

1 **Inhibition of Human Immunodeficiency Virus Type 1 Assembly and Release by the**  
2 **Cholesterol-binding Compound Amphotericin B Methyl Ester: Evidence for Vpu**  
3 **Dependence**  
4

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31 **ABSTRACT**

32

33 We investigate the mechanism by which the cholesterol-binding compound,  
34 amphotericin B methyl ester (AME) inhibits human immunodeficiency virus type 1  
35 (HIV-1) particle production. We observe no significant effect of AME on Gag binding to  
36 the plasma membrane, Gag association with lipid rafts, or Gag multimerization,  
37 indicating that the mechanism of inhibition by AME is distinct from that imposed by  
38 cholesterol depletion. Electron microscopy analysis indicates that AME significantly  
39 disrupts virion morphology. Interestingly, we found that AME does not inhibit the release  
40 of Vpu-defective HIV-1 or Vpu(-) retroviruses such as murine leukemia virus and simian  
41 immunodeficiency virus. We demonstrate that the ability of Vpu to counter the activity  
42 of CD317/BST-2/tetherin is markedly reduced by AME. These results indicate that AME  
43 interferes with the anti-CD317/BST-2/tetherin function of Vpu.

44 A number of studies have demonstrated that cholesterol-enriched plasma membrane  
45 microdomains known as lipid rafts (3, 9, 28) play important roles in the replication of a  
46 number of enveloped viruses including HIV-1 (22). Lipid rafts appear to function in both  
47 virus entry and particle egress and the HIV-1 lipid bilayer itself exhibits a raft-like lipid  
48 composition (2, 4). We previously reported that cholesterol depletion interferes with  
49 HIV-1 particle production by impairing the association of Gag with membrane (21, 24).  
50 Adding further support to the concept that membrane cholesterol plays an important role  
51 in HIV-1 biology, we recently demonstrated that the cholesterol-binding compound  
52 amphotericin B methyl ester (AME), a water-soluble, relatively non-cytotoxic derivative  
53 of amphotericin B, potently inhibits HIV-1 replication (34). The antiviral activity of  
54 AME is due to profoundly impaired viral infectivity as well as defective virus particle  
55 production (34). Interestingly, passaging of HIV-1 in the presence of AME led to viral  
56 escape from this compound; mutations that conferred resistance mapped to the  
57 cytoplasmic tail of gp41 (34, 35). This analysis revealed a novel mechanism of resistance  
58 whereby the gp41 mutations conferred resistance to AME by creating PR cleavage sites  
59 in the gp41 cytoplasmic tail, leading to truncation of gp41 after Env incorporation into  
60 virions (35). While the resistant mutants overcame the entry defect imposed by AME,  
61 they remained sensitive to the AME-imposed disruption of particle assembly and release  
62 (34, 35). In this study, we investigated the mechanism by which AME inhibits HIV-1  
63 assembly and release by evaluating the effect of this compound on the specific steps of  
64 the assembly and release pathway and involvement of viral proteins other than Gag in the  
65 AME-imposed inhibition of particle production.

66

67 **AME inhibits HIV-1 particle production with no significant effect on Gag-**  
68 **membrane binding, raft association, or Gag multimerization.** We previously  
69 demonstrated that AME inhibits HIV-1 replication in T-cell lines and primary cell types  
70 (34). The inhibitory effect of AME on viral replication appeared to be due predominantly  
71 to a 50-100-fold reduction in viral infectivity. However, we also noted a significant (~ 4-  
72 fold) impairment in HIV-1 particle production from infected Jurkat cells. To understand  
73 the mechanism by which AME inhibits virus release, in this study we first examined the  
74 effect of AME on particle production from HeLa cells following transfection with the  
75 full-length, infectious HIV-1 molecular clone pNL4-3. Transfected cells were  
76 metabolically radiolabeled, and cell- and virus-associated proteins were  
77 immunoprecipitated and quantified. Virus release efficiency was reduced in a  
78 concentration-dependent manner; treating virus-producing cells with 5  $\mu$ M AME reduced  
79 virus production by approximately three-fold, whereas 10  $\mu$ M AME reduced virus  
80 production over five-fold compared with untreated controls (Fig. 1A). Having established  
81 that AME treatment impairs HIV-1 particle production in multiple cell types, we next  
82 determined whether AME has any effect on Gag binding to membrane or on Gag  
83 association with detergent-resistant membrane (DRM), a commonly used biochemical  
84 surrogate for raft association. The steady-state distribution of Gag in membrane and  
85 DRM was monitored by immunoblotting. Virus-expressing HeLa cells were  
86 homogenized, divided into two aliquots, treated with or without a 0.25% final  
87 concentration of cold Triton X-100, and subjected to equilibrium flotation centrifugation  
88 on sucrose gradients as described previously (18, 19, 21, 25). The distribution of Gag in  
89 membrane and DRM fractions was determined by immunoblotting. We observed no

