The thrombopoietin receptor agonist eltrombopag inhibits human cytomegalovirus replication via iron chelation

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Abstract

The thrombopoietin receptor agonist eltrombopag was successfully used against human cytomegalovirus (HCMV)-associated thrombocytopenia refractory to immunomodulatory and antiviral drugs. These effects were ascribed to effects of eltrombopag on megakaryocytes. Here, we tested whether eltrombopag may also exert direct antiviral effects. Therapeutic eltrombopag concentrations inhibited HCMV replication in human fibroblasts and adult mesenchymal stem cells infected with six different virus strains and drug-resistant clinical isolates. Eltrombopag also synergistically increased the anti-HCMV activity of the mainstay drug ganciclovir. Time-of-addition experiments suggested that eltrombopag interferes with HCMV replication after virus entry. Eltrombopag was effective in thrombopoietin receptor-negative cells, and addition of Fe^{3+} prevented the anti-HCMV effects, indicating that it inhibits HCMV replication via iron chelation. This may be of particular interest for the treatment of cytopenias after haematopoietic stem cell transplantation, as HCMV reactivation is a major reason for transplantation failure. Since therapeutic eltrombopag concentrations are effective against drug-resistant viruses and synergistically increase the effects of ganciclovir, eltrombopag is also a drug repurposing candidate for the treatment of therapy-refractory HCMV disease.

Key words: human cytomegalovirus, antiviral therapy, eltrombopag, thrombopietin receptor agonist, drug resistance, iron chelation

Introduction

Eltrombopag is a thrombopoietin receptor (also known as c-Mpl or MPL) agonist that is used for the treatment of thrombocytopenia [1-3]. Its use has also been suggested for the treatment of cytopenias after haematopoietic stem cell transplantations and case reports support its safety and efficacy [4-9].

Human cytomegalovirus (HCMV) reactivation and HCMV-associated disease are leading reasons for the failure of haematopoietic stem cell transplantations [10-12]. Anti-HCMV drugs including ganciclovir, cidofovir, and foscarnet are available, but their use is associated with severe side effects [13]. In particular, the use of ganciclovir (and its prodrug valganciclovir), the mainstay treatment for cytomegalovirus disease, is associated with severe haematological side effects including thrombocytopenia [14-16].

A case report described the use of eltrombopag in an immunocompetent patient who suffered from human cytomegalovirus (HCMV)-associated thrombocytopenia [17]. Immunosuppressive treatment for thrombocytopenia (prednisone, intravenous immunoglobulin, dapsone) in combination with antiviral therapy (ganciclovir/ valganciclovir, HCMV hyperimmune globulin) only resulted in a temporary platelet response with subsequent relapse. A change to eltrombopag intended to increase platelet counts without immunosuppressive therapy resulted in a durable increase in platelet levels, no evidence of HCMV viraemia, and the resolution of symptoms [17]. The observed effects were attributed to eltrombopag overcoming HCMV-induced suppression of platelet production [17]. However, we hypothesised that direct antiviral effects may also have contributed to the beneficial outcome in the case report of the patient with HCMV-associated thrombocytopenia [17]. Indeed, we found that eltrombopag exerts anti-HCMV effects via iron chelation.

Materials and Methods

Drugs

Eltrombopag (as its orally active ethanolamine salt eltrombopag olamine) was purchased from Selleck Chemicals (via Absource Diagnostics GmbH, Munich Germany), deferasirox and ganciclovir from MedChemExpress (via Hycultec, Beutelsbach, Germany), and cidofovir from Cayman Chemical (via Biomol GmbH, Hamburg, Germany).

Cells and viruses

Primary human foreskin fibroblasts (HFFs) and adipose-derived adult mesenchymal stem cells (ASCs) were cultivated as previously described [18,19].

The wild type HCMV strain Hi91 was isolated from the urine of an AIDS patient with HCMV retinitis as described previously [20]. HCMV strains Davis and Towne were received from ATCC (Manassas, VA, USA). Virus stocks were prepared in HFFs maintained in minimal essential medium (MEM) supplemented with 4% FCS. U1, U59, and U75 are patient isolates, which were isolated as previously described [20,21]. Virus stocks were prepared in HFFs maintained in minimal essential medium (MEM) supplemented with 4% FCS.

Murine cytomegalovirus (Smith strain, catalogue number VR-1399) was obtained from ATCC and cultivated in NIH/3T3 mouse fibroblasts (ATCC).

DNA isolation, amplification, and sequencing were performed as previously described [21], using established primers [22].

