

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

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23

24 **Abstract**

25 The thrombopoietin receptor agonist eltrombopag was successfully used
26 against human cytomegalovirus (HCMV)-associated thrombocytopenia refractory to
27 immunomodulatory and antiviral drugs. These effects were ascribed to effects of
28 eltrombopag on megakaryocytes. Here, we tested whether eltrombopag may also
29 exert direct antiviral effects. Therapeutic eltrombopag concentrations inhibited HCMV
30 replication in human fibroblasts and adult mesenchymal stem cells infected with six
31 different virus strains and drug-resistant clinical isolates. Eltrombopag also
32 synergistically increased the anti-HCMV activity of the mainstay drug ganciclovir.
33 Time-of-addition experiments suggested that eltrombopag interferes with HCMV
34 replication after virus entry. Eltrombopag was effective in thrombopoietin receptor-
35 negative cells, and addition of Fe³⁺ prevented the anti-HCMV effects, indicating that it
36 inhibits HCMV replication via iron chelation. This may be of particular interest for the
37 treatment of cytopenias after haematopoietic stem cell transplantation, as HCMV
38 reactivation is a major reason for transplantation failure. Since therapeutic
39 eltrombopag concentrations are effective against drug-resistant viruses and
40 synergistically increase the effects of ganciclovir, eltrombopag is also a drug
41 repurposing candidate for the treatment of therapy-refractory HCMV disease.

42

43 **Key words:** human cytomegalovirus, antiviral therapy, eltrombopag, thrombopoietin
44 receptor agonist, drug resistance, iron chelation

45

46

47 **Introduction**

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

52 Human cytomegalovirus (HCMV) reactivation and HCMV-associated disease
53 are leading reasons for the failure of haematopoietic stem cell transplantations [10-
54 12]. Anti-HCMV drugs including ganciclovir, cidofovir, and foscarnet are available, but
55 their use is associated with severe side effects [13]. In particular, the use of ganciclovir
56 (and its prodrug valganciclovir), the mainstay treatment for cytomegalovirus disease,
57 is associated with severe haematological side effects including thrombocytopenia [14-
58 16].

59 A case report described the use of eltrombopag in an immunocompetent patient
60 who suffered from human cytomegalovirus (HCMV)-associated thrombocytopenia
61 [17]. Immunosuppressive treatment for thrombocytopenia (prednisone, intravenous
62 immunoglobulin, dapsone) in combination with antiviral therapy (ganciclovir/
63 valganciclovir, HCMV hyperimmune globulin) only resulted in a temporary platelet
64 response with subsequent relapse. A change to eltrombopag intended to increase
65 platelet counts without immunosuppressive therapy resulted in a durable increase in
66 platelet levels, no evidence of HCMV viraemia, and the resolution of symptoms [17].
67 The observed effects were attributed to eltrombopag overcoming HCMV-induced
68 suppression of platelet production [17]. However, we hypothesised that direct antiviral
69 effects may also have contributed to the beneficial outcome in the case report of the
70 patient with HCMV-associated thrombocytopenia [17]. Indeed, we found that
71 eltrombopag exerts anti-HCMV effects via iron chelation.

72 **Materials and Methods**

73 **Drugs**

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

79

80 **Cells and viruses**

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

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

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

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

94

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 Calcsyn
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 $(CI_{50} + 2 \times CI_{75} + 3 \times$
117 $CI_{90} + 4 \times CI_{95}) / 10$. 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}

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

121

122 **Viability assay**

123 Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-
124 diphenyltetrazolium bromide (MTT) dye reduction assay as described previously [23].
125 Confluent cell cultures in 96-well microtiter plates were incubated with culture medium
126 containing serial dilutions of the indicated substances. After five days of incubation
127 MTT (1 mg/ml) was added and after an additional four hours, cells were lysed in a
128 buffer containing 20% (w/v) SDS and 50% N,N-dimethylformamide adjusted to pH 4.5.
129 Absorbance was determined at 570 nm for each well using a 96-well multiscanner.
130 After subtracting background absorbance, cell viability was expressed in per cent
131 relative to untreated control cells. Drug concentrations that reduced cell viability by
132 50% (CC_{50}) were calculated using CalcuSyn (Biosoft, Cambridge, United Kingdom).

