of host translation as well as viral RNA and protein synthesis.

In this paper, we describe the biological properties of mutants resistant to and dependent on 3(2H)-isoflaven. The results obtained demonstrate that the drug interferes with viral RNA synthesis in both mutants that, in the dependent mutant, is also correlated with a blockage of virion maturation. Both mutants may be useful tools for studying the steps of poliovirus translation, replication and capsid maturation.

### Methods

**Compounds.** 3(2H)-isoflaven, 6-chloro-3(2H)-isoflaven and 4′-chboro-6-cyanoflavan were synthesized as previously described (Burali et al., 1987; Conti et al., 1990b). Stock solutions in ethanol (1 mg/ml) were stored at 4 °C before dilution with tissue culture medium. WIN51711 (5-[7-[4-(4,5-dihydro-2-oxazolyl)phenoxo]heptyl]-3-methylisoxazole) was kindly supplied by the Sterling-Winthrop Research Institute (Rensselaer, NY, USA) and was used as a control at a final concentration of 1 µg/ml (2.9 µM) (Fox et al., 1986; Zeichhardt et al., 1987).

**Cells and viruses.** HeLa cell monolayers were grown in Eagle’s minimum essential medium (MEM) supplemented with 10% foetal calf serum, 200 U/ml penicillin and 200 U/ml streptomycin (growth me-

dium). Poliovirus type 2 Sabin (ATCC VR-1003; referred to as Sabin 2) reference strain was used for the study. Infection of confluent HeLa cell monolayers was performed at an m.o.i. of 5 p.f.u. per cell. After virus adsorption for 30 min at 36 °C, MEM with 2% foetal calf serum (maintenance medium) was added. When the virus cytopathic effect (CPE) was complete (about 18–24 h), cells were harvested and titration of virus suspensions was performed by plaque assay in 35 mm Falcon dishes.

**Isolation of mutants.** Subconfluent monolayers of HeLa cells were infected with Sabin 2 virus at an m.o.i. of 0.001 p.f.u. per cell in the presence of 5 µM 3(2H)-isoflaven. The virus was recovered when the CPE was evident. Tenfold dilutions from 10⁻³ to 10⁻⁴ of the recovered virus were used for reinfection of HeLa cells and 3(2H)-isoflaven was added at a concentration of 10 µM. The highest dilution of virus able to induce a CPE was passaged serially in HeLa cells in the presence of increasing concentrations (up to 40 µM) of 3(2H)-isoflaven. After the seventh passage, virus mutants were plaque-isolated in the presence of 30 µM 3(2H)-isoflaven. The virus was released by freezing and thawing. Differentiation between drug-resistant and drug-dependent mutants was achieved by comparing the titres in the presence and in the absence of 3(2H)-isoflaven. Revertants of the dependent mutant were obtained by two cycles of plaque isolation in the absence of the drug.

**Plaque assay.** HeLa cell monolayers grown on 36 mm dishes were infected with 0.2 ml tenfold virus dilutions. After adsorption for 30 min at 36 °C, excess virus was removed and monolayers were overlaid with 2 ml MEM without phenol red containing 2% foetal calf serum and 1% agar. Incubation was performed at various temperatures, depending on the experiment, in a humidified atmosphere with 5% CO₂ in air. After 48 h, the cells were stained with 0.01% neutral red.

**Heat inactivation of virus infectivity.** The virus suspension (5 x 10⁶ p.f.u./ml) was incubated for 1 h at 4 °C with 20 µM 3(2H)-isoflaven and then for 15 min at different temperatures. The mixtures were diluted and titres were determined by plaque assay.

**One-step growth curve.** Monolayers of HeLa cells were infected with virus (10 p.f.u. per cell) in the presence or in the absence of 3(2H)-isoflaven for 30 min at 36 °C. After the virus inoculum was washed out, fresh medium with 20 µM 3(2H)-isoflaven was added. After different times of incubation at 36 °C, infected cultures were freeze-thawed three times and the titre of virus in the clarified supernatants was determined by plaque assay.