95 **Virus infectivity assay**

96 In 96-well microtiter plates, confluent cultures of HFFs or ASCs cells were
97 incubated with HCMV at the indicated multiplicities of infection (MOIs). After incubation
98 for one hour, cells were washed with PBS and incubated in MEM containing 4% FCS
99 and serial dilutions of the indicated substances.

100 As described previously [18,23], cells producing HCMV specific antigens were
101 detected 24h post infection by immunoperoxidase staining using monoclonal
102 antibodies directed against the UL123-coded 72 kDa immediate early antigen 1 (IEA1)
103 (Mouse Anti CMV IEA, MAB8131, Millipore, Temecula, CA, USA) and 120h post-
104 infection by immunoperoxidase staining using monoclonal antibodies directed against
105 UL55-encoded late antigen gB (LA) (kindly provided by K. Radsak, Institut für
106 Virologie, Marburg, Germany) as previously described. Drug concentrations that
107 reduced HCMV antigen expression by 50% (IC_{50}) were calculated using Calcusyn
108 (Biosoft, Cambridge, United Kingdom).

109

110 **Drug combination studies**

111 Drugs were combined at equimolar concentrations and single agent as well as
112 combined effects were determined by staining for HCMV LA. Combination indices
113 (CIs) were calculated at different levels of inhibition (50% inhibition, CI_{50}; 75%
114 inhibition, CI_{75}; 90% inhibition, CI_{90}; 95% inhibition, CI_{95}) by the method of Chou and
115 Talalay [24] using CalcuSyn software version 1.0 (Biosoft, Cambridge, United
116 Kingdom). Weighted average CI values (CI_{wt}) were calculated as (CI_{50} + 2 × CI_{75} + 3 ×
117 CI_{90} + 4 × CI_{95}) / 10. CI_{wt} values ≤0.7 indicate synergistic effects, CI_{wt} values >0.7 and
118 ≤0.9 moderately synergistic effects, CI_{wt} values >0.9 and ≤1.2 additive effects, CI_{wt}

values >1.2 and ≤1.45 moderately antagonistic effects, and CI_{wt} values >1.45 antagonistic effects [24].

Viability assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay as described previously [23]. Confluent cell cultures in 96-well microtiter plates were incubated with culture medium containing serial dilutions of the indicated substances. After five days of incubation MTT (1 mg/ml) was added and after an additional four hours, cells were lysed in a buffer containing 20% (w/v) SDS and 50% N,N-dimethylformamide adjusted to pH 4.5. Absorbance was determined at 570 nm for each well using a 96-well multiscanner. After subtracting background absorbance, cell viability was expressed in per cent relative to untreated control cells. Drug concentrations that reduced cell viability by 50% (CC_{50}) were calculated using Calcusyn (Biosoft, Cambridge, United Kingdom).

Virus yield assay

The amount of infectious virus was determined by virus yield assay in a single-cycle assay format as previously described [23]. Virus titres were expressed as 50% of tissue culture infectious dose ($TCID_{50}$/ mL) 120 h post infection.

Immunoblotting

Immunoblotting was performed as described previously [23]. In brief, cells were lysed in Triton X-100 sample buffer and proteins separated by sodium dodecyl sulfate (SDS) SDS-PAGE. Proteins were detected using specific antibodies against ß-actin (3598R-100-BV, BioVision via BioCat, Heidelberg, Germany) or HCMV 45 kDa late

antigen (MBS320051, MyBioSource via Biozol, Echingen, Germany) and were visualized by enhanced chemiluminescence using a commercially available kit (Thermo Scientific, Schwerte, Germany).

Statistics

Values presented are the mean ± S.D. of three independent biological repeats. Comparisons between two groups were performed using Student's t-test, three and more groups were compared by ANOVA followed by the Student-Newman-Keuls test. Data groups were considered significantly different at $P < 0.05$.

Results

Eltrombopag inhibits HCMV replication in human foreskin fibroblasts by interference with late processes of the replication cycle

Eltrombopag did not affect HCMV Hi91-induced immediate early antigen (IEA) expression, but inhibited HCMV Hi91-induced late antigen (LA) expression with an IC_{50} of 415 nM in HFFs (Figure 1A, 1B). Eltrombopag concentrations of up to 25µM did not reduce the viability of confluent or proliferating HFFs by 50%. Hence, the selectivity index CC_{50}/IC_{50} is higher than 60.2 (Figure 1A). Higher multiplicities of infection (MOIs) were associated with higher IC_{50} values (Figure 1C). At MOI 1, the highest MOI investigated in HFFs, the eltrombopag IC_{50} was 3844 nM. The observed eltrombopag concentrations are within the range of therapeutic plasma concentrations which have been described to exceed 45µM [25,26].