133

134 **Virus yield assay**

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

138

139 **Immunoblotting**

140 Immunoblotting was performed as described previously [23]. In brief, cells were
141 lysed in Triton X-100 sample buffer and proteins separated by sodium dodecyl sulfated
142 (SDS) SDS-PAGE. Proteins were detected using specific antibodies against β -actin
143 (3598R-100-BV, BioVision via BioCat, Heidelberg, Germany) or HCMV 45 kDa late

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

147

148 **Statistics**

149 Values presented are the mean \pm S.D. of three independent biological repeats.

150 Comparisons between two groups were performed using Student's t-test, three and

151 more groups were compared by ANOVA followed by the Student-Newman-Keuls test.

152 Data groups were considered significantly different at $P < 0.05$.

153

154

155 **Results**

156

157 **Eltrombopag inhibits HCMV replication in human foreskin fibroblasts by** 158 **interference with late processes of the replication cycle**

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

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

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

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

191

192 **Eltrombopag inhibits HCMV expression via iron chelation**

193 Eltrombopag was developed as thrombopoietin receptor agonist [1-3].
194 However, it is unlikely that eltrombopag inhibits HCMV replication via thrombopoietin
195 receptor activation, because fibroblasts do not express the thrombopoietin receptor
196 [28]. In agreement, eltrombopag also inhibited murine cytomegalovirus replication in
197 murine NIH/3T3 fibroblasts (Figure 3A), although eltrombopag does not target the
198 murine thrombopoietin receptor [29].

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

204 mechanism by which eltrombopag exerts its anti-HCMV effects (Figure 3B). Since
205 equimolar Fe(III)Cl₃ concentrations prevented the anti-HCMV effects of eltrombopag
206 (Figure 3B), we concluded that iron chelation is the main mechanism of eltrombopag's
207 anti-HCMV activity.

208

209 **Eltrombopag exerts synergistic effects with ganciclovir**

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

216

217 **Eltrombopag is effective in different cell types and against different virus strains** 218 **and isolates including drug-resistant ones**

219 Finally, we investigated the effects of eltrombopag against a broader range of
220 laboratory virus strains and clinical isolates in HFFs and primary adipose-derived adult
221 mesenchymal stem cells (ASCs), another cell type that supports HCMV replication
222 [39]. The laboratory HCMV strains included Davis [40] and Towne [41] in addition to
223 Hi91. The clinical isolates U1, U59, and U75 were isolated from the urine of patients
224 as previously described [20,21]. U1 and U59 harbour a A987G mutation in the HCMV
225 DNA polymerase UL54 (Table 1), which is known to confer combined ganciclovir and
226 cidofovir resistance [42,43]. U1 also displays a C607Y mutation in the HCMV kinase
227 UL97 (Table 1), which is associated with ganciclovir resistance [44,45]. In agreement,
228 U1 and U59 were characterised by high ganciclovir and cidofovir IC₅₀s (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 IC₅₀s 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)Cl₃. The presence of equimolar
241 Fe³⁺ 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

245 Discussion

246 Here, we show that the approved thrombopoietin receptor agonist eltrombopag
247 exerts anti-HCMV effects in various cell types infected with a range of different virus
248 strains and clinical isolates including drug-resistant ones. The observed IC₅₀ values
249 ranged from 99nM to 4331nM, which is in the range of therapeutic plasma
250 concentrations that have been reported to exceed 45µM [25,26]. Eltrombopag also
251 synergistically increased the activity of the approved anti-HCMV drug ganciclovir.

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

261 The anti-HCMV effects of eltrombopag are unlikely to be caused by action on
262 the thrombopoietin receptor, since eltrombopag was effective in cell types that do not
263 express the thrombopoietin receptor, which is expressed in haematopoietic cells
264 [28,29]. In agreement, eltrombopag also exerted antiviral effects in mouse fibroblasts
265 infected with murine CMV, although the haematological effects of eltrombopag are
266 known to be species-specific and to not affect mice [28,29].

267 Eltrombopag is also known to be an iron chelator [30,31]. Addition of Fe³⁺
268 prevented the eltrombopag-mediated anti-HCMV effects in strain Hi91- and clinical

269 isolate U1-infected cells. Hence, our data suggest that eltrombopag inhibits HCMV
270 replication via Fe³⁺ chelation.