**RNA transfection.** HeLa cell monolayers in 19 mm plastic dishes were pre-treated for 1 h at 36 °C with 1.5 mg/ml DEAE-dextran (molecular mass 500000) in PBS with calcium and magnesium ions. The cells were transfected with viral RNA (5 µg per dish) in the presence or in the absence of 3(2H)-isoflaven for 30 min at 36 °C. After the virus inoculum was washed out, fresh medium with 20 µM 3(2H)-isoflaven was added. After different times of incubation at 36 °C, infected cultures were freeze-thawed three times and centrifuged at low speed to remove cell debris. Titres were determined by plaque assay in HeLa cells in the absence of 3(2H)-isoflaven for Sabin 2 virus and in the presence of 3(2H)-isoflaven for both resistant and dependent mutants.

**Viral RNA synthesis.** HeLa cell monolayers in 36 mm dishes were infected at an m.o.i. of 25 p.f.u. per cell. After the adsorption period (30 min at 36 °C), the inoculum was removed and 500 µl maintenance medium with actinomycin D (2 µg/ml) was added to inhibit cellular RNA synthesis. At different times after infection, 5 µCi [5,6-³H]uridine per plate was added and the cells were incubated for 1 h at 36 °C, before lysis. Samples (50 µl) were counted in a scintillation spectrometer to measure the incorporation of uridine into trichloroacetic acid (TCA)-precipitable material.
Determination of viral and cellular protein synthesis. HeLa cell monolayers were infected at an m.o.i. of 10 p.f.u. per cell and incubated for 30 min at 36 °C. The inoculum was removed and methionine-free medium was added. After 90 min starvation, 40 µCi/ml [35S]methionine was added and cells were incubated for a further 150 min. The monolayers were washed three times with PBS and the cells were lysed in situ using 0.5 ml 1% NP-40. Incorporation of [35S]methionine into newly synthesized proteins was determined by precipitation of cell lysates using 10% TCA. TCA-insoluble material was collected by filtration onto nitrocellulose filters and radioactivity was determined in a Beckman β-counter.

For electrophoretic analysis, cell lysates were precipitated with 10 vols acetone (20 min at room temperature) and proteins were washed twice with acetone, dried and dissolved in Laemmli buffer. Proteins were then denatured for 3 min at 100 °C and immediately placed on ice. Peptides were resolved by electrophoresis in a 12.5% SDS-PAGE gel in Tris–glycine buffer at 130 V for 16–18 h at 16 °C. Pre-stained proteins were used as molecular mass markers. Gels were fixed, dried and autoradiographed.

In pulse-chase experiments, cells were labelled for 10 min. A 100-fold excess of unlabelled methionine was then added and the cells were harvested at the indicated time-points.

Sucrose gradient analysis. Suspensions of infected HeLa cells (5 x 10⁶) labelled with [35S]methionine were lysed in TNM (10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 1.5 mM MgCl₂) containing 1% sodium deoxycholate, 1% Brij 58 and 0.05 mM PMSF and incubated at 4 °C for 20 min. Cell nuclei were pelleted at 900 g and equal volumes of the resultant cell extracts were analysed on a 15–30% sucrose gradient (Moscufo et al., 1991). The gradients were centrifuged at 4 °C in a Beckman SW 40 rotor for 2.5 h at 39000 r.p.m. Fractions were collected from the top and the radioactivity of each fraction was determined in a scintillation spectrophotometer.

Results

Selection and characterization of drug-resistant mutants

As previously reported (Conti et al., 1990a), the inhibitory effect of 3(2H)-isoflavene on replication of Sabin 2 poliovirus is dose dependent and at a concentration of 20 µM the virus yield is reduced by about 98%. The drug reduced the plaque titre of Sabin 2 virus by more than 10⁴-fold, as measured by the drug-plating index (plaque titre in the presence of drug divided by that in the absence of drug). Under these conditions, rescued viruses were still as drug sensitive as the parental strain and showed the same plaque phenotype. To isolate virus mutants resistant to 3(2H)-isoflavene, subconfluent monolayers of HeLa cells were infected with the progeny from a single plaque of Sabin 2 poliovirus in the presence of the compound, as described in Methods. Seven passages were necessary for plaque isolation of the resistant mutants. Thirty-four plaques were selected randomly from the agar overlay. Virus stocks were prepared in the presence of 3(2H)-isoflavene and virus titres were determined with or without the compound.