90 significant effect of AME treatment on the distribution of Gag in total membrane or  
91 DRM (data not shown). This finding was confirmed by analyzing Gag association with  
92 membrane and DRM following pulse-chase labeling. We detected ~36% and ~43% of  
93 Pr55<sup>Gag</sup> in membrane fractions without or with AME treatment, respectively (Fig. 1B).  
94 After cold Triton X-100 treatment, ~25% of Pr55<sup>Gag</sup> was associated with DRM without  
95 AME treatment and ~32% of Gag was DRM associated following treatment with 10  $\mu$ M  
96 AME (Fig. 1B). These results indicate that the defect in virus release caused by AME is  
97 not due to disrupted association of Gag with total membrane or with DRM. This is in  
98 contrast to results obtained with cholesterol-depleting agents, which significantly impair  
99 Gag-membrane binding (24). We also observed that AME treatment did not affect the  
100 distribution of the raft marker caveolin, or the nonraft marker transferrin receptor (TfR),  
101 with membrane or DRM (data not shown). To determine whether AME treatment of  
102 virus-producer cells affects higher-order Gag multimerization, we used a cell-based assay  
103 that measures assembly-induced masking of epitopes recognized by anti-Gag Abs (25).  
104 As reported in our earlier study (25), the effect of sample denaturation on  
105 immunoprecipitation efficiency provides a measure of higher-order Gag multimerization.  
106 Gag-expressing cells treated or not with AME were metabolically radiolabeled and cell  
107 lysates were immunoprecipitated with or without prior denaturation. We observed that  
108 ~30% of membrane-bound Gag is epitope-exposed and that AME treatment does not  
109 have a significant effect on this value (Fig. 1C). Similarly, the degree of epitope exposure  
110 of DRM-associated Gag was not affected by AME treatment (Fig. 1C). These data  
111 indicate that the higher-order multimerization of membrane-bound or DRM-associated  
112 Gag measured in this assay is not affected by treatment of Gag-expressing cells with

113 AME. We previously reported (34) that propagation of HIV-1 in the presence of AME  
114 leads to the emergence of AME-resistant variants. Mutations responsible for AME  
115 resistance (gp41 mutations P203L and S205L) map to a region of the gp41 cytoplasmic  
116 tail close to the membrane-spanning domain. To determine whether AME-resistant  
117 mutants overcome the defect in particle production caused by AME, we measured the  
118 release of AME-resistant mutants in the presence and absence of AME. We observed  
119 that the release of the AME-resistant mutants was inhibited by AME to an extent similar  
120 to that of WT (Fig. 1D). Thus, the mutations in gp41 that induce resistance to AME in the  
121 context of virus replication and single-cycle infectivity assays do not reverse the effects  
122 of AME on virus particle production.

123  
124 **AME does not affect the subcellular localization of Gag but alters the morphology**  
125 **and density of released HIV-1 particles.** Standard membrane flotation assays do not  
126 distinguish between Gag bound to the plasma membrane and Gag associated with  
127 intracellular membrane. To determine whether AME treatment affects the trafficking of  
128 Gag to the plasma membrane, we compared the localization of Gag in AME-treated vs.  
129 untreated cells by immunostaining. HeLa cells transiently transfected with pNL4-3 were  
130 treated overnight with AME, fixed, immunostained with anti-MA antibodies, and  
131 analyzed by fluorescence microscopy. Gag in cells either treated or not treated with AME  
132 displayed a predominantly punctate, plasma membrane localization pattern (data not  
133 shown) indicating that AME did not induce a major change in Gag trafficking.

134 Because AME treatment does not affect Gag binding to the plasma membrane or  
135 Gag multimerization, we examined whether this cholesterol-binding compound might

136 disrupt virion budding from the cell surface. HeLa cells transfected with pNL4-3 were  
137 treated with 10  $\mu$ M AME or were left untreated and were subsequently fixed and  
138 examined by transmission EM. In both AME-treated and untreated cells, numerous  
139 released mature particles were observed, with no striking accumulation of immature  
140 particles at the plasma membrane detected in treated cells (data not shown). These  
141 results indicate that AME does not act by disrupting HIV-1 late domain function.

142 It has been reported that the lipid bilayer of HIV-1 virions is enriched in  
143 cholesterol relative to the host cell plasma membrane (2, 4), raising the possibility that, as  
144 a cholesterol-binding compound, AME could bind preferentially to the viral vs. cellular  
145 membrane. To address this issue, we examined the impact on virion morphology of  
146 treating purified virions with AME *in vitro*. Intriguingly, the morphology of AME-treated  
147 viral particles differed significantly from that of untreated virions, with severely distorted  
148 viral membrane observed (Fig. 2A). The extent of virion distortion was quantified by  
149 measuring the deviation from circularity of 100 treated and untreated particles. This  
150 analysis was performed by drawing a circle around each virion and measuring the  
151 distance between the viral membrane and the periphery of the circle along the radius.  
152 Virions whose circularity deviated by more than 20% of the radius were scored as  
153 distorted. Approximately 80% of the AME-treated virions were classified as being  
154 distorted whereas only 11% of untreated virions were distorted. To examine the density  
155 of virions treated with AME *in vitro* we sedimented particles treated or not with AME on  
156 linear sucrose gradients. A marked increase in virion density was observed in the AME-  
157 treated samples (Fig. 2B). The perturbation of virion morphology and density induced by  
158 AME treatment may be a major contributor to the previously reported (34, 35) infectivity

159 defect imposed by this cholesterol-binding compound and is likely to result from direct  
160 binding of AME to cholesterol in the viral membrane.

161

162 **Release of the Fyn(10)fullMA Gag chimera is inhibited by AME.** We reported  
163 recently that the inhibition of virus release caused by cholesterol depletion is due to  
164 disrupted Gag–membrane binding and impaired higher-order Gag multimerization (24).  
165 We observed that fusing the Fyn membrane-binding signal to the N-terminus of Gag  
166 [Fyn(10)fullMA] reverses the impact of cholesterol depletion on virus production. To  
167 determine whether the same effect is seen with the cholesterol-binding compound AME,  
168 we performed virus release assays with Fyn(10)fullMA Gag in the presence or absence of  
169 AME. Interestingly, the release of Fyn(10)fullMA Gag is reduced by AME to an extent  
170 similar to that of the WT (Fig. 3). These results, together with the findings presented  
171 above that AME does not inhibit Gag–membrane binding or Gag multimerization,  
172 indicate that the mechanism by which AME inhibits HIV-1 particle production is distinct  
173 from the mechanism by which cholesterol-depleting agents disrupt virus assembly and  
174 release.