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

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

294 In conclusion, therapeutic eltrombopag concentrations inhibit HCMV replication
295 via chelation of Fe³⁺ 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.

300

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465 **Figure legends**

466

467 **Figure 1.** Effects of eltrombopag on HCMV late antigen (LA) expression in primary
468 human foreskin fibroblasts (HFFs). A) Representative dose response curves showing
469 the effects of eltrombopag on HCMV LA expression and HFF viability (as determined
470 after 120h of incubation). Eltrombopag concentrations that reduce HCMV LA
471 expression by 50% (IC₅₀) and the viability of proliferating HFFs by 50% (CC₅₀) relative
472 to untreated controls are also provided. Eltrombopag was continuously present from
473 the time of virus infection. B) Representative photographs and Western blots
474 demonstrating the effects of eltrombopag on HCMV LA expression. In A) and B), HFFs
475 were infected with HCMV strain Hi91 (MOI 0.02). HCMV late antigen (LA) expression
476 was detected 120h post infection. C) Representative dose-response curves and IC₅₀
477 values indicating effects of eltrombopag on HCMV LA expression in HFFs infected
478 with different MOIs of HCMV strain Hi91 as detected 120h post infection.

479

480 **Figure 2.** Effects of eltrombopag on HCMV replication and at different stages of the
481 viral replication cycle. HFFs were infected with HCMV strain Hi91 (MOI 0.02). HCMV
482 late antigen (LA) expression and virus titres were detected 120h post infection. A)
483 Virus titres in the absence or presence of eltrombopag. B) Representative dose-
484 response curves and IC₅₀ values indicating the effects of eltrombopag on
485 HCMV LA expression after 24h of pre-treatment, after treatment during the 1h
486 adsorption period, after drug addition post infection following the 1h virus adsorption
487 period, after drug addition 24h post infection, and after drug addition 48h post infection.

488

489 **Figure 3.** Eltrombopag inhibits HCMV infection by iron depletion. A) Representative
490 dose response curve indicating the effects of eltrombopag on cytopathogenic effect
491 (CPE) formation (detected 120h post infection) in murine cytomegalovirus (MOI 1)-
492 infected murine NIH/3T3 fibroblasts and eltrombopag concentration that reduces CPE
493 formation by 50% (IC₅₀) relative to untreated control. The findings indicate that
494 eltrombopag interferes with cytomegalovirus replication by thrombopoietin receptor-
495 independent effects, since eltrombopag does not activate the murine thrombopoietin
496 receptor. The investigated eltrombopag concentrations did not affect NIH/3T3 cell
497 viability. B) Representative growth curve indicating the effects of equimolar
498 concentrations of Fe(III)Cl₃ on the anti-HCMV effects of eltrombopag as indicated by
499 HCMV LA expression in HCMV Hi91 (MOI 0.02)-infected human foreskin fibroblasts
500 (HFFs) 120h post infection. Equimolar Fe(III)Cl₃ concentrations circumvent the anti-
501 HCMV effects exerted by eltrombopag.

502

503 **Figure 4.** Antiviral effects of eltrombopag in combination with ganciclovir. A) Effects of
504 equimolar drug concentrations on HCMV LA expression in HCMV Hi91 (MOI 0.02)-
505 infected human foreskin fibroblasts (HFFs) 120h post infection. *P < 0.05 compared
506 to either single treatment; B) Combination indices (CIs) at different levels of inhibition
507 and weighted average CI values (CI_{wt}) calculated as $(CI_{50} + 2 \times CI_{75} + 3 \times CI_{90} + 4 \times$
508 $CI_{95}) / 10$ [24]. CI_{wt} values ≤ 0.7 indicate synergistic effects [24].