Of the 34 mutants isolated, 31 were resistant and three were dependent on 3(2H)-isoflavene. The mutants were grouped into three distinct categories. Twenty-five had approximately the same plaque titre in the presence and in the absence of the drug, yielding drug-plating indices of approximately 1, and were defined as highly resistant. Six had a drug-plating index of 0.001 to 0.01 and were defined as slightly resistant. Their behaviour differed from that of the remaining three mutants, which showed a drug-plating index of 1000 to > 10000; these mutants were defined as drug dependent. One drug-dependent mutant (D1) and one drug-resistant mutant (R1) were characterized in more detail.

Virus growth at different temperatures

As a first step towards determining the target of the antiviral compound, the ability of the mutants D1 and R1 to grow in vitro at different temperatures was tested. Different dilutions of resistant and dependent mutants, and of Sabin 2 virus as a control, were grown in HeLa cells at 33, 36 and 39 °C in the presence and in the absence of 3(2H)-isoflavene. As shown in Table 1(a), each strain tested grew with equal efficiency at 33 and 36 °C, depending on the presence or absence of the drug. None of them grew at 39 °C. In the presence and in the absence of the drug, Sabin 2 and R1 strains showed a smaller plaque phenotype at 33 °C compared with growth at 36 °C. In the absence of the drug the dependent mutant did not show an increase in titre at 33 °C compared with 36 °C. In the presence of the drug, the plaque phenotype of the mutant D1 was smaller than the reference strain Sabin 2, both at 33 and 36 °C.

Revertant mutants of D1 virus grew with the same efficiency in the presence or in the absence of 3(2H)-isoflavene and showed a normal plaque phenotype. Viruses recovered by plating the dependent mutant in the absence of the drug were still dependent but to a lesser extent; the difference in titre in the presence or in the absence of the compound was between 1.5 and 2 log₁₀ p.f.u./ml.

Heat inactivation of virus infectivity

One of the characteristics of most anti-enterovirus compounds, such as the well-studied WINs, is their ability to bind to the virus shell and to inhibit the uncoating step. Indeed, virus mutants resistant to these compounds can escape the binding of the drug to the viral capsid (Heinz et al., 1989, 1990). As we have previously demonstrated, 3(2H)-isoflavene does not inhibit Sabin 2 virus binding, penetration or uncoating (Conti et al., 1988). The ability of the compound to bind to the capsid of D1 and R1 mutants was analysed by incubating the resistant, dependent and parental strains with 20 µM 3(2H)-isoflavene for 1 h at 4 °C and then for 15 min at 36, 42 and 48 °C. As a positive control, the capsid-binding compound WIN51711 was used. As shown in Table 1(b), 3(2H)-isoflavene had no effect on the slight inactivation of the resistant and parental strains at higher temperatures. The dependent mutant was susceptible to heat inactivation and this was strictly correlated to the temperature. At 48 °C in the absence of the drug, a reduction in virus titre of approximately 3 log₁₀ p.f.u./ml was observed compared with the parental
Table 1. Effect of 3(2H)-isoflavene on virus growth and heat inactivation of Sabin 2 virus and drug-dependent (D1) and -resistant (R1) mutants

Data represent titres expressed as p.f.u./ml and are means of three separate experiments. Drug, 3(2H)-isoflavene; WIN, WIN51711.

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>33 °C</th>
<th>36 °C</th>
<th>39 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>− Drug + Drug</td>
<td>− Drug + Drug</td>
<td>− Drug + Drug</td>
</tr>
<tr>
<td>Virus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sabin 2</td>
<td>7·27 × 10⁸</td>
<td>1·09 × 10⁸</td>
<td>8·68 × 10⁸</td>
</tr>
<tr>
<td>D1</td>
<td>8·18 × 10⁸</td>
<td>3·45 × 10⁷</td>
<td>5·00 × 10⁸</td>
</tr>
<tr>
<td>R1</td>
<td>1·18 × 10⁷</td>
<td>8·33 × 10⁷</td>
<td>1·54 × 10⁷</td>
</tr>
</tbody>
</table>

