Hydantoin: the mechanism of its *in vitro* anti-enterovirus activity revisited

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Abstract

It has been generally accepted that hydantoin [5-(3,4-dichlorophenyl)methylhydantoin] exerts its anti-enterovirus activity by solely inhibiting viral assembly. However, we here show that hydantoin inhibits enteroviral RNA synthesis as well as subgenomic replication in a dose-dependent manner. We demonstrate that inhibition of RNA synthesis is the predominant mechanism of action at relatively high concentrations of hydantoin. However, at lower concentrations inhibition of viral morphogenesis is the main mechanism of action. Thus, hydantoin inhibits enteroviral replication by two distinct mechanisms.

Body text

5-(3,4-dichlorophenyl) methylhydantoin (hydantoin) was first described in 1974 as an *in vitro* inhibitor of coxsackievirus-A21 (CV-A21), poliovirus (PV), infectious canine hepatitis and canine distemper virus by researchers from Eli Lilly (1). The compound was also shown to offer some level of protection against CV-A21 induced paralysis and mortality in mice. The antiviral effect of hydantoin on PV-1 (strain Mahoney) replication was later studied in more detail (2). The spectrum of antiviral activity was determined in a single cycle replication assay and activity was demonstrated against the three PV serotypes and CV-A21, whereas coxsackievirus-B3 (CV-B3) and human rhinovirus (HRV)-B14 and -A16 proved less susceptible. Time-of-addition studies with end-point titration as read-out, suggested that hydantoin was an inhibitor of late stage PV replication. Furthermore, it was concluded from Northern blot analysis that hydantoin, unlike the RNA replication inhibitor guanidine HCl, had no effect on positive-strand viral RNA synthesis. Sucrose density gradient fractionation studies of PV-1-infected cultures treated with hydantoin revealed an accumulation of the 14S pentamer and 75S pro-capsid assembly intermediates as well as the presence of a new 110S encapsidation intermediate. In this 110S fraction VP0 proved to be only partially cleaved into VP2 and VP4, a process known to occur after RNA encapsidation has taken place (3). These data let the authors to conclude that hydantoin affected viral encapsidation and maturation of the 110S intermediate. Accumulation of the 110S PV encapsidation intermediate following treatment with hydantoin was also observed in another study (4). By contrast, in yet another study the appearance of this intermediate particle was not observed albeit an effect on PV assembly was further proposed based on data obtained from a cell-free system (5).

We demonstrated recently that a glutathione-depleting molecule (TP219) efficiently blocks enterovirus morphogenesis (6). In some of our (preliminary) studies hydantoin was used as a reference compound. Using this molecule, we obtained results that challenged the above mentioned lack of inhibitory activity on poliovirus RNA replication (which was previously demonstrated by means of Northern blot) (2). To investigate this further, we studied the effect of a concentration series of hydantoin on viral RNA synthesis in a single round of viral replication. Briefly, cells were infected with either CV-B3 Nancy, EV-A71 BrCr or PV-1 Sabin (MOI >10) and were treated with four different concentrations (5, 25, 50, 100 µg/ml) of hydantoin or 150 µg/ml of guanidine HCl, a well-known inhibitor of viral RNA replication (7). Following a seven hours incubation period, intracellular RNA was isolated (Qiagen kit) and viral RNA levels were quantified by means of a RT-qPCR probe-based assay. In parallel, potential

cytostatic/cytotoxic effects of hydantoin on cell viability was assessed by means of an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium,innersalt] assay according to published methods (8). Hydantoin was shown to inhibit in a dose-dependent manner the accumulation of viral RNA of all three viruses (Fig. 1A-C) without apparent toxicity (Fig. 1D). However, differences in susceptibility of the different viruses was observed. CV-B3 proved to be relative resistant to the antiviral effect of hydantoin. EV-A71 however was the most susceptible of the viruses studied as RNA synthesis was already completely inhibited by hydantoin at a concentration of 25 µg/ml. It was previously shown that a Q65R amino acid substitution in the PV-1 2C protein conferred partial resistance to hydantoin (2). Similarly, we here show that hydantoin inhibited wild-type PV-1 RNA replication more efficiently than replication of the PV-1 2C Q65R variant. Interestingly, this mutation also resulted in a partial resistance to the viral RNA replication inhibitor Gua HCl, suggesting that this amino acid might also play a role during viral RNA synthesis which further corroborates the observation that hydantoin affects viral RNA replication. Thus, whereas it was earlier reported that hydantoin at 50 µg/ml does not affect viral RNA replication of PV-1 Mahoney (2, 5), we here now demonstrate, using RT-qPCR analysis in single cycle assays, that hydantoin inhibits enterovirus viral RNA replication in a dose dependent manner.