175

176 **Inhibition of virus release by AME is Vpu-dependent.** The experiments described  
177 above were performed in the context of a full-length, infectious HIV-1 molecular clone  
178 encoding all HIV-1 proteins. To gain further insights into the mechanism by which AME  
179 inhibits HIV-1 particle production, we examined a potential role for viral proteins other  
180 than Gag in the ability of AME to disrupt the late stages of the viral replication cycle. We  
181 evaluated the effect of AME on the assembly and release of virus particles in the context

182 of clones defective for PR, Env, Nef or Vpu. As shown in Fig. 4A, the effect of AME on  
183 the release of HIV-1 mutants defective for PR, Env or Nef was comparable to that  
184 observed for WT, indicating that expression of these proteins is not required for the  
185 AME-imposed inhibition of particle production. In contrast, we observed that mutation of  
186 Vpu largely abrogated the ability of AME to interfere with HIV-1 assembly and release  
187 (Fig. 4B). As reported (8, 13, 30, 31), deletion of Vpu reduces particle production ~10  
188 fold ( $9.3 \pm 2.4\%$  relative to WT). AME treatment at 5  $\mu\text{M}$  had no effect on the release of  
189 Vpu-defective HIV-1 and at 10  $\mu\text{M}$  caused a reduction in Vpu(-) particle release of only  
190 ~30%, compared to the 70% reduction observed with the WT at 10  $\mu\text{M}$  AME (Fig. 4B).

191 To examine the possibility that the release of Vpu-defective HIV-1 is so low that  
192 further reductions imposed by AME treatment are not detectable, we measured the effect  
193 of AME on the release of a late-domain-deficient (PTAP<sup>-</sup>) p6 mutant (11). The release of  
194 the PTAP<sup>-</sup> mutant was reduced by >10-fold (to  $7.2 \pm 1.2\%$  WT levels), yet, unlike the  
195 Vpu-defective mutant, AME treatment further reduced the release of this mutant by  
196 ~four-fold (Fig. 4C). We also observed that AME treatment significantly impaired the  
197 production of a pNL4-3 derivative bearing mutations in the MA domain of Gag  
198 (29KE/31KE) (23) (Fig. 4C). The 29KE/31KE mutant exhibits a ~four-fold defect in  
199 virus release efficiency due to the retargeting of Gag to multivesicular bodies (20, 23).  
200 Thus, the inability of AME to potently inhibit the release of Vpu-defective HIV-1 is not  
201 due simply to the inefficient release of this mutant.

202 To confirm the requirement for Vpu expression in the ability of AME to inhibit  
203 particle production, we examined the effect of AME on the release of two retroviruses  
204 that do not encode Vpu: SIVmac239 and MLV. We observed that 5  $\mu\text{M}$  treatment did not

205 reduce SIVmac239 release and that 10  $\mu$ M AME reduced particle production by only  
206 ~20% (Fig. 4D). Similarly, MLV release was only minimally affected by AME treatment  
207 (Fig. 4D). These results are consistent with a connection between Vpu expression and the  
208 AME-imposed defect in virus particle production. To support the finding that Vpu  
209 deletion reverses the ability of AME to inhibit HIV-1 particle production, we constructed  
210 a Vpu-deficient variant of the Fyn(10)fullMA molecular clone [Fyn(10)fullMA $\Delta$ Vpu].  
211 We observed that the release of the Fyn(10)fullMA $\Delta$ Vpu mutant was not significantly  
212 reduced by AME (Fig. 3). These results again highlight that inhibition of HIV-1 particle  
213 production by AME exhibits a clear Vpu dependence.

214  
215 **AME inhibits the ability of Vpu to counter the host factor CD317/BST-2/tetherin.**

216 Two recent studies demonstrated that Vpu promotes virus release by counteracting the  
217 ability of CD317/BST-2/tetherin to retain HIV-1 virions at the cell surface (15, 32).  
218 Because the data presented above provide evidence that the effect of AME on virus  
219 release is Vpu-dependent, we tested whether AME might prevent Vpu from counteracting  
220 the virus-tethering activity of CD317/BST-2/tetherin. To investigate the relationship  
221 between AME inhibition and the counteraction of CD317/BST-2/tetherin activity by  
222 Vpu, we analyzed virus release in the 293T cell line, which does not express appreciable  
223 levels of endogenous tetherin. First, we confirmed that CD317/BST-2/tetherin  
224 overexpression in 293T cells induced a strong (~10-fold) inhibition of Vpu-defective  
225 HIV-1 release but had little effect on the release of WT HIV-1 (data not shown). We  
226 next tested the effect of AME on virus release in this context. We observed that in the  
227 presence of CD317/BST-2/tetherin overexpression, AME had no significant effect on the

228 production of Vpu-defective particles (Fig. 5). This result recapitulates the lack of a  
229 major effect of AME on Vpu-defective virus release in HeLa cells, which constitutively  
230 express CD317/BST-2/tetherin. Coexpression of Vpu reversed the block in Vpu-defective  
231 virus release (Fig. 5), consistent with previous reports (15, 32). Interestingly, this Vpu-  
232 induced rescue of virus release was to a large extent inhibited by AME; in the context of  
233 Vpu-defective HIV-1 with exogenous Vpu and CD317/BST-2/tetherin expression, AME  
234 inhibited virus release by ~5-fold (Fig. 5). These results suggest a model whereby AME  
235 inhibits HIV-1 particle production at least in part by interfering with the ability of Vpu to  
236 counter the virus-retaining function of CD317/BST-2/tetherin. The absence of an  
237 accumulation of mature virions tethered to the cell surface in AME-treated cells suggests  
238 that AME does not fully block Vpu function. In addition, we observed some reduction  
239 (~2-fold) in particle release from pNL4-3-transfected 293T cells (data not shown), which  
240 are not Vpu-responsive (15, 33), suggesting that in this cell line AME may impose  
241 additional defects in particle production not directly related to CD317/BST-2/tetherin.