|                  |       |       |       |       |       |       |
| Sabin 2          | 1·68 × 10⁸ | 9·37 × 10⁷ | 1·97 × 10⁸ | 5·30 × 10⁷ | 7·50 × 10⁷ | 8·93 × 10⁷ |
| D1               | 6·04 × 10⁷ | 8·83 × 10⁷ | 8·98 × 10⁷ | 3·11 × 10⁷ | 4·05 × 10⁷ | 8·15 × 10⁷ |
| R1               | 7·65 × 10⁷ | 7·76 × 10⁷ | 8·13 × 10⁷ | 7·25 × 10⁷ | 7·62 × 10⁷ | 7·81 × 10⁷ |

|                  |       |       |       |       |       |       |
|                  | − Drug + Drug + WIN | − Drug + Drug + WIN | − Drug + Drug + WIN |
| Sabin 2          | 1·87 × 10⁷ | 2·47 × 10⁷ | 3·06 × 10⁷ |
| D1               | 2·73 × 10⁷ | 1·95 × 10⁷ | 8·18 × 10⁷ |
| R1               | 6·71 × 10⁶ | 6·81 × 10⁶ | 6·13 × 10⁷ |

Table 2. Single-step growth curves of Sabin 2 virus and drug-dependent (D1) and -resistant (R1) mutants

Data represent means of three separate experiments. Drug, 3(2H)-isoflavene.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Sabin 2 (− Drug + Drug)</th>
<th>D1 (− Drug + Drug)</th>
<th>R1 (− Drug + Drug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6·109</td>
<td>5·680</td>
<td>6·159</td>
</tr>
<tr>
<td>2</td>
<td>5·610</td>
<td>5·530</td>
<td>5·410</td>
</tr>
<tr>
<td>4</td>
<td>7·333</td>
<td>6·290</td>
<td>6·691</td>
</tr>
<tr>
<td>6</td>
<td>7·858</td>
<td>6·468</td>
<td>7·295</td>
</tr>
<tr>
<td>8</td>
<td>7·963</td>
<td>6·468</td>
<td>7·345</td>
</tr>
<tr>
<td>10</td>
<td>8·130</td>
<td>6·550</td>
<td>7·668</td>
</tr>
</tbody>
</table>

The titre of the dependent mutant recovered approximately tenfold in the presence of the drug. Under the same conditions, WIN51711 had a completely protective effect.

Virus yield in single-step growth conditions

The effect of 3(2H)-isoflavene on the yield of virus mutants in single-step growth conditions was determined by infecting HeLa cells in the presence or in the absence of the compound at a concentration of 20 μM. The total yield of the viruses was assessed by plaque assays. In the presence of 3(2H)-isoflavene, the growth of the parental strain decreased by between 1·2 and 1·8 log₁₀ units compared with growth in the absence of the compound. Under the same conditions, the yield of the resistant mutant was unaffected. As expected, the dependent mutant showed a reduction in growth of approximately 4 log₁₀ units when the compound was not added (Table 2). During the first hours of infection the reduction in titre of the resistant and Sabin 2 strains was not affected by the presence of 3(2H)-isoflavene, suggesting that the drug is unable to block the reduction in infectivity (eclipse) that normally occurs immediately after infection.
### Table 3. Effect of 3(2H)-isoflavene on RNA infectivity after transfection and RNA synthesis of Sabin 2 virus and drug-dependent (D1) and -resistant (R1) mutants

<table>
<thead>
<tr>
<th>Virus</th>
<th>RNA infectivity (p.f.u./ml)</th>
<th>2 h p.i.</th>
<th>4 h p.i.</th>
<th>6 h p.i.</th>
<th>8 h p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sabin 2</td>
<td>$5 \times 10^8$</td>
<td>$2 \times 10^4$</td>
<td>$7 \times 10^3$</td>
<td>$12 \times 10^2$</td>
<td>$5 \times 10$</td>
</tr>
<tr>
<td>D1</td>
<td>$1 \times 10^5$</td>
<td>$3 \times 10^3$</td>
<td>$7 \times 10^2$</td>
<td>$3 \times 10$</td>
<td>$3 \times 10$</td>
</tr>
<tr>
<td>R1</td>
<td>$1 \times 10^5$</td>
<td>$8 \times 10^2$</td>
<td>$8 \times 10^1$</td>
<td>$7 \times 10$</td>
<td>$7 \times 10$</td>
</tr>
</tbody>
</table>

* RNA synthesis was measured as total TCA-precipitable radioactivity. Data are $10^{-4} \times [5,6^2H]$uridine incorporated (c.p.m.).