To further corroborate the RT-qPCR analysis, we next studied the potential antiviral effect of hydantoin on PV-1, EV-A71 and CV-B3 subgenomic replicon replication (9-11). Given that in these replicons the viral structural genes have been replaced by a luciferase gene, any antiviral effect can per definition not be caused by an effect on assembly. In such case that hydantoin would solely act by blocking viral morphogenesis and not by targeting viral RNA replication, no effect on replicon replication will be observed. Briefly, monolayer cultures of BGM or RD cells were transfected with the different replicons constructs and incubated at 37°C in standard tissue culture medium, supplemented with a particular concentration of the antiviral compound. Following 12 h of incubation, firefly luciferase activity was measured with the Steady-Glo Luciferase Assay System (Promega) (Fig. 2). Hydantoin inhibited the luciferase signal in cells transfected with the EV-A71, PV-1 and CV-B3 constructs in a dose dependent manner. In analogy with the RT-qPCR analysis, hydantoin inhibited replicons in a comparable manner whereby the CV-B3 replicon proved to be least sensitive to the antiviral effect of hydantoin and the EV-A71 replicon the most sensitive. Taken together, these data clearly show that hydantoin is an inhibitor of enteroviral RNA replication.

Since it was previously shown that hydantoin inhibits viral morphogenesis, it might be possible that hydantoin inhibits enterovirus replication by a dual mechanism of action. To investigate this possibility, we studied the inhibitory effect of hydantoin on PV-1 replication by measuring intracellular (Fig. 3A) and extracellular (Fig. 3B) RNA levels and infectious virus particles (Fig. 3C) in a single cycle assay. Gua HCl completely blocked intracellular viral RNA replication whereas geldanamycin (a known inhibitor of heat shock protein 90 that was previously reported to inhibit morphogenesis without affecting viral RNA replication (12)) did not reduce intracellular viral RNA levels (Fig. 3A). In line with the observations above, both concentrations of 50 and 100 µg/ml hydantoin inhibited viral RNA synthesis albeit to a different extent. Whereas 50 µg/ml of hydantoin had only little effect on viral RNA synthesis, 100 µg/ml hydantoin markedly decreased intracellular levels of viral RNA. In parallel, we also assessed the effect of these compounds on extracellular RNA levels and the formation of infectious virus particles (Fig. 3B and 3C). As expected, geldanamycin treatment clearly resulted in a comparable reduction of both extracellular RNA and infectious virus particles, indicating that despite having no effect on intracellular RNA replication, the formation of infectious virus particles is severely hampered. Similar to Gua HCl treatment, a concentration of 100 µg/ml hydantoin completely blocked both extracellular RNA levels and infectious virus particles as well. Thus, at this concentration the predominant mechanism of action is inhibition of viral RNA replication. In contrast, 50 µg/ml hydantoin clearly reduced extracellular RNA levels and the amount of infectious virus particles despite having only little effect on intracellular RNA replication. Thus, at 50 µg/ml hydantoin, the predominant mechanism of action of hydantoin appears to be inhibition of virus morphogenesis. Interestingly, at this concentration the effect on extracellular RNA levels was less pronounced than the effect on infectious virus particles production, suggesting that the extracellular RNA detected might not have originated form infectious virus.

Taken together, we provide conclusive evidence that the antiviral compound hydantoin has a dual mechanism of action. At 50 µg/ml, the predominant mechanism of action is the inhibition of morphogenesis of infectious virus particles and RNA synthesis is little affected. This likely explains why it was earlier reported that hydantoin exclusively inhibits viral morphogenesis, since all the experiments were performed using concentrations of 50 µg/ml. However, we now show that at higher concentrations hydantoin also inhibits viral RNA synthesis. A tight link between viral RNA replication and the process of morphogenesis has been described before. For instance, a quasi-infectious chimeric virus that replicates it’s genome with wildtype kinetics but has an impaired encapsidation phenotype, could be

rescued by a single mutation in protein 2C, a protein that is an essential part of the viral replication complex (13). In the same study, biochemical assays provided strong evidence for a direct interaction between protein 2C and the capsid protein VP3. In a later study by the same laboratory encapsidation defective PV was generated by means of alanine-scanning mutagenesis of 2C, demonstrating the role of this viral protein in morphogenesis (14). Moreover, the defect could be rescued by suppressor mutations in either 2C itself or in VP1/VP3, demonstrating a functional interaction between 2C and the capsid proteins. Within this context, it might not be surprising that hydantoin might affect both RNA replication and virus morphogenesis. The precise mechanisms by which the compound inhibits viral RNA synthesis and how it exerts its dual mode of action remain to be studied.