Eltrombopag-induced inhibition of HCMV LA translated into reduced virus replication as indicated by virus yield assay (Figure 2A). At a concentration of 10µM, eltrombopag reduced virus titres by 1.8 x 10^4-fold and at 500nM still by 15-fold.

The HCMV replication cycle is divided into three phases characterised by the expression of immediate early, delayed early, and late viral genes. Immediate early genes are transcribed immediately after infection and do not depend on synthesis of viral DNA or transcription of proteins. Delayed early proteins are represented by the viral DNA polymerase and other viral functions required for viral DNA synthesis and some viral structural proteins. Late genes encode mostly structural proteins used in viral assembly and packaging, and are generally expressed subsequent to delayed early genes [27].

To better define which phases of the viral replication cycle are affected by eltrombopag, the drug was added at different time points (Figure 2B, Suppl. Table 1). Pre-incubation and drug addition during the one-hour virus adsorption period did not or only modestly affect virus replication. This shows that eltrombopag does not primarily interfere with virus binding to host cells and virus internalisation but needs to be present during virus replication to exert its anti-HCMV effects. Drug addition one hour or 24h post infection was sufficient to achieve maximum inhibition of HCMV LA expression (Figure 2B, Suppl. Table 1). This, together with the observed lack of inhibition of HCMV IEA expression, indicates that eltrombopag inhibits the late stages of the HCMV replication cycle characterised by LA expression. Drug addition 48h post infection resulted in reduced effects compared to drug addition one hour or 24h post infection (Figure 2B, Suppl. Table 1).

Eltrombopag inhibits HCMV expression via iron chelation

Eltrombopag was developed as thrombopoietin receptor agonist [1-3]. However, it is unlikely that eltrombopag inhibits HCMV replication via thrombopietin receptor activation, because fibroblasts do not express the thrombopoietin receptor [28]. In agreement, eltrombopag also inhibited murine cytomegalovirus replication in murine NIH/3T3 fibroblasts (Figure 3A), although eltrombopag does not target the murine thrombopoietin receptor [29].

Eltrombopag is also an iron chelator [2,30,31], and iron chelators have been shown to inhibit HCMV replication [32-38]. The addition of equimolar Fe^{3+} concentrations was shown to inhibit pharmacological action of eltrombopag that are caused via iron chelation [31]. Hence, we investigated eltrombopag in combination with equimolar $Fe(III)Cl_3$ concentrations to investigate whether iron chelation is the

mechanism by which eltrombopag exerts its anti-HCMV effects (Figure 3B). Since equimolar Fe(III)Cl_3 concentrations prevented the anti-HCMV effects of eltrombopag (Figure 3B), we concluded that iron chelation is the main mechanism of eltrombopag's anti-HCMV activity.

Eltrombopag exerts synergistic effects with ganciclovir

Next, we tested eltrombopag in combination with ganciclovir, the mainstay of anti-HCMV therapies [13]. The combination of equimolar eltrombopag and ganciclovir concentrations resulted in synergistic anti-HCMV effects (Figure 4), which is illustrated by a weighted average combination index (CI_{WT}) of 0.17 ± 0.03 as determined by the method of Chou and Talalay [24]. According to this method, combined effects are considered to be synergistic at a CI_{WT} for <0.7 [24].

Eltrombopag is effective in different cell types and against different virus strains and isolates including drug-resistant ones

Finally, we investigated the effects of eltrombopag against a broader range of laboratory virus strains and clinical isolates in HFFs and primary adipose-derived adult mesenchymal stem cells (ASCs), another cell type that supports HCMV replication [39]. The laboratory HCMV strains included Davis [40] and Towne [41] in addition to Hi91. The clinical isolates U1, U59, and U75 were isolated from the urine of patients as previously described [20,21]. U1 and U59 harbour a A987G mutation in the HCMV DNA polymerase UL54 (Table 1), which is known to confer combined ganciclovir and cidofovir resistance [42,43]. U1 also displays a C607Y mutation in the HCMV kinase UL97 (Table 1), which is associated with ganciclovir resistance [44,45]. In agreement, U1 and U59 were characterised by high ganciclovir and cidofovir IC_{50}s (Table 1), which

229 are typically considered to indicate resistance [46-48]. U75 also displayed resistance
230 to ganciclovir and cidofovir (Table 1), although it does not harbour known resistance
231 mutations.

232 The eltrombopag IC_{50}s ranged from 99nM (U1 in HFFs) to 4331nM (Hi91 in
233 ASCs) (Figure 5A, Suppl. Table 2). When compared across the two cell types, the
234 different HCMV strains and clinical isolates displayed similar eltrombopag sensitivity,
235 apart from U1, which appeared to be particularly sensitive to eltrombopag in HFFs and
236 ASCs (Figure 5B). The average HCMV sensitivity to eltrombopag was very similar in
237 both cell types (Figure 5C).