### Cross-reactivity with other flavonoids

Cross-resistance and cross-dependence of both mutants with other flavonoid compounds (derivatives of 4′,6-dichloroflavan) known to be effective against enterovirus infection were studied by plaque assay. The presence of 6-chloro-3(2H)-isoflavene, which is closely related to 3(2H)-isoflavene, did not lead to a change in phenotype of either dependent or resistant mutants. Conversely, both mutants maintained only the resistant phenotype toward 4′-chboro-6-cyanoflavan (data not shown).

### RNA transfection

The lack of effect of 3(2H)-isoflavene on virus binding, penetration and uncoating was confirmed further by experiments on poliovirus RNA transfection. As shown in Table 3, the Sabin 2 parental strain showed a reduction in titre of approximately 4 $log_{10}$ p.f.u./ml when 3(2H)-isoflavene was added. It is interesting to note that the resistant virus R1 showed an increase in titre of about 2 $log_{10}$ p.f.u./ml in the presence of the drug. The difference between each of the three experiments carried out was $\pm 0.2$ $log_{10}$ p.f.u./ml. When the drug was added during the transfection, the dependent mutant D1 showed a rise in virus titre of approximately 5 $log_{10}$ p.f.u./ml compared with when the compound was absent.

### Viral RNA synthesis

To study the effect of 3(2H)-isoflavene on RNA synthesis in the mutants, the kinetics of [5,6,3H]uridine incorporation were measured in cells infected with Sabin 2, D1 and R1, in the presence and in the absence of 3(2H)-isoflavene. Actinomycin D was used to prevent cellular RNA synthesis (Table 3). Viral RNA synthesis was inhibited in cells infected with the Sabin 2 strain in the presence of the drug, while in untreated infected cells, it reached a peak 4 h post-infection (p.i.). Synthesis of RNA occurred in cells infected with the resistant mutant with and without 3(2H)-isoflavene addition. In the absence of the compound, a decrease of approximately 30% in total uridine incorporation was observed (at 6 h p.i., which represents the time of maximum RNA synthesis for these mutants). Interestingly, in the absence of the compound, the dependent mutant showed a low level of RNA synthesis. When 3(2H)-isoflavene was added, RNA synthesis reached levels comparable to those of the resistant mutant and Sabin 2 reference strains. In the presence of the compound, a delay of about 2 h in the peak of uridine incorporation was observed in the dependent and resistant mutants compared with the parental virus. At the concentration of 20 $\mu$M 3(2H)-isoflavene, cellular RNA synthesis was not affected (data not shown).

### Virus-induced shut-off of host translation

Protein translation in cells infected by both mutants was monitored by measuring the total [35S]methionine incorporation at different times p.i., in the presence or in the absence of 3(2H)-isoflavene. In cells infected with the Sabin 2 parental strain, addition of the compound at the beginning of infection completely prevented the shut-off induced by the virus (Fig. 1).
When the drug was added soon after the infection this effect was reduced, and it progressively decreased, completely disappearing at 90 min p.i. In contrast, the D1 and R1 mutants induced a complete inhibition of cellular protein synthesis, irrespective of when the drug was added. Notably, $[^{35}\text{S}]$-methionine incorporation in D1-infected cells was 50 and 60\% lower than that found in Sabin 2 and the mutant R1, respectively (Fig. 1).

