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Mode of action of 2-furylmercury chloride, an anti-rhinovirus compound

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Abstract

2-Furylmercury chloride (2-FMC), an organic mercury derivative, has been found to inhibit the replication of all tested human rhinovirus (HRV) serotypes belonging to the antiviral group B and a limited number of HRV serotypes belonging to the antiviral group A. The mechanism of action of 2-FMC was tested against HRV-2 (antiviral group B, minor receptor group), and compared with an antiviral compound for which the viral target was already determined (enviroxime). 2-FMC was found to bind reversibly to virus particles. However, time-dependent plaque reduction assays revealed that 2-FMC did not interfere with early events of HRV-2 replication.

Using a quantitative RT-PCR ELISA assay, we were able to prove that 2-FMC inhibits the synthesis of viral RNA. However, the mode of action of 2-FMC is not identical to that of enviroxime, another inhibitor of viral RNA synthesis. Time-of-addition and time-of-withdrawal experiments demonstrated that 2-FMC acted during a broader time interval than enviroxime.

Keywords: Anti-rhinovirus drug; 2-Furylmercury chloride; Viral RNA synthesis inhibitor

1. Introduction

Human rhinoviruses (HRVs) are a major cause of the common cold in humans. They represent a large genus (more than 100 serotypes) within the picornaviridae, a virus family that contains many human and animal pathogens. Several antiviral compounds, such as pirodavir and pleconaril, have been shown to inhibit members of the picornavirus family by binding to viral capsid proteins (Andries et al., 1992; Pevear et al., 1999). Other anti-picornaviral compounds, however, seem to exert their antiviral activity through interactions with non-structural viral components. The array of possible targets and mechanisms used by different picornaviral inhibitors have already been extensively reviewed (Korant et al., 1983; Carrasco, 1994; Andries, 1995), but constantly new compounds have been emerged.

In preparation of a Phase I study using pirodavir, we tested the clinical samples and were surprised to discover some antiviral activity in a placebo preparation. Upon analysis of all individual components, we could attribute the antiviral activity to thimerosal, a mercury-containing compound used as a preservative in many pharmaceutical preparations. Upon testing of a few other mercury-containing compounds from chemical suppliers, we identified a more potent inhibitor, 2-furylmercury chloride (2-FMC). In this work, we have studied the antiviral spectrum of this compound and were able to delineate its mechanism of action.

2. Materials and methods

2.1. Viruses and cells

HRV-2 (D. Blaas, University of Vienna, Austria) was grown on HeLa monolayer cells as described elsewhere (Skern et al., 1984).

2.2. Antiviral compounds

2-Furylmercury chloride and enviroxime were kindly provided by the Johnson and Johnson Pharmaceutical R&D (Beerse, Belgium). Stock solutions (1 mg/ml) of the antiviral compounds were made in dimethyl sulfoxide (DMSO) and were subsequently diluted in the appropriate culture media. The final DMSO concentration was maximum 0.1% and it was shown that this concentration had no effect on the cell cultures. Therefore, 0.1% DMSO was also added to all no-drug control samples. Unless stated otherwise, final concentrations of 0.1 μg/ml 2-furylmercury chloride (2-FMC) and 1 μg/ml enviroxime (ENV) were used in all experi-

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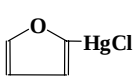
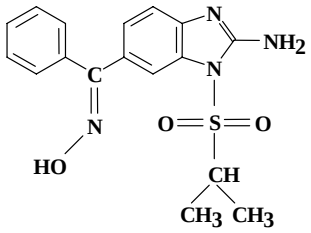


2-Furylmercury Chloride



Enviroxime

Fig. 1. Chemical formulas.

ments. For the chemical formulas of the compounds, see Fig. 1.

2.3. Time-of-addition assay

HeLa cell monolayer cultures were infected with HRV-2 (60 plaque forming units/petri-dish) and immediately incubated in drug-free medium at 34 °C. At specified times following infection, the drug-free medium was replaced with medium containing either 2-FMC (0.1 μg/ml), ENV (1 μg/ml) or no drug. All cell cultures were further incubated at 34 °C for the remaining time of the viral replication cycle, i.e., until 7 h 30 min post-infection (p.i.). To remove the drugs, the cultures were immediately rinsed three times with cold PBS buffer (pH 7.4) after incubation. Finally, an overlay was applied in order to measure the viral infectivity via a plaque assay.