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Bullet points

- Hydantoin was earlier reported to solely inhibit enterovirus morphogenesis.
- Hydantoin has a dual mechanism of antiviral action that involves inhibition of morphogenesis and RNA replication
- The predominant mechanism of inhibition is determined by the concentration of hydantoin.

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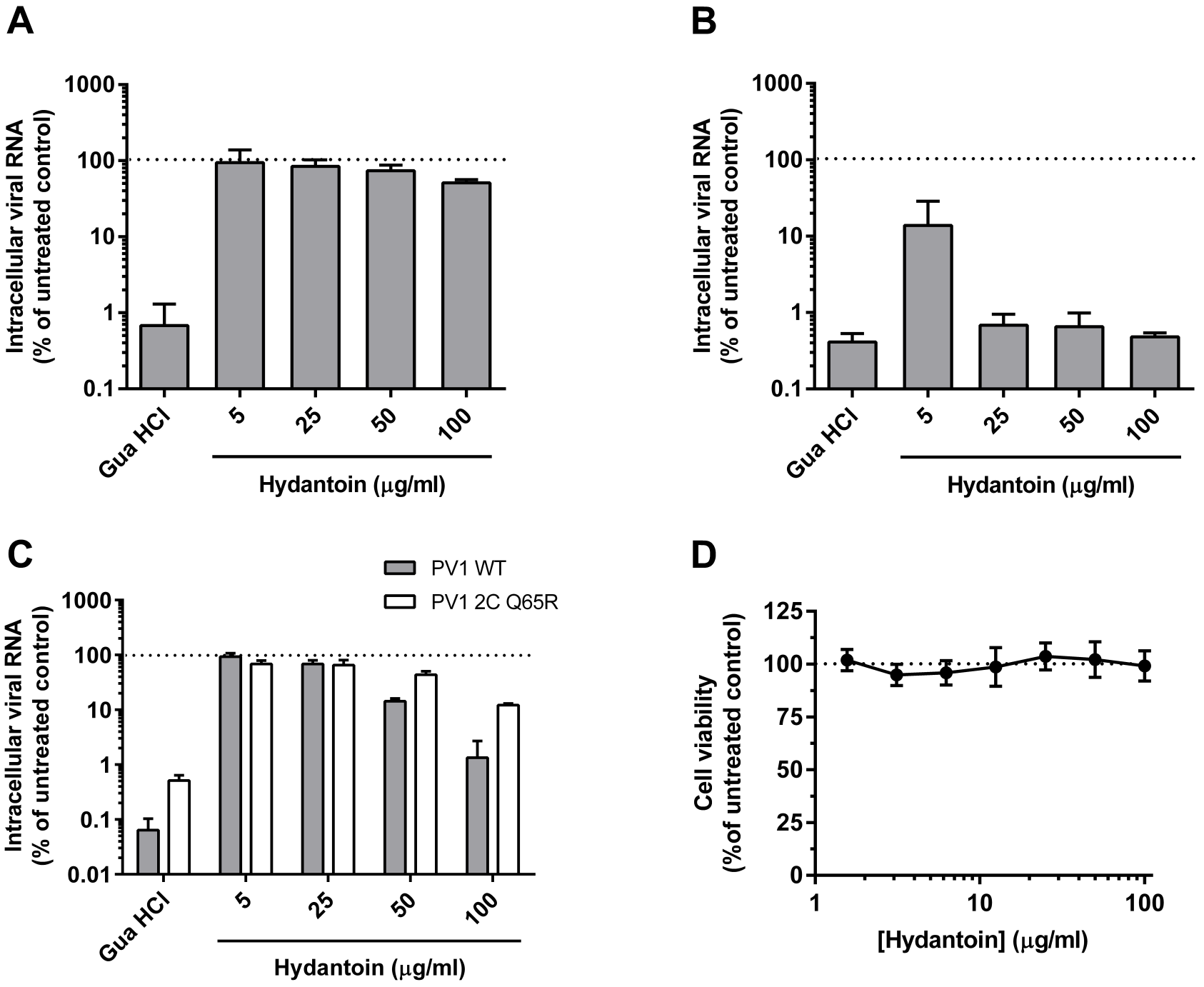
Legend to Figures

Figure 1. Hydantoin inhibits viral RNA replication. Cell cultures were infected with (A) CV-B3, (B) EV-A71 or (C) PV-1 WT and PV-1 2C Q65R and treated with 150 µg/ml Gua HCl or the indicated concentration of hydantoin. Intracellular viral RNA was isolated seven hours post infection and quantified by means of RT-qPCR. (D) BGM cell viability with MTS assay after incubation with a concentration series of hydantoin. The dotted line indicates the untreated control. All data are mean values ± SD of ≥3 individual measurements.