238 To confirm the relevance of iron chelation as mechanism of the anti-HCMV
239 action of eltrombopag using a clinical virus isolate, U1-infected HFFs were treated with
240 equimolar concentrations of eltrombopag and $Fe(III)Cl_3$. The presence of equimolar
241 Fe^{3+} concentrations prevented the eltrombopag-induced inhibition of HCMV LA
242 expression in U1-infected cells in a comparable fashion (Figure 5D) as in Hi91-infected
243 cells (Figure 3B).

244

Discussion

Here, we show that the approved thrombopoietin receptor agonist eltrombopag exerts anti-HCMV effects in various cell types infected with a range of different virus strains and clinical isolates including drug-resistant ones. The observed IC_{50} values ranged from 99nM to 4331nM, which is in the range of therapeutic plasma concentrations that have been reported to exceed 45µM [25,26]. Eltrombopag also synergistically increased the activity of the approved anti-HCMV drug ganciclovir.

Our findings are in agreement with a case report on an immunocompetent patient, who suffered from HCMV-associated thrombocytopenia and recovered after eltrombopag therapy [17]. This response had originally been attributed to effects of eltrombopag on platelet production [17]. The possibility that eltrombopag may exert antiviral affects was not considered. Our current data show that therapeutic eltrombopag levels interfere with HCMV replication, which may have contributed to the beneficial clinical outcome. Notably, eltrombopag has also been shown to inhibit the replication of severe fever with thrombocytopenia syndrome virus, a member of the genus Banyangvirus (Phenuiviridae) [49].

The anti-HCMV effects of eltrombopag are unlikely to be caused by action on the thrombopoietin receptor, since eltrombopag was effective in cell types that do not express the thrombopoietin receptor, which is expressed in haematopoietic cells [28,29]. In agreement, eltrombopag also exerted antiviral effects in mouse fibroblasts infected with murine CMV, although the haematological effects of eltrombopag are known to be species-specific and to not affect mice [28,29].

Eltrombopag is also known to be an iron chelator [30,31]. Addition of Fe^{3+} prevented the eltrombopag-mediated anti-HCMV effects in strain Hi91- and clinical

isolate U1-infected cells. Hence, our data suggest that eltrombopag inhibits HCMV replication via Fe^{3+} chelation.

A number of different iron chelators including desferrioxamine, diethylenetriaminepeantaacetic acid (DTPA), and ethylenediaminedisuccinic acid (EDDS) were shown to inhibit HCMV replication [32-38]. However, the iron chelators tiron and ciclopirox olamine were not found to inhibit HCMV strain AD169 replication in MRC5 cells [50]. The experimental set-up differed, as MRC5 cells were infected at a high MOI of 3 and no dose-response relationships were determined. Hence, a direct comparison is not possible. Notably, specific antiviral activity can easily be missed if the therapeutic window between antiviral and cytotoxic effects is relatively small. For example, desferrioxamine was found to inhibit HCMV replication at concentrations that did not decrease the viability of confluent fibroblasts but affected dividing cells [32]. In contrast, eltrombopag inhibits HCMV replication in concentrations that do not affect cell proliferation. Hence, the size of the therapeutic window that discriminates between anti-HCMV activity and antiproliferative and cytotoxic effects substantially differs among iron chelators, and eltrombopag seems to be an iron chelator that possesses a particularly preferential therapeutic window in terms of its anti-HCMV activity.

Eltrombopag has been suggested for the treatment of cytopenias after haematopoietic stem cell transplantations and case reports support its safety and efficacy [4-9]. Since HCMV reactivation and HCMV-associated disease are leading reasons for the failure of haematopoietic stem cell transplantations [10-12], antiviral effects exerted by eltrombopag may also contribute to improved therapy outcome. Notably, eltrombopag was effective against resistant clinical HCMV isolates, and resistance formation to the approved drugs is a major challenge after stem cell transplantation [11,12].