2.4. Time-of-removal assay

HeLa cell monolayer cultures were infected with HRV-2 (60 plaque forming units/petri-dish) and immediately incubated at 34 °C in medium containing either 2-FMC (0.1 μg/ml), ENV (1 μg/ml) or no drug. At various times p.i., the cell cultures were rinsed three times with cold PBS buffer (pH 7.4) in order to remove the drugs, and were further incubated at 34 °C in drug-free medium. After one replication cycle (approximately at 7 h 30 min p.i.), the medium was replaced by an overlay and viral infectivity was measured via a plaque assay.

2.5. Reversibility of inhibition and comparison of 2-FMC activity with enviroxime activity

To determine whether the effects of 2-FMC and enviroxime were readily reversible, 2-FMC (0.1 μg/ml) and ENV (1 μg/ml) were added to HRV-2-infected HeLa cell monolayer cultures (MOI of 10) immediately after adsorption (i.e., 1 h 30 min p.i.). At 4 h 30 min p.i., cultures were rinsed three times with cold PBS buffer (pH 7.4) and the medium was replaced with either drug-free or drug-containing medium. Virus was harvested at 9 h p.i. and yields were determined by a plaque assay. To define whether 2-FMC blocks an earlier or a later step in the viral life cycle than ENV, infections were carried out as described for the reversal of inhibition except that instead of receiving drug-free medium, 2-FMC-treated cultures received medium containing ENV and vice versa. Drug-free cultures that received either 2-FMC or ENV at 4 h 30 min were also included in the experiment.

2.6. Analysis of viral protein synthesis and post-synthetic cleavages

HeLa cell monolayer cultures in methionine-deficient medium were infected with HRV-2 at a MOI of 0.5. Immediately after infection, 2-FMC was added in different concentrations. At 4 h p.i., cell cultures were radiolabeled with (Tran^{35}S-label^{TM}) and incubation was continued at 34 °C for another 20 h. Cell lysates were prepared by adding a cell lysis buffer (0.1% Nonidet P-40, 0.5% desoxycholate and 0.05% bovine serum albumine in PBS buffer, pH 7.4) to the cultures. Cellular debris was removed by a low-speed centrifugation and viral proteins in the supernatant were immunoprecipitated with rabbit antiserum against HRV-2. The samples were boiled in Laemmli sample buffer and the proteins were analysed by SDS-PAGE in 12.5% slabgels (0.1% SDS). Viral proteins were finally visualised by fluorography. Immunoprecipitation, SDS-PAGE and fluorography were as described previously (Vrijsen et al., 1983; Verlinden et al., 2000).

2.7. Positive-strand RNA synthesis detection

An in-house developed quantitative RT-PCR ELISA detection method was used to measure positive-strand HRV-2 RNA synthesis in virus-infected cells (Lauwers et al., 2001, 2002; Verheyden et al., 2003).

Briefly, HeLa cell monolayer cultures were infected with HRV-2 (MOI: 225) and incubated at 34 °C in drug-free or drug-containing (1 μg/ml ENV or 0.1 μg/ml 2-FMC) medium. At different time points (i.e., at 0, 2, 4, 6, 8 and 10 h p.i.), cell cultures were rinsed three times with cold PBS buffer (pH 7.4) and cell lysates were subsequently prepared by adding a cell lysis buffer (0.1% Nonidet P-40, 0.5% desoxycholate and 0.05% bovine serum albumine in PBS buffer, pH 7.4) to the cultures. After removing the cellular debris, total RNA was extracted from the cell lysates by using a RNA isolation kit (PURESCRIPT® RNA Isolation Kit, Gentra systems, Minneapolis, MN). The obtained RNA pellets were resuspended and diluted in RNase and DNase free water (Sigma Chemical Co., St. Louis, MO). A quantitative RT-PCR ELISA analysis was performed as described previously (Verheyden et al., 2003) with a HRV-2 specific primer set and probe. The sequences of both primers and probe used, are all situated in a 120-bp segment within the 5′ untranslated region of the HRV-2 genome, a region known to be highly conserved among HRVs (Halonen et al., 1995). The downstream primer (5′-GAAACACGGACACCCAAAG-3′) and the 3′

digoxigenin-labeled capture probe (5′-GCTGCAGGGTT-AAGGTTAGCC-3′) are antisense to genomic HRV-2 RNA. The 5′ biotin-labeled upstream primer (5′-TCCTCCGGCC-CCTGAATG-3′) is sense to genomic HRV-2 RNA. Both primers and probe were purchased at Pharmacia Biotech (Uppsala, Sweden).