**Viral protein synthesis**

To determine whether 3(2H)-isoflavene affected the RNA translation of the mutants, protein synthesis was examined by SDS–PAGE of extracts from infected-cell cultures continuously labelled with $[^{35}\text{S}]$methionine (2.5–5 h p.i.) in the absence or in the presence of the compound. The results are shown in Fig. 2. By comparing lanes 4, 6 and 8, it can be seen that 3(2H)-isoflavene completely inhibited the protein synthesis of Sabin 2 poliovirus but it did not affect the protein synthesis of either mutant, and normal processing of protein precursors was clearly noted. Interestingly, in the absence of the drug, the capsid protein VP2 was not present in the dependent mutant (lane 5).

Protein processing of the mutants and the Sabin 2 reference strain was investigated further by $[^{35}\text{S}]$methionine pulse–chase labelling of infected cells harvested at different times p.i. The results in Fig. 3 show that, in the absence of the drug, a characteristic pattern of virus-specific proteins was synthesized in Sabin 2 virus-infected cells (Fig. 3A, lanes 5–9): normal proteolytic processing of P1, P2 and P3 precursors occurred and the mature capsid proteins were formed. Lanes 10–15 clearly show that, in the presence of 3(2H)-isoflavene, complete inhibition of viral protein synthesis occurred. Furthermore, the compound abolished the shut-off of host-cell protein synthesis. As shown in Fig. 3 (B), lanes 4–9, the dependent mutant D1 showed normal proteolytic processing of the P2 and P3 precursors in the absence of the drug. The precursor P1 was cleaved to VP0, VP1 and VP3, but VP0 was not cleaved to form its final products, VP2 and VP4; this led to an accumulation of VP0, as revealed by gel electrophoresis. When 3(2H)-isoflavene was present (lanes 10–15), proteolytic processing was unaffected. The resistant mutant R1 showed normal synthesis of all viral proteins and a clear proteolytic processing, both in the absence (Fig. 3C, lanes 4–9) and in the presence (lanes 10–15) of 3(2H)-isoflavene.

The effect of the timing of drug addition on polyprotein processing of the D1 mutant is shown in Fig. 4. Addition of the drug at times from the beginning of infection until 220 min p.i. resulted in normal synthesis of viral proteins and correct proteolytic processing (lanes 4–9). VP0 was not cleaved when the drug was added 4 h p.i. or later (lanes 10–12). This is consistent with data on the recovery of virus yield, as measured under the same experimental conditions (data not shown).

**Sucrose gradient analysis**

The lack of cleavage of precursor VP0 in the dependent mutant in the absence of 3(2H)-isoflavene was further evidenced by sucrose gradient analysis of assembly intermediates with extracts from HeLa cells infected with this mutant in the presence or in the absence of the compound. The resistant and parental strains were used as controls. The profile of TCA-precipitated radioactivity found in the 160S and 75S peaks of the sucrose gradient is shown in Fig. 5. Both resistant and Sabin 2 reference strains displayed a pattern of mature virus in the absence of the compound, with only 160S particles being
Resistant and dependent poliovirus mutants

Fig. 3. Effect of 3(2H)-isosflavene on the processing of viral proteins in poliovirus-infected cells. HeLa cells were infected with poliovirus Sabin 2 reference strain (A), dependent mutant D1 (B) and resistant mutant R1 (C) in the absence (lanes 4–9) or in the presence (lanes 10–15) of 3(2H)-isosflavene. Proteins (lanes 4–15) were pulse-labelled for 10 min with [35S]methionine (40 µCi/ml) at 3–5 h p.i., after which a 100-fold excess of 20 mM methionine was added. Cells were lysed with 1% NP-40 after 0 (lanes 4 and 10), 30 (5 and 11), 60 (6 and 12), 90 (7 and 13), 120 (8 and 14) or 150 min (9 and 15). Samples representing equivalent volumes of cell lysates were resolved on a 12–5% SDS–PAGE gel and autoradiographed as described in Methods. Lanes 1, mock-infected cells; 2, mock-infected cells with 20 µM 3(2H)-isosflavene; 3, infected-cell control.

Discussion

Studies on the mechanisms of action of antiviral compounds and the analysis of drug-resistant mutants play a key role in molecular virology. Indeed, identification of the antiviral target has been very useful in understanding some steps of virus infection and replication (Pincus et al., 1986; Pincus & Wimmer, 1986; Mosser & Rueckert, 1993; Shepard et al., 1993). In this study, we have analysed the target of 3(2H)-isosflavene, known to be an inhibitor of the early stages of picornavirus infection, through the isolation and biological characterization of poliovirus type 2 dependent and resistant mutants.