Figure 2. Hydantoin inhibits subgenomic replicons. Cell cultures were transfected with viral replicons of PV-1, EV-A71 and CV-B3 and treated with 96 µg/ml Guanidine HCl or 10, 50 or 100 µg/ml hydantoin. Following 12 hours of incubation luciferase activity was measured. All data are mean values ± SD of ≥3 individual measurements.

Figure 3. Hydantoin has a dual mechanism of action. BGM cell cultures were infected with PV-1 at an MOI >10. After an incubation period of one hour, the virus was aspirated and replaced with medium containing the appropriate compound. At 8 hours post infection both the intracellular (A) and extracellular (B) PV-1 RNA content was determined using qPCR. (C) In parallel, cell cultures were infected with PV-1 at a MOI of 5. After an incubation period of one hour, the virus was aspirated and replaced with medium containing the appropriate compound. At 8 hours post infection, cultures were frozen and subjected to three rounds of freeze-thawing. Virus titers were determined using end-point titration. The dotted line indicates the untreated control. All data are mean values ± SD of ≥3 individual measurements


A
Intracellular viral RNA
(% of untreated control)
1000
100
10
1
0.1
Gua HCl
5
25
50
100
Hydantoin (μg/ml)
B
Intracellular viral RNA
(% of untreated control)
1000
100
10
1
0.1
Gua HCl
5
25
50
100
Hydantoin (μg/ml)
C
PV1 WT
PV1 2C Q65R
Intracellular viral RNA
(% of untreated control)
1000
100
10
1
0.1
0.01
Gua HCl
5
25
50
100
Hydantoin (μg/ml)
D
Cell viability
(%of untreated control)
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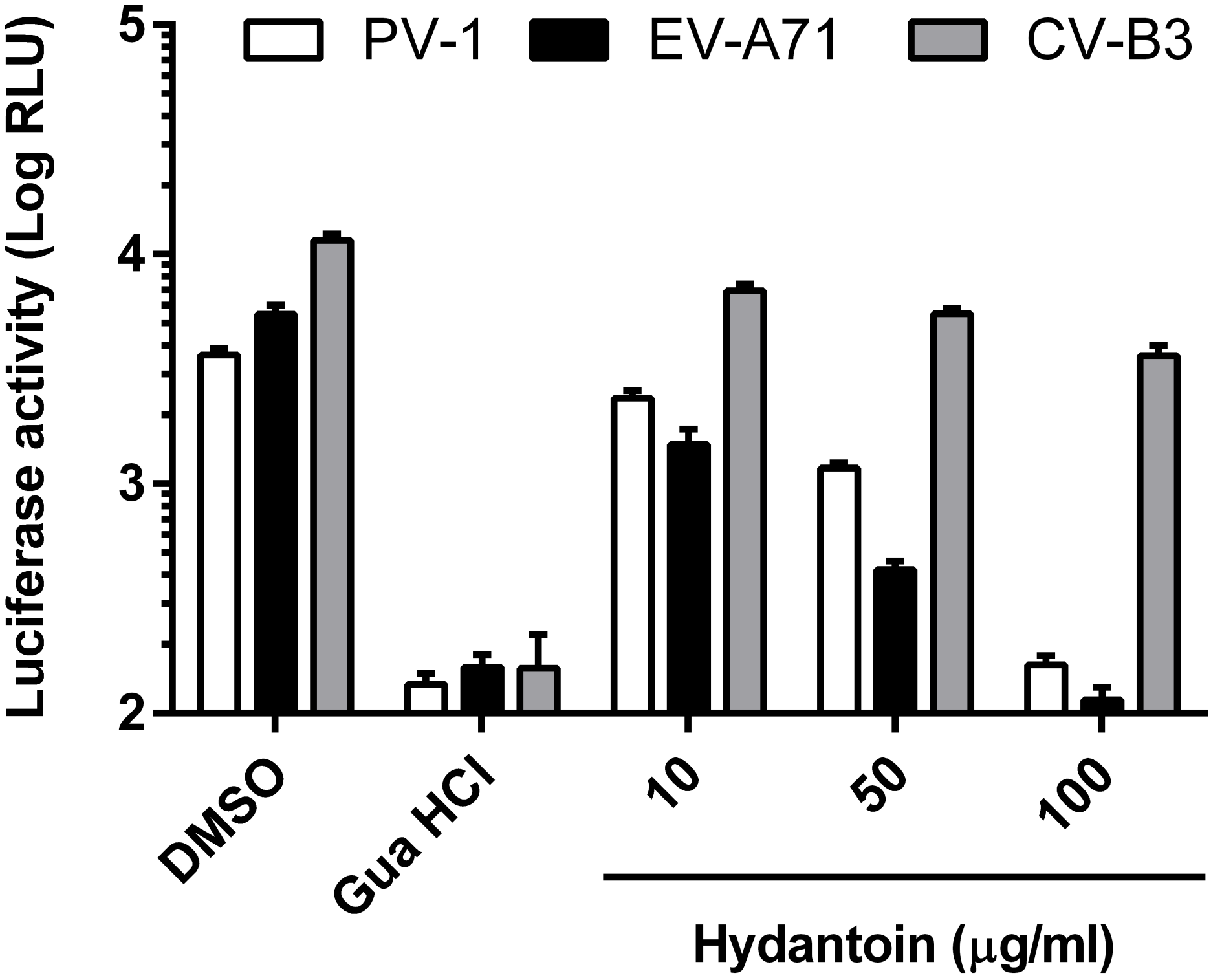
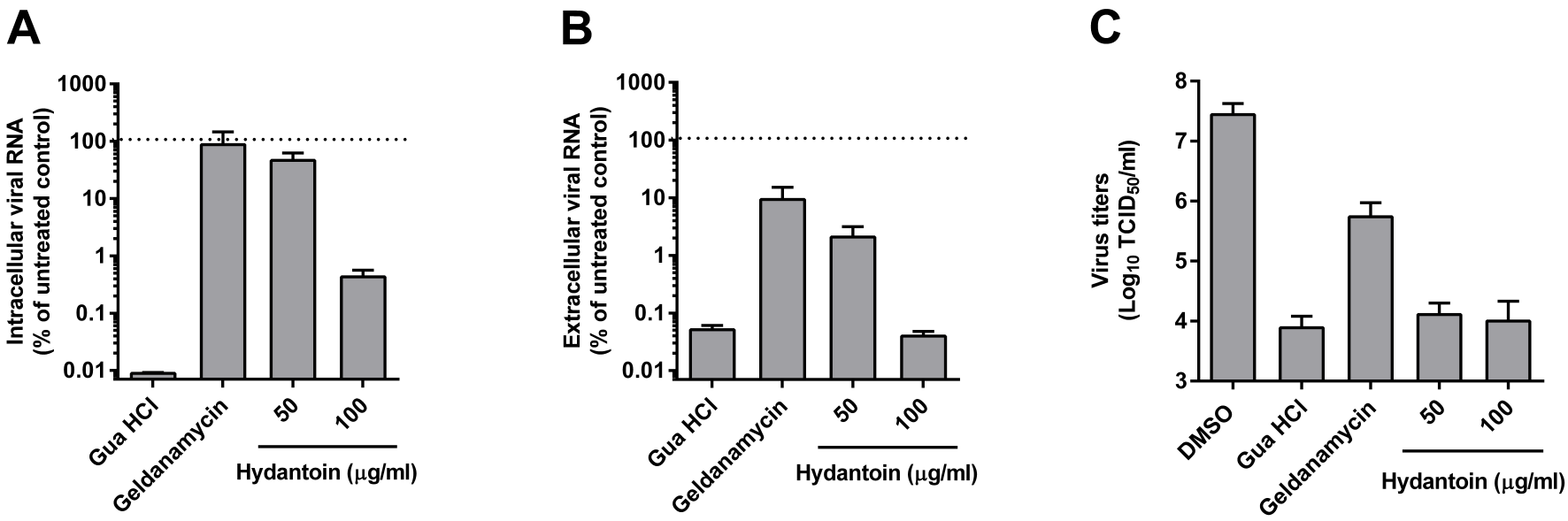
100
75
50
25
0
1
10
100
[Hydantoin] (μg/ml)


PV-1
EV-A71
CV-B3
Luciferase activity (Log RLU)
5
4
3
2
DMSO
Gua HCl
10
50
100
Hydantoin (μg/ml)


A
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(% of untreated control)
1000
100
10
1
0.1
0.01
Gua HCl
Geldanamycin
50
100
Hydantoin (μg/ml)
B
Extracellular viral RNA
(% of untreated control)
1000
100
10
1
0.1
0.01
Gua HCl
Geldanamycin
50
100
Hydantoin (μg/ml)
C
Virus titers
(Log10 TCID50/ml)
8
7
6
5
4
3
DMSO
Gua HCl
Geldanamycin
50
100
Hydantoin (μg/ml)