3. Results

3.1. Spectrum of activity of 2-FMC

A systematic evaluation of a group of capsid-binding agents (fifteen compounds) against all serotyped HRV revealed the existence of two antiviral groups, A and B, based upon differential susceptibility to these antiviral compounds (Andries et al., 1990). Seventeen representative rhinovirus serotypes can be used to discriminate between antiviral groups A and B (Andries, 1995). The activity of 2-FMC against these seventeen serotypes is summarised in Table 1.

The data show clearly that 2-FMC is primarily active against HRV serotypes belonging to the antiviral group B (IC_{50} values $\leq 0.050\ \mu M$). Only one serotype (i.e., HRV-45) belonging to the antiviral group A shows a low IC_{50} value, indicating that 2-FMC preferentially exerts its antiviral activity against antiviral group B HRVs.

The high selectivity was also confirmed by testing 2-FMC against other picornaviruses (such as poliovirus and Coxsackie virus), and non-picornaviruses such as herpes simplex virus (HSV), respiratory syncytial virus (RSV) and influenzavirus. No antiviral activity was found (results not shown).

Table 1
Activity of 2-FMC against a representative panel of HRV serotypes

	Antiviral activity of 2-FMC[a,b]
Antiviral group A	
HRV-14	>158
HRV-42	>158
HRV-45	0.025
HRV-70	>158
HRV-72	>158
HRV-86	2.510
Antiviral group B	
HRV-2	0.020
HRV-9	0.020
HRV-15	0.025
HRV-29	0.025
HRV-39	0.050
HRV-41	0.025
HRV-51	0.025
HRV-59	0.032
HRV-63	0.025
HRV-85	0.040
HRV-89	0.025

[a] The antiviral activity of 2-FMC is expressed as IC_{50} (μM).
[b] Each IC_{50} is the mean of two to four experiments.

In further experiments, HRV-2 was chosen as target virus because this serotype is very sensitive to 2-FMC (Table 1). It is also the best studied virus within the minor receptor group HRVs, and serotypes belonging to the minor receptor group HRVs are all part of antiviral group B (Andries et al., 1990).

The final concentration of 2-FMC used in all experiments was 0.1 μg/ml (0.39 μM). This concentration was chosen, because it fitted well between the IC_{50} value of 2-FMC against HRV-2 (0.020 μM, see Table 1) and the 50% cytotoxic concentration of 2-FMC on HeLa cells, which was approximately 3.9 μM. Below this 3.9 μM concentration, no cytostatic effects or inhibition of macromolecular synthesis in the uninfected HeLa cells were detected (results not shown). This suggests that non-specific inhibition does not account for the antiviral activity of 2-FMC.

3.2. Effect of antiviral compounds on HRV-2 replication steps

We first decided to determine the time period within the viral replication cycle in which 2-FMC showed antiviral activity. Therefore, two independent experiments were performed: (i) a time-of-addition assay in which the antiviral compound was added to HRV-2-infected cell cultures at different time points p.i. and was subsequently removed after the completion of one viral replication cycle (Fig. 2A), and (ii) a time-of-removal assay in which 2-FMC was added at the onset of viral infection and was subsequently removed at various times p.i. (Fig. 2B). In both assays, ENV was included because it was already shown that this drug targets a step in the middle of the replication cycle of HRV-14, i.e., the viral RNA replication (Heinz and Vance, 1995).