294 In conclusion, therapeutic eltrombopag concentrations inhibit HCMV replication
295 via chelation of Fe^{3+} ions. Eltrombopag is effective against drug-resistant viruses and
296 synergistically increases the effects of the mainstay anti-HCMV drug ganciclovir. The
297 anti-HCMV activity of eltrombopag may be of particular interest for its use for the
298 treatment of cytopenias after haematopoietic stem cell transplantation, as HCMV
299 reactivation and disease is a major reason for transplantation failure.
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Figure legends

Figure 1. Effects of eltrombopag on HCMV late antigen (LA) expression in primary human foreskin fibroblasts (HFFs). A) Representative dose response curves showing the effects of eltrombopag on HCMV LA expression and HFF viability (as determined after 120h of incubation). Eltrombopag concentrations that reduce HCMV LA expression by 50% (IC_{50}) and the viability of proliferating HFFs by 50% (CC_{50}) relative to untreated controls are also provided. Eltrombopag was continuously present from the time of virus infection. B) Representative photographs and Western blots demonstrating the effects of eltrombopag on HCMV LA expression. In A) and B), HFFs were infected with HCMV strain Hi91 (MOI 0.02). HCMV late antigen (LA) expression was detected 120h post infection. C) Representative dose-response curves and IC_{50} values indicating effects of eltrombopag on HCMV LA expression in HFFs infected with different MOIs of HCMV strain Hi91 as detected 120h post infection.

Figure 2. Effects of eltrombopag on HCMV replication and at different stages of the viral replication cycle. HFFs were infected with HCMV strain Hi91 (MOI 0.02). HCMV late antigen (LA) expression and virus titres were detected 120h post infection. A) Virus titres in the absence or presence of eltrombopag. B) Representative dose-response curves and IC_{50} values indicating indicating the effects of eltrombopag on HCMV LA expression after 24h of pre-treatment, after treatment during the 1h adsorption period, after drug addition post infection following the 1h virus adsorption period, after drug addition 24h post infection, and after drug addition 48h post infection.

Figure 3. Eltrombopag inhibits HCMV infection by iron depletion. A) Representative dose response curve indicating the effects of eltrombopag on cytopathogenic effect (CPE) formation (detected 120h post infection) in murine cytomegalovirus (MOI 1)-infected murine NIH/3T3 fibroblasts and eltrombopag concentration that reduces CPE formation by 50% (IC_{50}) relative to untreated control. The findings indicate that eltrombopag interferes with cytomegalovirus replication by thrombopoietin receptor-independent effects, since eltrombopag does not activate the murine thrombopoietin receptor. The investigated eltrombopag concentrations did not affect NIH/3T3 cell viability. B) Representative growth curve indicating the effects of equimolar concentrations of $Fe(III)Cl_3$ on the anti-HCMV effects of eltrombopag as indicated by HCMV LA expression in HCMV Hi91 (MOI 0.02)-infected human foreskin fibroblasts (HFFs) 120h post infection. Equimolar $Fe(III)Cl_3$ concentrations circumvent the anti-HCMV effects exerted by eltrombopag.

Figure 4. Antiviral effects of eltrombopag in combination with ganciclovir. A) Effects of equimolar drug concentrations on HCMV LA expression in HCMV Hi91 (MOI 0.02)-infected human foreskin fibroblasts (HFFs) 120h post infection. *P < 0.05 compared to either single treatment; B) Combination indices (CIs) at different levels of inhibition and weighted average CI values (CI_{wt}) calculated as (CI_{50} + 2 × CI_{75} + 3 × CI_{90} + 4 × CI_{95}) / 10 [24]. CI_{wt} values $\leq$0.7 indicate synergistic effects [24].

Figure 5. Antiviral effects of eltrombopag determined in different cell types infected by different human cytomegalovirus (HCMV) strains and clinical isolates. Human foreskin fibroblasts (HFFs) were infected at an MOI of 0.02 and adipose-derived adult mesenchymal stem cells (ASCs) at an MOI of 5. HCMV late antigen (LA) expression

514 was determined 120h post infection. A) Eltrombopag concentrations that reduce
515 HCMV LA expression by 50% (IC_{50}). Numerical values are provided in Suppl. Table
516 2. The investigated eltrombopag concentrations did not affect cell viability. B) Average
517 eltrombopag IC_{50}s for each virus strain and isolate in HFFs and ASCs. C) Average
518 eltrombopag IC_{50}s across virus strains and isolates in HFFs and ASCs. D)
519 Representative growth curve indicating the effects of equimolar concentrations of
520 $Fe(III)Cl_3$ on the anti-HCMV effects of eltrombopag as indicated by HCMV LA
521 expression in U1 (MOI 0.02)-infected HFFs 120h post infection.
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Figure 1