509

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

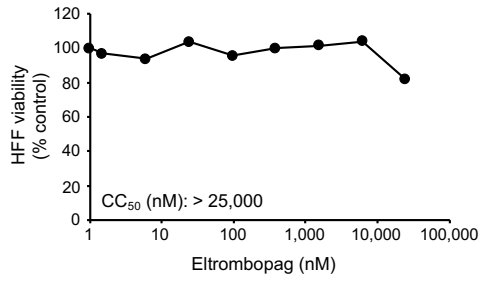
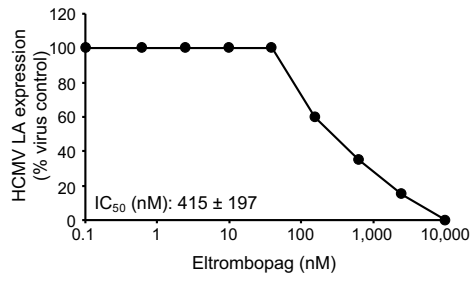
514 was determined 120h post infection. A) Eltrombopag concentrations that reduce
515 HCMV LA expression by 50% (IC₅₀). Numerical values are provided in Suppl. Table
516 2. The investigated eltrombopag concentrations did not affect cell viability. B) Average
517 eltrombopag IC₅₀s for each virus strain and isolate in HFFs and ASCs. C) Average
518 eltrombopag IC₅₀s across virus strains and isolates in HFFs and ASCs. D)
519 Representative growth curve indicating the effects of equimolar concentrations of
520 Fe(III)Cl₃ on the anti-HCMV effects of eltrombopag as indicated by HCMV LA
521 expression in U1 (MOI 0.02)-infected HFFs 120h post infection.

522

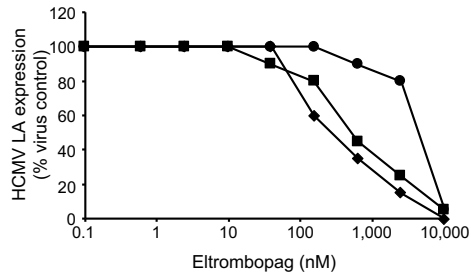
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Figure 1

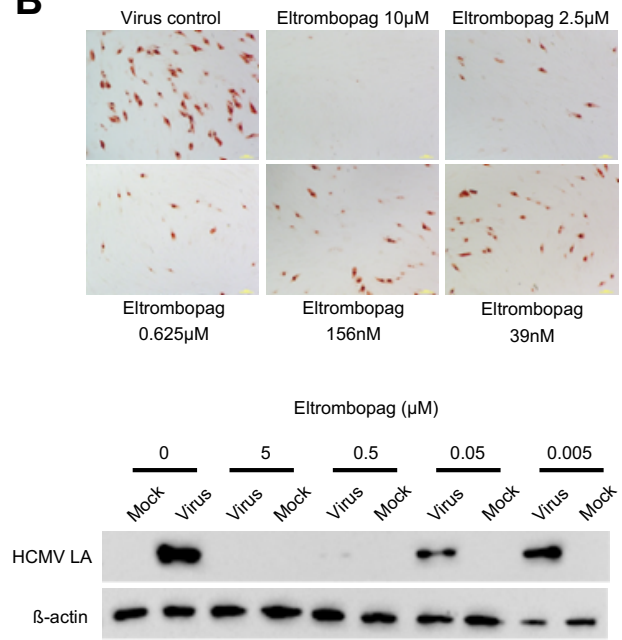
A



C



B



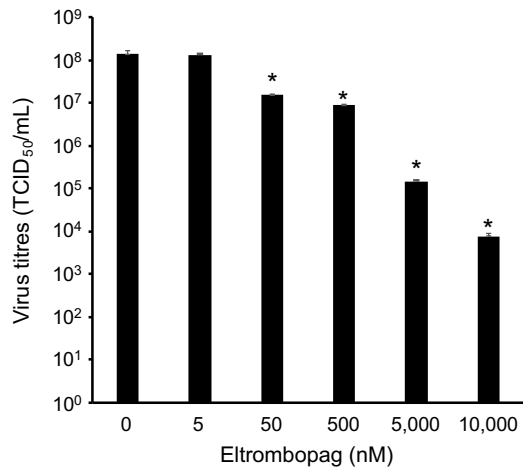
- MOI 1: IC_{50} (nM): 3844 ± 705
- MOI 0.1: IC_{50} (nM): 855 ± 429
- ◆ MOI 0.01: IC_{50} (nM): 251 ± 135