A feature of poliovirus and other single-stranded RNA viruses is the accumulation of spontaneous mutations at a frequency of $10^{-4}$ to $10^{-5}$ per round of replication in cell culture (Holland et al., 1982). Single amino acid substitutions, which can induce resistance to an antiviral compound, are normally selected after a single round of growth in the presence of the selecting agent (Sherry et al., 1986; Heinz et al., 1989). In the case of 3(2H)-isosflavene, which almost completely inhibits replication of Sabin 2 virus at a concentration of 20 µM (Conti et al., 1990a), none of the residual virus was resistant to the drug after a single passage. Consequently, we used a multi-step procedure consisting of serial reinfections by the virus grown in the presence of increasing concentrations of the drug. This procedure allows the enrichment of virus variants carrying multi-site mutations and stimulates the production of drug-dependent mutants (Heinz & Vance, 1995). We were able to isolate several mutants, two of which (i.e. R1, highly resistant, and D1, dependent) were biologically characterized to clarify the target of the drug further.

We have shown previously that 3(2H)-isosflavene does not bind to the poliovirus capsid or stabilize capsid conformation (Conti et al., 1990a). This is consistent with the observation that the reduction in stability of the dependent mutant during heat-inactivation experiments was only slightly recovered by the presence of 3(2H)-isosflavene. In contrast, WIN51711, a compound that stabilizes the virus particle by inhibiting
Fig. 4. Effect of 3(2H)-isoflavene, added at different times p.i. at 20 µM, on protein synthesis and processing in the dependent mutant D1. Proteins were radiolabelled as described in the legend to Fig. 1. Samples representing equivalent volumes of cell lysates were resolved on a 12.5% SDS–PAGE gel. Lanes 1, mock-infected cells; 2, mock-infected cells with 20 µM 3(2H)-isoflavene; 3, infected cells without 3(2H)-isoflavene; 4–12, infected cells with 20 µM 3(2H)-isoflavene added at the times indicated.

Revertant viruses of the dependent mutant, which recovered their ability to grow in the absence of the drug, presented an almost normal plaque phenotype. Investigation of cross-reactivity of the mutants with other flavonoids related to 3(2H)-isoflavene indicated that dependence was strictly related to chemical residues present in the compounds that affect conformation. The planarity of the isoflavene molecule, due to the presence of a double bond in the oxygenated ring, probably allows these compounds to counterbalance the effect of the mutations responsible for the dependent phenotype.

Studies of virus yields after a single growth cycle in the dependent mutant demonstrated a link between viral RNA synthesis and production of infectious virus particles. In the
absence of the drug, a drastic reduction was observed both in terms of RNA (approximately 70%) and virus particle production (about 4 log_{10} p.f.u./ml). In the resistant mutant, no differences were noted in the production of virus particles after a single growth cycle in the absence or in the presence of the drug, even though a 30% reduction in RNA synthesis was observed in the absence of the drug. The amount of RNA synthesized was probably sufficient to induce the maturation of virus particles. It is interesting to note that D1 (in the presence of the drug) and R1 (both in its presence and absence) showed a delay of about 2 h in the peak of uridine incorporation compared with the Sabin 2 virus. This suggests the possible involvement of the viral polymerase in the establishment of the resistant and dependent phenotypes, although other virus-encoded proteins and some cellular proteins have been reported to be implicated in viral genome replication (Johnson & Sarnow, 1995; Roehl et al., 1997).

Rescued viruses from the D1 mutant grown in the absence of the drug still showed a dependent phenotype, indicating that multiple mutations are probably present in this mutant.