242 In conclusion, in this report we evaluated the mechanism by which the  
243 cholesterol-binding compound AME disrupts HIV-1 particle production. Our  
244 biochemical analyses indicated that treating virus-producing cells with AME had no  
245 significant impact on Gag-membrane binding, DRM association, or higher-order Gag  
246 multimerization. Subcellular Gag localization was also not substantially affected by  
247 AME. However, AME treatment induced a shift in the density of membrane-associated  
248 Gag and caused distortions in the lipid bilayer of treated virions, suggesting that AME  
249 binding alters the properties of the viral membrane. Experiments designed to test whether  
250 AME-induced disruption of virus release was linked to the expression of virally encoded

251 proteins other than Gag revealed a requirement for Vpu expression in AME-imposed  
252 virus release inhibition. We speculate that AME binding could directly block the ion  
253 channel activity of Vpu (5, 27) or could indirectly alter Vpu function via  
254 cholesterol/membrane binding. We are currently investigating the cell surface  
255 localization, lipid raft association, and co-localization of CD317/BST-2/tetherin with  
256 HIV-1 Gag in presence of AME when Vpu is coexpressed. Given that Vpu plays an  
257 important role in lentiviral pathogenesis in vivo (10), this accessory protein may  
258 represent a viable target for the development of antiretroviral agents.

259

ACCEPTED

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261

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275 **References**

- 276 1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and**  
277 **M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-  
278 associated retrovirus in human and nonhuman cells transfected with an infectious  
279 molecular clone. *J Virol* **59**:284-91.
- 280 2. **Aloia, R. C., H. Tian, and F. C. Jensen.** 1993. Lipid composition and fluidity of  
281 the human immunodeficiency virus envelope and host cell plasma membranes.  
282 *Proc Natl Acad Sci U S A* **90**:5181-5.
- 283 3. **Brown, D. A., and E. London.** 2000. Structure and function of sphingolipid- and  
284 cholesterol-rich membrane rafts. *J Biol Chem* **275**:17221-4.
- 285 4. **Brugger, B., B. Glass, P. Haberkant, I. Leibrecht, F. T. Wieland, and H. G.**  
286 **Krausslich.** 2006. The HIV lipidome: A raft with an unusual composition. *Proc*  
287 *Natl Acad Sci U S A.*
- 288 5. **Ewart, G. D., T. Sutherland, P. W. Gage, and G. B. Cox.** 1996. The Vpu  
289 protein of human immunodeficiency virus type 1 forms cation-selective ion  
290 channels. *J Virol* **70**:7108-15.
- 291 6. **Freed, E. O., and M. A. Martin.** 1994. Evidence for a functional interaction  
292 between the V1/V2 and C4 domains of human immunodeficiency virus type 1  
293 envelope glycoprotein gp120. *J Virol* **68**:2503-12.
- 294 7. **Freed, E. O., and M. A. Martin.** 1995. Virion incorporation of envelope  
295 glycoproteins with long but not short cytoplasmic tails is blocked by specific,  
296 single amino acid substitutions in the human immunodeficiency virus type 1  
297 matrix. *J Virol* **69**:1984-9.

- 298 8. **Gottlinger, H. G., T. Dorfman, J. G. Sodroski, and W. A. Haseltine.** 1991.  
299 Effect of mutations affecting the p6 gag protein on human immunodeficiency  
300 virus particle release. *Proc Natl Acad Sci U S A* **88**:3195-9.
- 301 9. **Hancock, J. F.** 2006. Lipid rafts: contentious only from simplistic standpoints.  
302 *Nat Rev Mol Cell Biol* **7**:456-62.
- 303 10. **Hill, M. S., A. Ruiz, E. Pacyniak, D. M. Pinson, N. Culley, B. Yen, S. W.**  
304 **Wong, and E. B. Stephens.** 2008. Modulation of the severe CD4(+) T-cell loss  
305 caused by a pathogenic simian-human immunodeficiency virus by replacement of  
306 the subtype B vpu with the vpu from a subtype C HIV-1 clinical isolate. *Virology*  
307 **371**:86-97.
- 308 11. **Huang, M., J. M. Orenstein, M. A. Martin, and E. O. Freed.** 1995. p6Gag is  
309 required for particle production from full-length human immunodeficiency virus  
310 type 1 molecular clones expressing protease. *J Virol* **69**:6810-8.
- 311 12. **Kiernan, R. E., A. Ono, G. Englund, and E. O. Freed.** 1998. Role of matrix in  
312 an early postentry step in the human immunodeficiency virus type 1 life cycle. *J*  
313 *Virol* **72**:4116-26.
- 314 13. **Klimkait, T., K. Strebel, M. D. Hoggan, M. A. Martin, and J. M. Orenstein.**  
315 1990. The human immunodeficiency virus type 1-specific protein vpu is required  
316 for efficient virus maturation and release. *J Virol* **64**:621-9.
- 317 14. **Landau, N. R., and D. R. Littman.** 1992. Packaging system for rapid production  
318 of murine leukemia virus vectors with variable tropism. *J Virol* **66**:5110-3.
- 319 15. **Neil, S. J., T. Zang, and P. D. Bieniasz.** 2008. Tetherin inhibits retrovirus release  
320 and is antagonized by HIV-1 Vpu. *Nature* **451**:425-30.