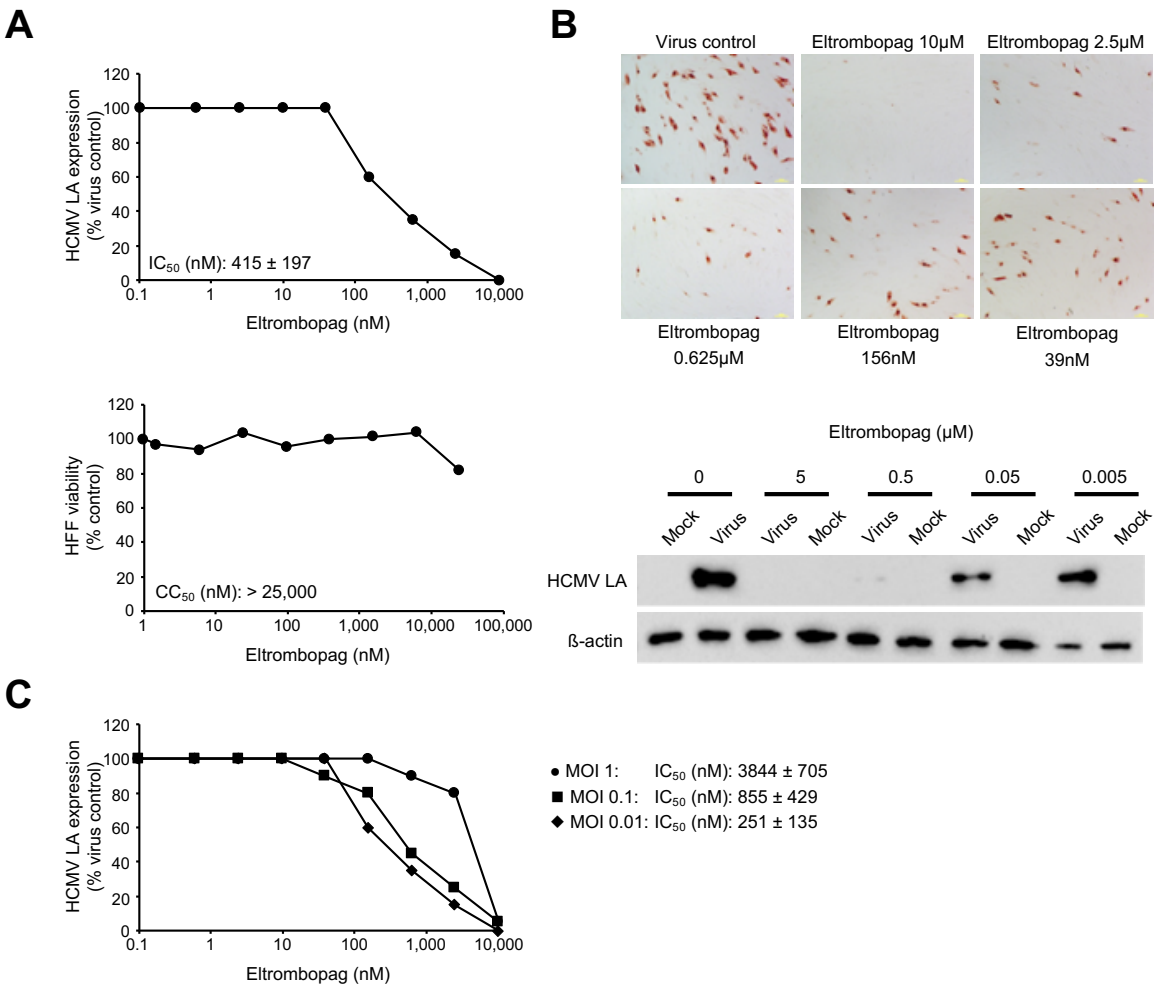

Figure 2

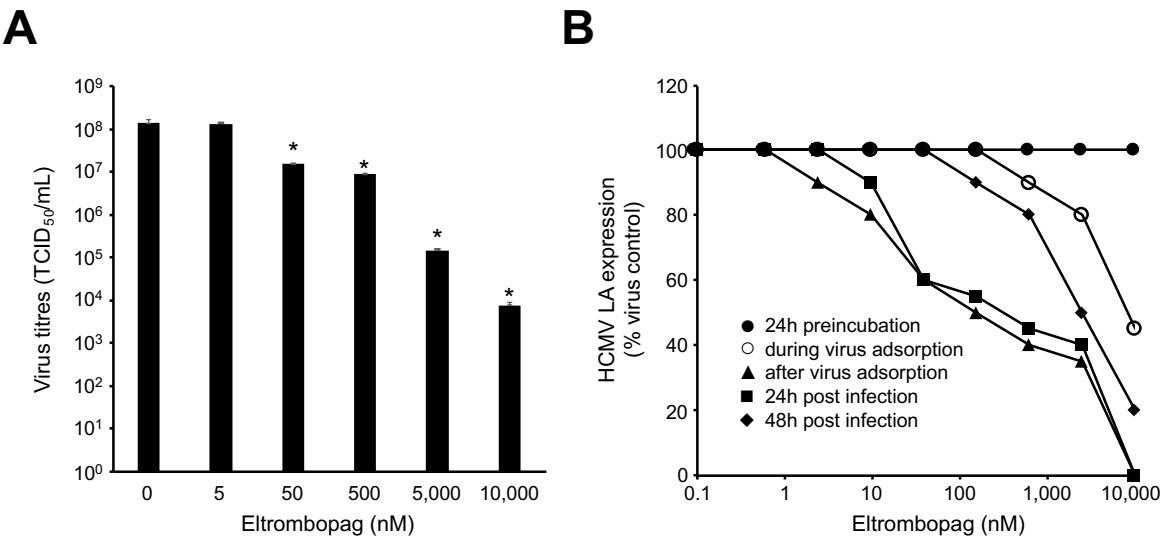