524

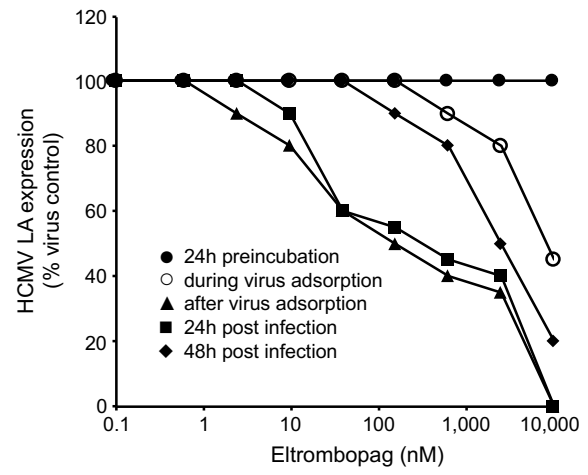
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Figure 2

A



B

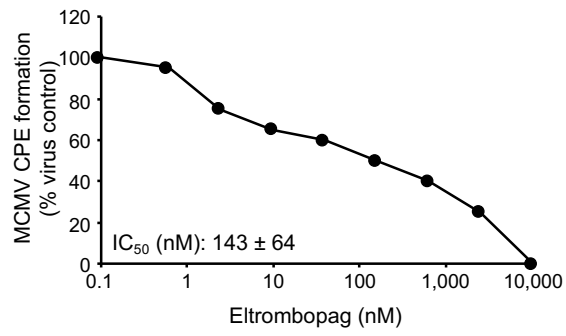


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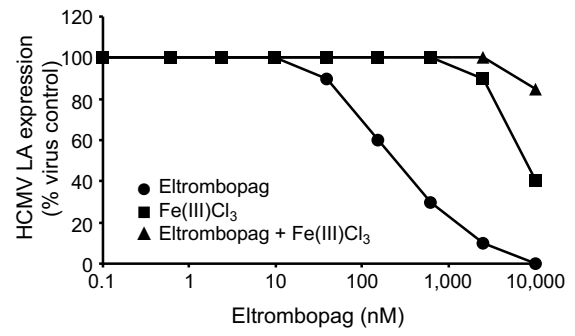
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Figure 3

A



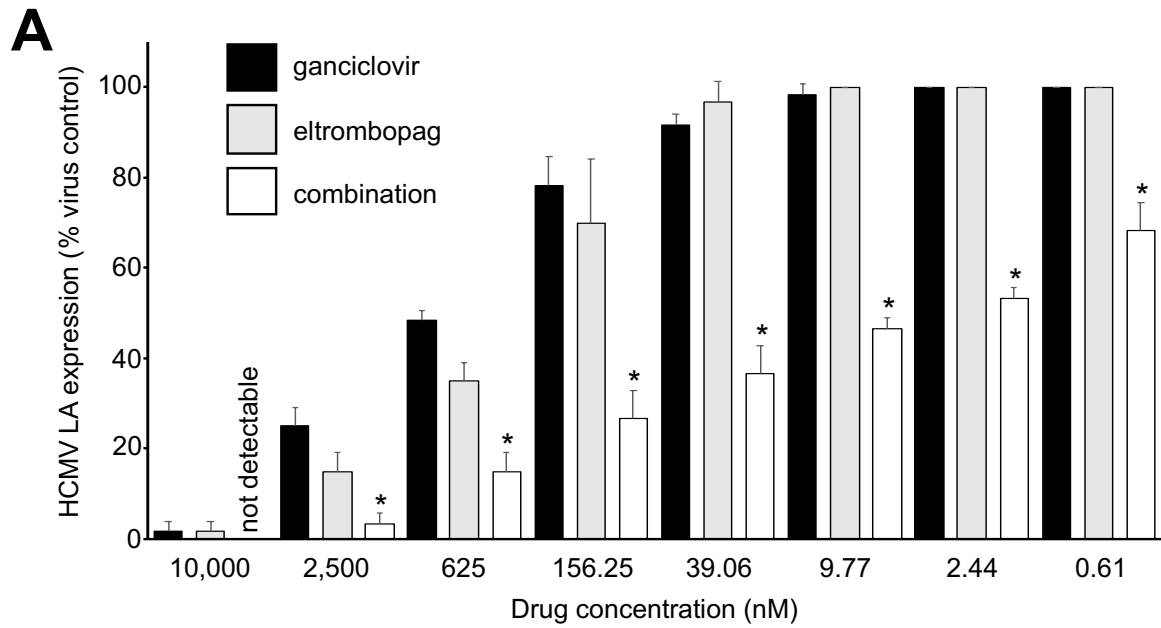
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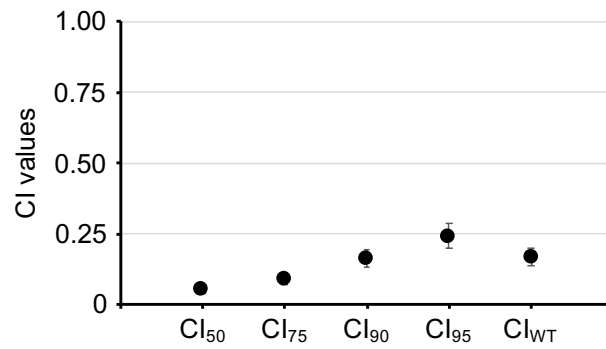
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Figure 4