The time-of-addition experiments showed that 2-FMC inhibits replication completely when added before 4 h 30 min p.i. However, 2-FMC still exerted activity when added 5 h 30 min p.i. (80% inhibition) and 6 h 30 min p.i. (60% inhi-

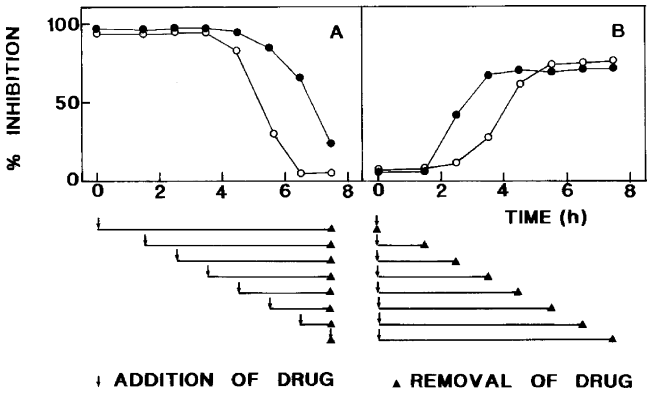


Fig. 2. Inhibitory effect of 2-FMC (●) and ENV (○) added to (A) or removed from (B) HRV-2-infected cells at different time points p.i. For more details on infection and incubation conditions, see Section 2. After one replication cycle, viral yields were determined by plaque assay. Values shown are relative to no-drug controls at each time point.

bition). ENV inhibited replication completely when added 3 h 30 min p.i. and showed also a significant activity at 4 h 30 min p.i. (80% inhibition) (Fig. 2A).

The time-of-removal experiments showed that 2-FMC exerted no activity when the compound is present between the time of infection until 1 h 30 min p.i. The first activity of 2-FMC was seen when the drug was present between the time of infection until 2 h 30 min p.i. and full inhibition was observed when the drug was present between the time of infection until 3 h 30 min p.i. ENV showed its first detectable activity when added between the time of infection until 2 h 30 min p.i. and showed almost complete activity when kept on the cells until 4 h 30 min p.i. (Fig. 2B).

Taken together, 2-FMC is active over a rather broad period of time, which is 2 h 30 min to 5 h 30 min p.i. This replication period includes the peak period of viral protein and RNA synthesis. Thus, 2-FMC does not act upon the early viral replication steps such as adsorption, entry and uncoating. The experiments with the control inhibitor ENV confirm that this drug targets a replication step in the middle of the replication cycle (activity in a time period between 3 h 30 min and 4 h 30 min p.i.). This time period is, however, narrower than for 2-FMC.

3.3. Reversal of inhibition and comparison of 2-FMC activity with ENV

As 2-FMC and ENV showed an antiviral activity within the same time period, we decided to compare the activity of 2-FMC and ENV more closely. First, we examined whether the effects of both drugs were reversible in culture. Midway through the infectious cycle (4 h 30 min p.i.), we rinsed the cells with buffer and we incubated the infected cells further in drug-free medium. Results clearly showed that the activities of both 2-FMC and ENV were indeed reversible under the conditions used (Table 2; compare results 2-FMC/2-FMC and ENV/ENV with, respectively, 2-FMC/control and ENV/control). Then, we investigated the relation between both compounds by determining whether 2-FMC blocks an earlier or a later step in the HRV-2 replication cycle than ENV. To this end, both compounds and the DMSO control were added to the virus-infected cells in different sequential order during one viral life cycle. The results for the control/2-FMC–2-FMC/control and control/ENV–ENV/control combinations indicated that 2-FMC exerts a higher antiviral activity during the first half of viral infection, whereas ENV exerts its antiviral activity mainly during the second half of the viral replication cycle (Table 2). These findings indicate that both compounds use a different mechanism to inhibit HRV-2 replication. The high drop in infectivity observed for combinations between 2-FMC and ENV even strengthens this hypothesis, because it suggests that both compounds have an additive effect.

Table 2
Reversibility of antiviral activity and comparison of 2-furylmercury chloride (2-FMC) activity with enviroxime (ENV) activity

Order of addition of antiviral compounds (initial compound/replacement compound)[a]	Infectivity ($\log_{10}$ PFU/ml)	Drop in infectivity ($\log_{10}$ PFU/ml) compared to control
Control/control	8.6	–
2-FMC/2-FMC	5.7	2.9
ENV/ENV	4.6	4.0
2-FMC/control	6.6	2.0
ENV/control	7.6	1.0
Control/2-FMC	7.0	1.6
control/ENV	5.4	3.2
2-FMC/ENV	4.5	4.1
ENV/2-FMC	4.7	3.9

[a] HRV-2-infected cells (MOI: 10) were incubated in medium containing either DMSO (0.1%), 2-FMC (0.1 μg/ml) or ENV (1.0 μg/ml) for 4 h 30 min at 34 °C. Cultures were rinsed three times with cold PBS buffer (pH 7.4) and the media were replaced as indicated. Virus yields were harvested at 9 h p.i. and quantified by a plaque assay.