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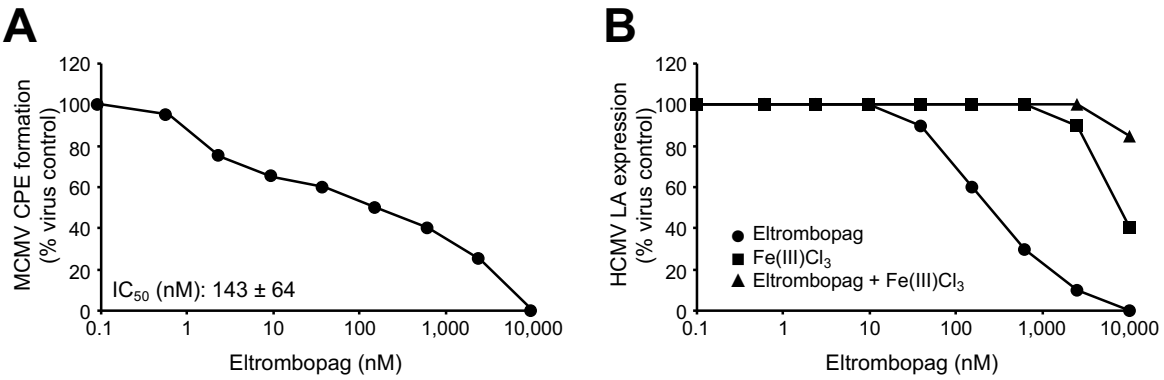
Figure 3