- 321 16. **Nguyen, K. L., M. Ilano, H. Akari, E. Miyagi, E. M. Poeschla, K. Strebel, and**  
322 **S. Bour.** 2004. Codon optimization of the HIV-1 vpu and vif genes stabilizes their  
323 mRNA and allows for highly efficient Rev-independent expression. *Virology*  
324 **319:163-75.**
- 325 17. **Ono, A., S. D. Ablan, S. J. Lockett, K. Nagashima, and E. O. Freed.** 2004.  
326 Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 Gag targeting to the  
327 plasma membrane. *Proc Natl Acad Sci U S A* **101:14889-94.**
- 328 18. **Ono, A., D. Demirov, and E. O. Freed.** 2000. Relationship between human  
329 immunodeficiency virus type 1 Gag multimerization and membrane binding. *J*  
330 *Virol* **74:5142-50.**
- 331 19. **Ono, A., and E. O. Freed.** 1999. Binding of human immunodeficiency virus type  
332 1 Gag to membrane: role of the matrix amino terminus. *J Virol* **73:4136-44.**
- 333 20. **Ono, A., and E. O. Freed.** 2004. Cell-type-dependent targeting of human  
334 immunodeficiency virus type 1 assembly to the plasma membrane and the  
335 multivesicular body. *J Virol* **78:1552-63.**
- 336 21. **Ono, A., and E. O. Freed.** 2001. Plasma membrane rafts play a critical role in  
337 HIV-1 assembly and release. *Proc Natl Acad Sci U S A* **98:13925-30.**
- 338 22. **Ono, A., and E. O. Freed.** 2005. Role of lipid rafts in virus replication. *Adv*  
339 *Virus Res* **64:311-58.**
- 340 23. **Ono, A., J. M. Orenstein, and E. O. Freed.** 2000. Role of the Gag matrix  
341 domain in targeting human immunodeficiency virus type 1 assembly. *J Virol*  
342 **74:2855-66.**

- 343 24. **Ono, A., A. A. Waheed, and E. O. Freed.** 2007. Depletion of cellular cholesterol  
344 inhibits membrane binding and higher-order multimerization of human  
345 immunodeficiency virus type 1 Gag. *Virology* **360**:27-35.
- 346 25. **Ono, A., A. A. Waheed, A. Joshi, and E. O. Freed.** 2005. Association of human  
347 immunodeficiency virus type 1 gag with membrane does not require highly basic  
348 sequences in the nucleocapsid: use of a novel Gag multimerization assay. *J Virol*  
349 **79**:14131-40.
- 350 26. **Regier, D. A., and R. C. Desrosiers.** 1990. The complete nucleotide sequence of  
351 a pathogenic molecular clone of simian immunodeficiency virus. *AIDS Res Hum*  
352 *Retroviruses* **6**:1221-31.
- 353 27. **Schubert, U., A. V. Ferrer-Montiel, M. Oblatt-Montal, P. Henklein, K.**  
354 **Strebel, and M. Montal.** 1996. Identification of an ion channel activity of the  
355 Vpu transmembrane domain and its involvement in the regulation of virus release  
356 from HIV-1-infected cells. *FEBS Lett* **398**:12-8.
- 357 28. **Simons, K., and D. Toomre.** 2000. Lipid rafts and signal transduction. *Nat Rev*  
358 *Mol Cell Biol* **1**:31-9.
- 359 29. **Smith, S. M., R. B. Markham, and K. T. Jeang.** 1996. Conditional reduction of  
360 human immunodeficiency virus type 1 replication by a gain-of-herpes simplex  
361 virus 1 thymidine kinase function. *Proc Natl Acad Sci U S A* **93**:7955-60.
- 362 30. **Strebel, K., T. Klimkait, F. Maldarelli, and M. A. Martin.** 1989. Molecular  
363 and biochemical analyses of human immunodeficiency virus type 1 vpu protein. *J*  
364 *Virol* **63**:3784-91.

- 365 31. **Terwilliger, E. F., E. A. Cohen, Y. C. Lu, J. G. Sodroski, and W. A.**  
366 **Haseltine.** 1989. Functional role of human immunodeficiency virus type 1 vpu.  
367 Proc Natl Acad Sci U S A **86**:5163-7.
- 368 32. **Van Damme, N., D. Goff, C. Katsura, R. L. Jorgenson, R. Mitchell, M. C.**  
369 **Johnson, E. B. Stephens, and J. Guatelli.** 2008. The interferon-induced protein  
370 BST-2 restricts HIV-1 release and is downregulated from the cell surface by the  
371 viral Vpu protein. Cell Host Microbe **3**:245-52.
- 372 33. **Varthakavi, V., R. M. Smith, S. P. Bour, K. Strebel, and P. Spearman.** 2003.  
373 Viral protein U counteracts a human host cell restriction that inhibits HIV-1  
374 particle production. Proc Natl Acad Sci U S A **100**:15154-9.
- 375 34. **Waheed, A. A., S. D. Ablan, M. K. Mankowski, J. E. Cummins, R. G. Ptak,**  
376 **C. P. Schaffner, and E. O. Freed.** 2006. Inhibition of HIV-1 replication by  
377 amphotericin B methyl ester: selection for resistant variants. J Biol Chem  
378 **281**:28699-711.
- 379 35. **Waheed, A. A., S. D. Ablan, J. D. Roser, R. C. Sowder, C. P. Schaffner, E.**  
380 **Chertova, and E. O. Freed.** 2007. HIV-1 escape from the entry-inhibiting effects  
381 of a cholesterol-binding compound via cleavage of gp41 by the viral protease.  
382 Proc Natl Acad Sci U S A **104**:8467-71.  
383

384 **FIGURE LEGENDS**

385

386 **Fig. 1. AME inhibits HIV-1 particle production with no significant effect on Gag**  
387 **binding to the plasma membrane, Gag association with lipid rafts, or Gag**

388 **multimerization.** (A) HeLa cells were transfected with pNL4-3 (1) and 6 h  
389 posttransfection treated with the indicated concentrations of AME for 20 – 24 h. One day  
390 posttransfection, cells were metabolically labeled for 2 h with [<sup>35</sup>S]Met/Cys, and labeled  
391 viral proteins in cell and virion lysates were immunoprecipitated with HIV-Ig and  
392 analyzed by SDS-PAGE followed by fluorography (6). Virus release efficiency was  
393 calculated as the amount of virion-associated p24 relative to total (cell plus virion) Gag.