3.4. Influence of 2-FMC on HRV-2 protein synthesis and post-synthetic cleavages

The interval in which 2-FMC exerts its antiviral activity, i.e., a time period between 2 h 30 min and 5 h 30 min p.i., correlates with the peak period of HRV-2 translation and transcription in HeLa cells. In a cell infected with a picornavirus, viral transcription and viral translation are synchronised (Koch and Koch, 1985; Verlinden et al., 2002). However, as viral translation (directly from the positive-strand RNA) is necessary to synthesise the RNA-dependent RNA polymerase (required for viral transcription), we decided first to study the effect of 2-FMC on viral protein synthesis and on post-synthetic cleavages. Protein synthesis was studied by radiolabeling proteins synthesised in HRV-2-infected cells. 2-FMC was added to the cells in different concentrations as fully described in Fig. 3. After radiolabeling, protein synthesis, as well as post-synthetic cleavages, were examined by polyacrylamide gel electrophoresis after the radiolabeled proteins were im-

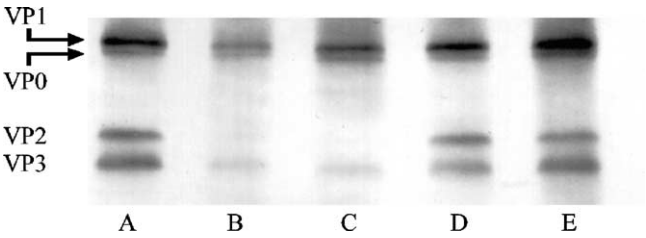


Fig. 3. Influence of 2-FMC on HRV-2 protein synthesis and post-synthetic cleavages in the infected cell. HeLa cell monolayer cultures were infected with HRV-2 at a MOI of 0.5. Immediately after infection, 2-FMC was added in different concentrations: 100 ng/ml (lane B), 50 ng/ml (lane C), 10 ng/ml (lane D) or 5 ng/ml (lane E). One cell culture received no 2-FMC (lane A). Radiolabel was added 4 h p.i. and incubation was continued at 34 °C. At 24 h p.i., cells were harvested. Lysates were prepared and de novo synthesised viral proteins were determined by immunoprecipitation and SDS-polyacrylamide gel electrophoresis followed by fluorography.

munoprecipitated with a polyclonal serum recognising all structural proteins (and precursors) of HRV-2.

The results are shown in the fluorogram (Fig. 3). HRV-2 protein synthesis is clearly, but not totally inhibited by 2-FMC, when 2-FMC is present in the virus-infected cell cultures at concentrations higher than the IC_{50} (Fig. 3, compare lanes B–D with lanes A and E).

It is also obvious that 2-FMC does not inhibit the post-synthetic cleavages, because whenever viral proteins are synthesised, end products of the post-synthetic cleavages, such as the capsid proteins, were detected (Fig. 3, lane B–D).

3.5. Inhibition of viral RNA synthesis

Several virus-encoded non-structural proteins are involved in HRV-2 RNA synthesis (Heinz and Vance, 1995). However, a key role is played by the viral-encoded polymerase $3D^{pol}$. This RNA-dependent RNA polymerase first copies input positive-strand RNA (viral genome) into negative strand RNA, which, in turn, serves as a template for the synthesis of viral progeny positive strands from a multistranded replicative intermediate (RI) (Barton and Flanegan, 1997). Within picornavirus-infected cells, transcription is normally heavily biased toward synthesis of positive-strand RNAs (both viral and genomic mRNA) and this greatly complicates the detection of negative strand RNA of positive-stranded RNA viruses (Novak and Kirkegaard, 1991).

The possible inhibitory effect of 2-FMC on the synthesis of plus strand HRV-2 RNA was examined by an in-house developed quantitative rhinovirus specific RT-PCR ELISA method (Lauwers et al., 2001, 2002; Verheyden et al., 2003). ENV was used as a positive control, because it has been shown previously that this drug preferentially inhibits synthesis of the positive strand by targeting the 3A coding region of rhinovirus and poliovirus (Heinz and Vance, 1995).