Co-infection experiments performed with the D1 and Sabin 2 virus strains with and without the antiviral compound showed that no complementation occurred between the two viruses, suggesting that viral and/or cellular cis-acting factors may play a role in the development of the dependent and/or resistant phenotype(s). This is further supported by transfection experiments that allowed the direct effect of the drug on the infectivity of RNA to be studied, bypassing the first stages of poliovirus infection (adsorption, penetration and uncoating). The results obtained from these experiments confirm that the RNA of the mutant D1 was only able to produce infectious particles when transfected in the presence of 3(2H)-isoflavene: conversely, in HeLa cells transfected with Sabin 2 RNA, virus production was reduced in the presence of the compound.

The clear shut-off of host translation observed during D1 and R1 infections, both in the presence and in the absence of 3(2H)-isoflavene, indicates that the synthesis of the viral polyprotein and its cleavage occurred normally. Indeed, viral protein 2A, which seems to be responsible for the inhibition of cellular protein synthesis through cleavage of the cellular factor elf-4G, was active with and without the compound (data not shown).

Analysis of the translation of D1 viral proteins demonstrated that in the absence of the drug the precursor VP0 was not cleaved, with the VP2 protein consequently being absent. This was confirmed by sucrose gradient analysis of the assembly intermediates, which showed the presence of only 75S empty capsids. Addition of the drug re-established normal processing of the polyprotein, leading to the formation of 160S mature virions. The kinetic study of drug addition showed that a normal proteolytic pattern and virus recovery occurred when 3(2H)-isoflavene was added within 220 min of the beginning of the infection. Addition of the compound later in infection inhibited the processing of VP0 protein and the development of infectious virus particles. Our results indicate that the reduction in RNA synthesis observed in D1-infected cells in the absence of the drug could be responsible for the prevention of maturation of virus particles, suggesting that a regulatory mechanism exists between RNA synthesis and encapsidation in the replicative intermediate complex. In fact, the mechanism of cleavage of VP0 into VP2 and VP4 is probably autocatalytic, since the cleavage site formed by the C-terminal asparagine residue of VP4 and the N-terminal serine of VP2 is not recognized by the viral proteases 2A or 3CD, and the cleavage probably involves basic groups from the viral RNA serving as proton acceptors (Arnold et al., 1987). The low level of RNA synthesis in the D1 mutant is probably not sufficient to trigger RNA encapsidation, which did occur in the resistant mutant. The dependent mutant could therefore be a useful model to study this mechanism more closely.

As expected, the resistant mutant maintained a normal rate of protein synthesis and processing, both in the presence and in the absence of 3(2H)-isoflavene, but the maturation of infectious virus particles (160S) was enhanced in the presence of the drug. This is further supported by the 30% increase in RNA synthesis observed in this mutant in the presence of the drug, as well as in the production of virus particles after RNA transfection.

The effect of 3(2H)-isoflavene on poliovirus Sabin 2 infection seems to take place in the very early stages of virus infection. The mechanism of action of 3(2H)-isoflavene on the poliovirus type 2 replication cycle could be through a blockage of poliovirus translation by interaction with viral RNA or with cellular or viral proteins involved in recognition and utilization of the internal ribosome entry site structural domains. Our results demonstrate that some step(s) in RNA production is involved in the establishment of the dependent phenotype. Virus replication cannot begin until the polymerase gene has been translated and the minimum time that elapses between uncoating and the beginning of viral RNA synthesis is about 15 min (Rueckert, 1996). Taking into account the observations that addition of the drug from the beginning of infection until 220 min p.i. led to complete development of infectious virus in the D1 mutant and that the translation of viral proteins was not involved in establishment of the dependent and resistant phenotypes, we hypothesize that some step(s) in the metabolic cascade between RNA translation and synthesis could be implicated in the development of these phenotypes. Sequencing of these resistant and dependent mutants is in progress and will elucidate the mechanism of action of the drug more clearly, through identification of the mutations responsible for conferring the resistant and dependent phenotypes and of the viral protein which interacts with the drug.

We would like to thank Y. V. Svitkin for critical reading of the manuscript. This study was partially supported by grants from the Istituto Superiore di Sanità ("Prevention of risk factors of maternal and
child health’, art. 12 D.L. 502/92) and the WHO (‘Characterization of polio and other enteroviruses associated with paralytic disease in Italy, Albania and Malta’, I8/181/211, BH/Ir 1996-1997).

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Received 12 May 1998; Accepted 2 September 1998