394 (B) HeLa cells transfected with pNL4-3/PR<sup>-</sup> (11) were treated (+) or not treated (-) with  
395 10 μM AME and were pulse-labeled for 5 min and chased in unlabeled medium for 15  
396 min. Post-nuclear supernatants were incubated in the absence or presence of 0.25%  
397 Triton X-100 and subjected to membrane flotation centrifugation (18, 19, 21). Gradient  
398 fractions were treated with RIPA buffer and membrane and DRM fractions (fractions 1-  
399 5) were pooled. Labeled Pr55<sup>Gag</sup> in each pooled fraction was recovered by

400 immunoprecipitation after denaturation, and the amount of Gag present in membrane and  
401 DRM fractions compared to total Gag in all ten fractions was quantified. (C) HeLa cells

402 transfected with pNL4-3/PR<sup>-</sup> were treated (+) or not treated (-) with 10 μM AME and  
403 subjected to the epitope-exposure assay for higher-order Gag multimerization (25). The  
404 percentage of Gag epitope exposure in membrane and DRM fractions was determined.

405 (D) HeLa cells were transfected with pNL4-3 or the AME-resistant mutants (P203L and

406 S205L) (34), treated (+) or not (-) with 10  $\mu$ M AME and virus release efficiency was  
407 calculated as in A. Data represent means  $\pm$ SD, n = 3-5.

408

409 **Fig. 2. AME treatment distorts the morphology and increases the density of purified**  
410 **virions.** (A) Virions collected from HeLa cells 24 h posttransfection with pNL4-3 were  
411 treated (+) or not (-) with AME for 2 h, pelleted by ultracentrifugation, fixed, and  
412 analyzed by EM. Bar = 100 nm. (B) HIV-1 virions purified as in panel A and treated with  
413 the indicated concentrations of AME were layered onto 20 to 70% (w/v) linear sucrose  
414 density gradients and subjected to ultracentrifugation (12). Ten fractions (Fr. 1-10) were  
415 collected from the top of the gradient and analyzed by Western blotting with HIV-Ig.

416

417 **Fig. 3. Insertion of the membrane-targeting signal from c-Fyn does not diminish the**  
418 **ability of AME to disrupt virus particle production.** HeLa cells transfected with  
419 pNL4-3, pNL4-3/Fyn(10)fullMA (17) or pNL4-3/Fyn(10)fullMA/delVpu [constructed by  
420 exchanging the BssHII-SphI (pNL4-3 nt 711-1443) fragment of Fyn(10)fullMA (24) with  
421 the corresponding fragment from pNL4-3delVpu] were treated with the indicated  
422 concentrations of AME and metabolically labeled with [<sup>35</sup>S]Met/Cys. Virus release  
423 efficiency was calculated as described in the Fig. 1 legend. The virus release efficiencies  
424 for WT and Fyn(10)fullMA Gag constructs were each normalized to 100%. The release  
425 efficiency for Fyn(10)fullMA was ~ 6-fold higher than that of WT. The Vpu-defective  
426 mutant Fyn(10)fullMA/delVpu displayed a ~ 5-fold defect in particle production relative  
427 to Fyn(10)fullMA. Data represent means  $\pm$ SD, n = 4.

428

429 **Fig. 4. Inhibition of virus release is Vpu-dependent.** HeLa cells were transfected with  
430 HIV-1 molecular clones defective for PR (pNL4-3/PR<sup>-</sup>), Env (pNL4-3KFS) (7), or Nef  
431 (pNL4-3/delNef) (29) (A), Vpu (pNL4-3delVpu) (13) (B), or the pNL4-3/PTAP<sup>-</sup> (11) or  
432 pNL4-3/MA 29KE/31KE mutants (23) (C). In (D), cells were transfected with pNL4-3  
433 (HIV-1), an SIVmac239 molecular clone (SIV) (26), or a vector expressing MLV GagPol  
434 (MLV) (14). Cells treated with the indicated concentrations of AME were metabolically  
435 labeled. Cell and viral lysates were immunoprecipitated with anti-SIVmac239 antiserum  
436 or goat anti-MLV Gag p30 antiserum (obtained from ViroMed Biosafety Laboratories,  
437 Camden, NJ) and virus release efficiency was calculated as described in the Fig. 1  
438 legend. In (A-C), virus release efficiencies for WT and mutant molecular clones were  
439 each normalized to 100%. The release efficiencies of mutants compared to WT (in %) are:  
440 pNL4-3/PR<sup>-</sup>, 65 ± 21; pNL4-3KFS, 184±108; pNL4-3/delNef, 120 ± 38; pNL4-  
441 3delVpu, 9 ± 2; pNL4-3/PTAP<sup>-</sup>, 7 ± 1; and pNL4-3/MA/29KE/31KE, 28.0±11.0. In (D),  
442 HIV-1, SIVmac239, and MLV release efficiencies were each normalized to 100%. Data  
443 represent means ±SD, n = 3-5.