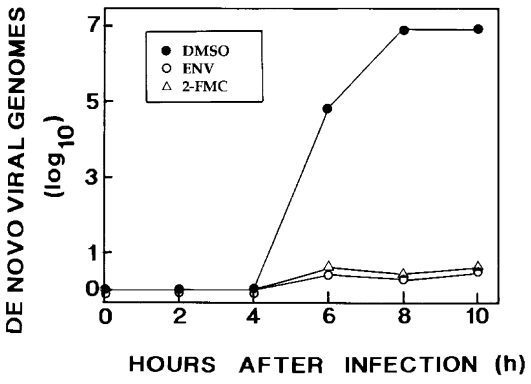


Fig. 4. Effect of 2-FMC and ENV on HRV-2 positive-strand RNA synthesis in function of time. HeLa monolayer cells were infected with HRV-2 (MOI: 225) and incubated at 34 °C in drug-free (0.1% DMSO) and drug-containing (0.1 μg/ml 2-FMC or 1.0 μg/ml ENV) medium. Total RNA was harvested at 2 h intervals (i.e., at 0, 2, 4, 6, 8 and 10 h p.i.) and subjected to RT-PCR. Detection of the PCR products was performed with an ELISA assay using a digoxigenin-labeled capture probe.

In Fig. 4, it is shown that in untreated infected cells, HRV-2 RNA synthesis starts between 4 and 6 h p.i. At 8 h p.i., a maximum of RNA copies is synthesised. In ENV-, but also in 2-FMC-treated infected cells, there is a complete inhibition of positive-strand viral RNA synthesis. This finding shows that 2-FMC exerts its antiviral action by inhibiting the viral RNA synthesis step during HRV-2 replication in cells.

4. Discussion

There are several approaches to identify antiviral compounds. Very attractive approaches are the screening of large series of chemical compounds in several in-vivo and in-vitro screening tests or the rational drug design. However, serendipity has always been an important factor in the development of science. Also in this case: the first compound (thiomersal) found to possess antiviral activity in this study, was incidentally identified in a placebo preparation. Subsequently, a screening of further mercury-containing compounds was undertaken that led to a more potent inhibitor, 2-FMC, for which the spectrum and antiviral target is studied in this paper.

As shown in Table 1, 2-FMC possesses an antiviral activity primarily against HRV serotypes belonging to the antiviral group B and serotypes belonging to this antiviral group account for five times as many colds, as compared to serotypes belonging to antiviral group A (Andries et al., 1990). 2-FMC possesses even an activity against one serotype (HRV-45) belonging to antiviral group A. This spectrum of activity was very promising, but given the presence of a mercury atom in this antiviral compound, 2-FMC itself has no chance of becoming a useful drug to treat a common cold. However, identification of a new antiviral target can lead to new approaches in the discovery of antiviral drugs. Moreover, antiviral compounds can be used as a tool in the molecular analysis of viral functions and even as an aid in virus classification.

The antiviral spectrum of 2-FMC on the one hand, and the reversible binding to virus particles on the other hand were suggestive for a mechanism of action similar to capsid-binding compounds (i.e., pirodavir or pleconaril). However, time-dependent plaque reduction assays made it clear that 2-FMC possessed no activity towards the first replication steps of HRV-2, such as adsorption or uncoating (Fig. 2). Moreover, results suggested that 2-FMC is active in the middle of the replication cycle and this over a rather broad time interval (2 h 30 min–5 h 30 min p.i.), a time period including the viral protein and viral RNA synthesis. ENV, which was used as a reference compound and which targets the viral positive-strand synthesis (Heinz and Vance, 1995), possessed an activity in the same time period, but a more narrow one (3 h 30 min–4 h 30 min p.i.).

Finally, using an in-house developed quantitative rhinovirus specific RT-PCR ELISA method (Verheyden et al.,

2003), we could demonstrate that 2-FMC inhibits the HRV-2 positive-strand RNA synthesis.

In further experiments, the exact antiviral target of 2-FMC will be elucidated using escape mutants selected against 2-FMC and also against ENV. We stress that the antiviral target of ENV is the viral protein 3A, a non-structural viral protein (Heinz and Vance, 1995).

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