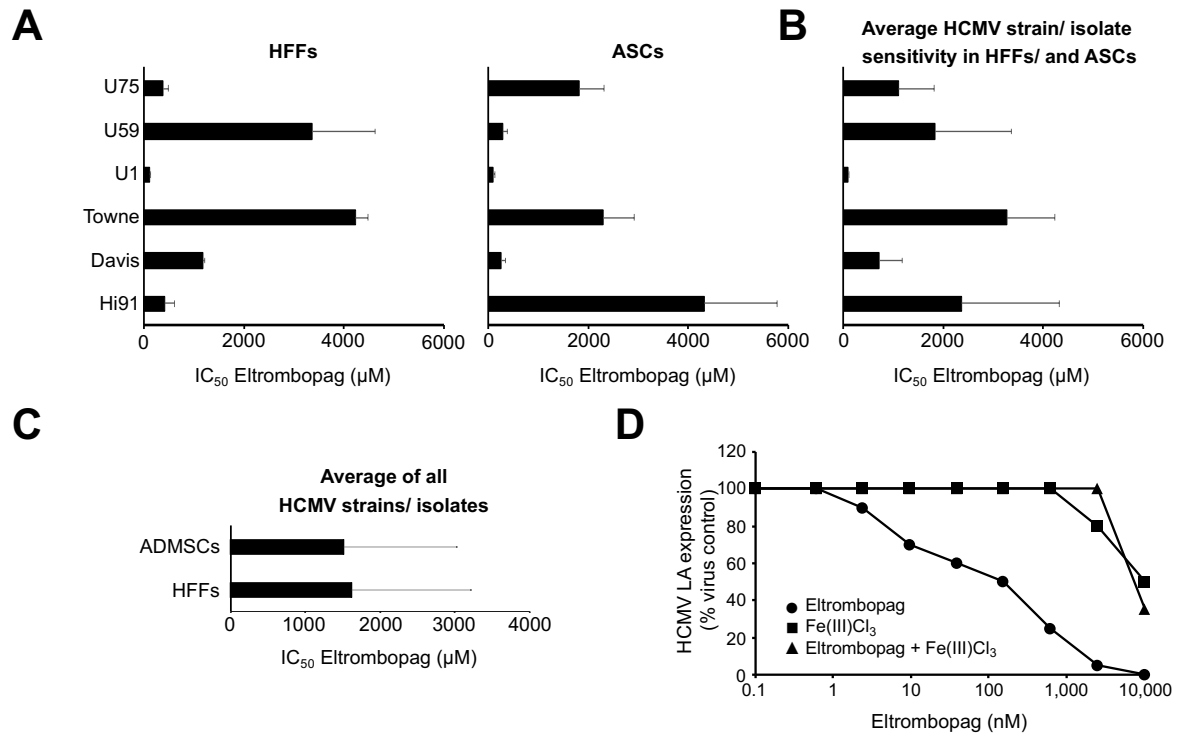
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Figure 5



532

533