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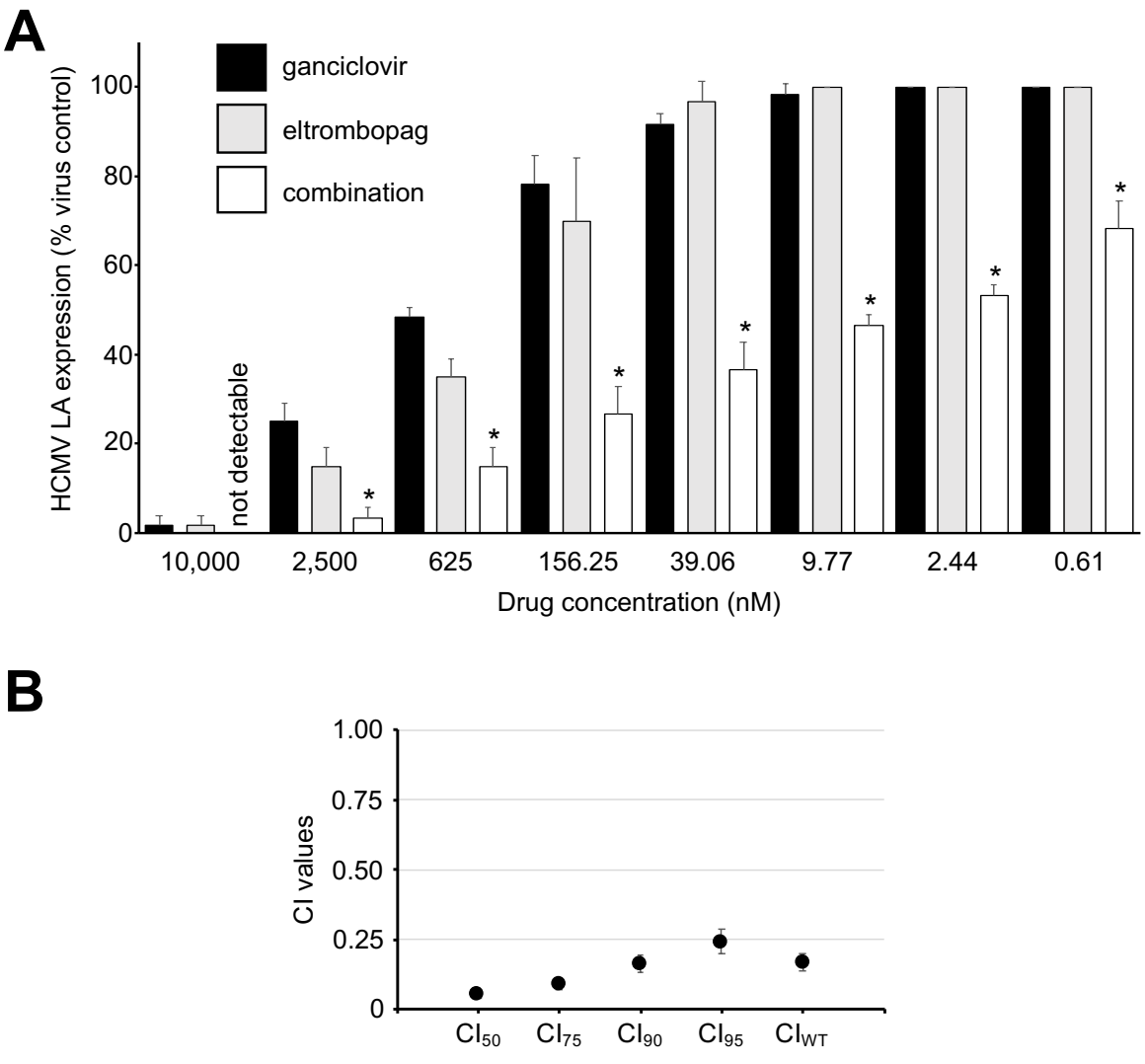
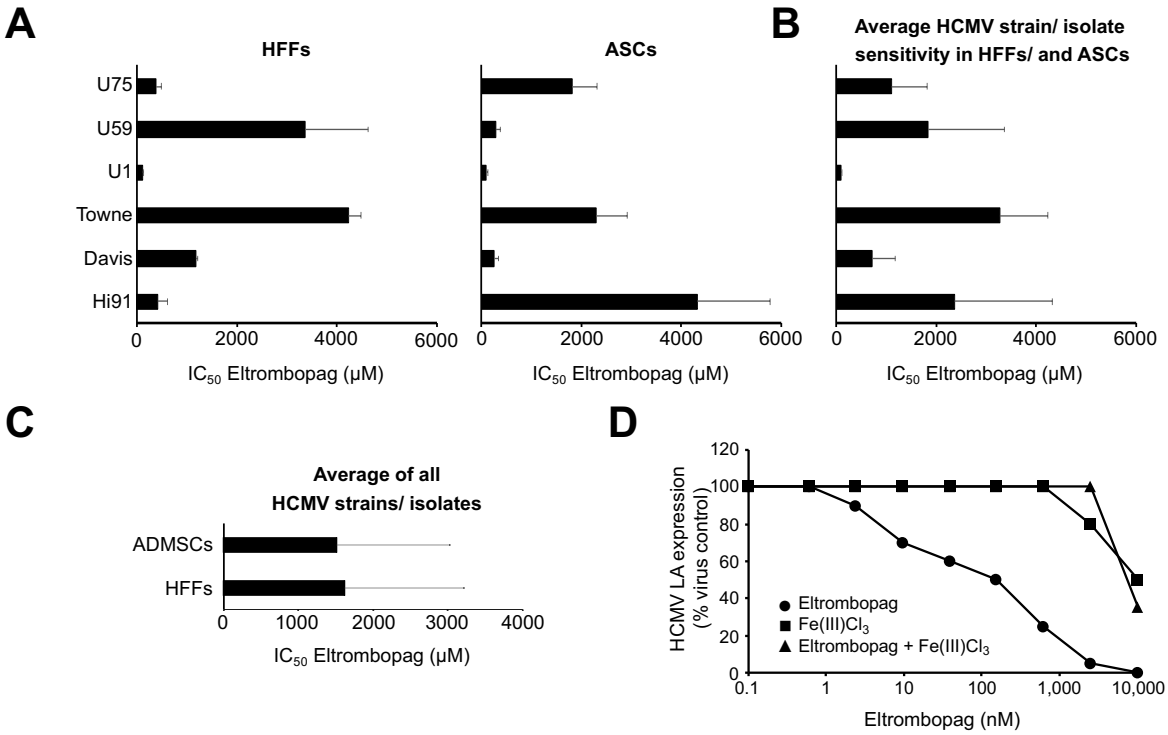


Figure 5



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