444  
445 **Fig. 5. AME disrupts the ability of Vpu to counter CD317/BST-2/tetherin.** 293T cells  
446 were cotransfected with a Vpu-defective pNL4-3 derivative (pNL4-3delVpu) (13) and an  
447 HA-tagged CD317/BST-2/tetherin expression vector (15), with or without the Vpu  
448 expression plasmid (pCMV-Vphu) (16). Transfected cells were treated (+) or not (-) with  
449 10 µM AME, metabolically labeled, and virus release efficiency calculated as described  
450 in the Fig. 1 legend. Data represent mean ±SD, n = 3.

451

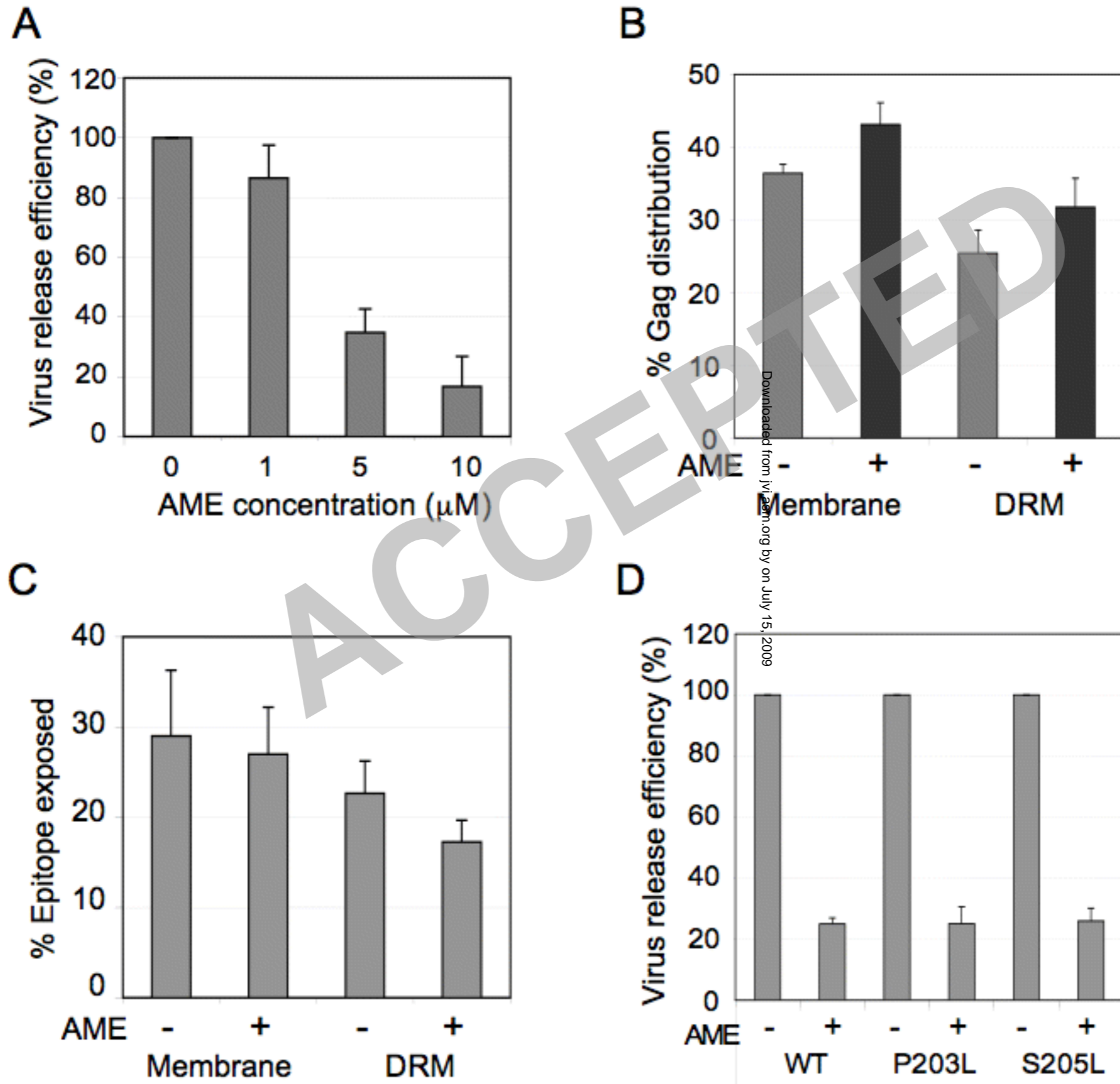


FIG. 1

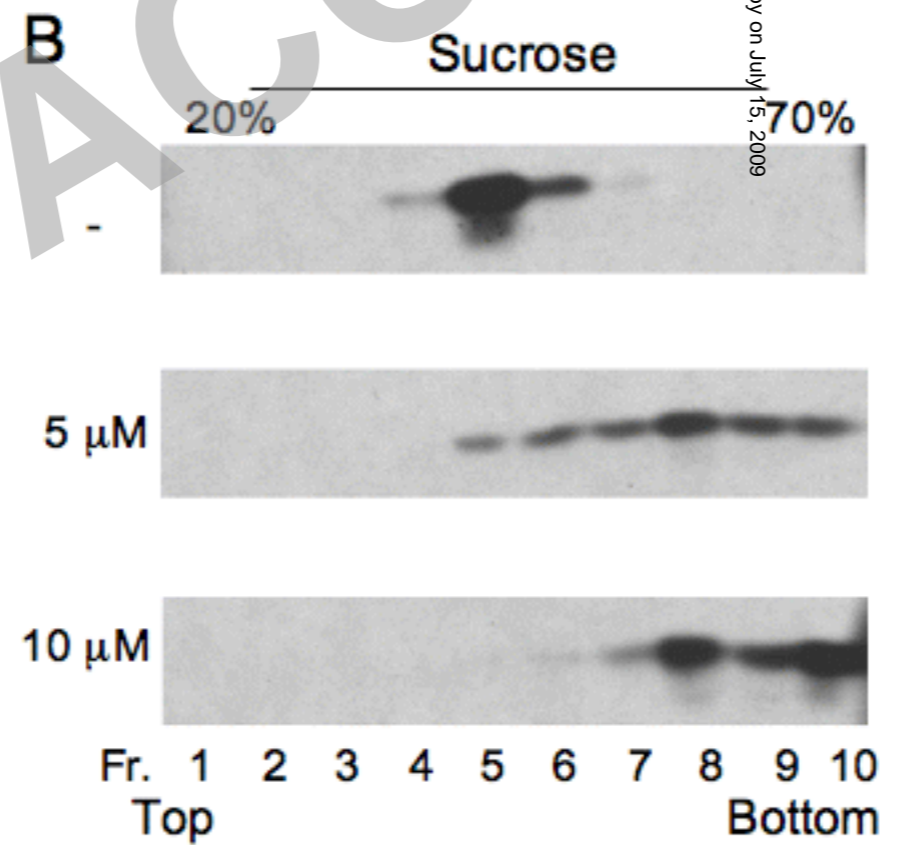
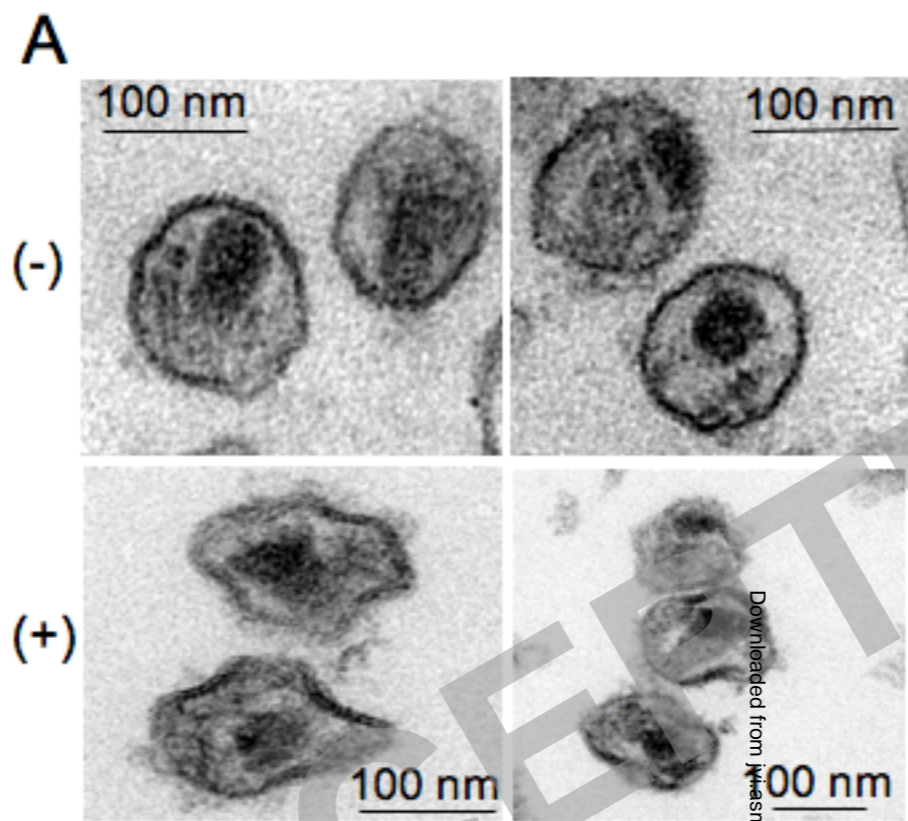


FIG. 2

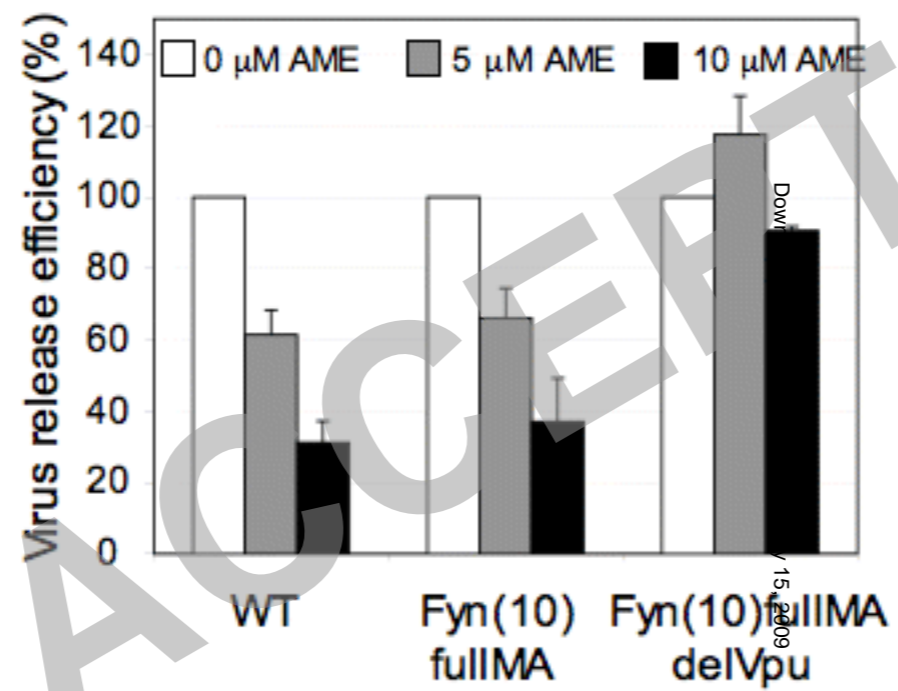


FIG. 3

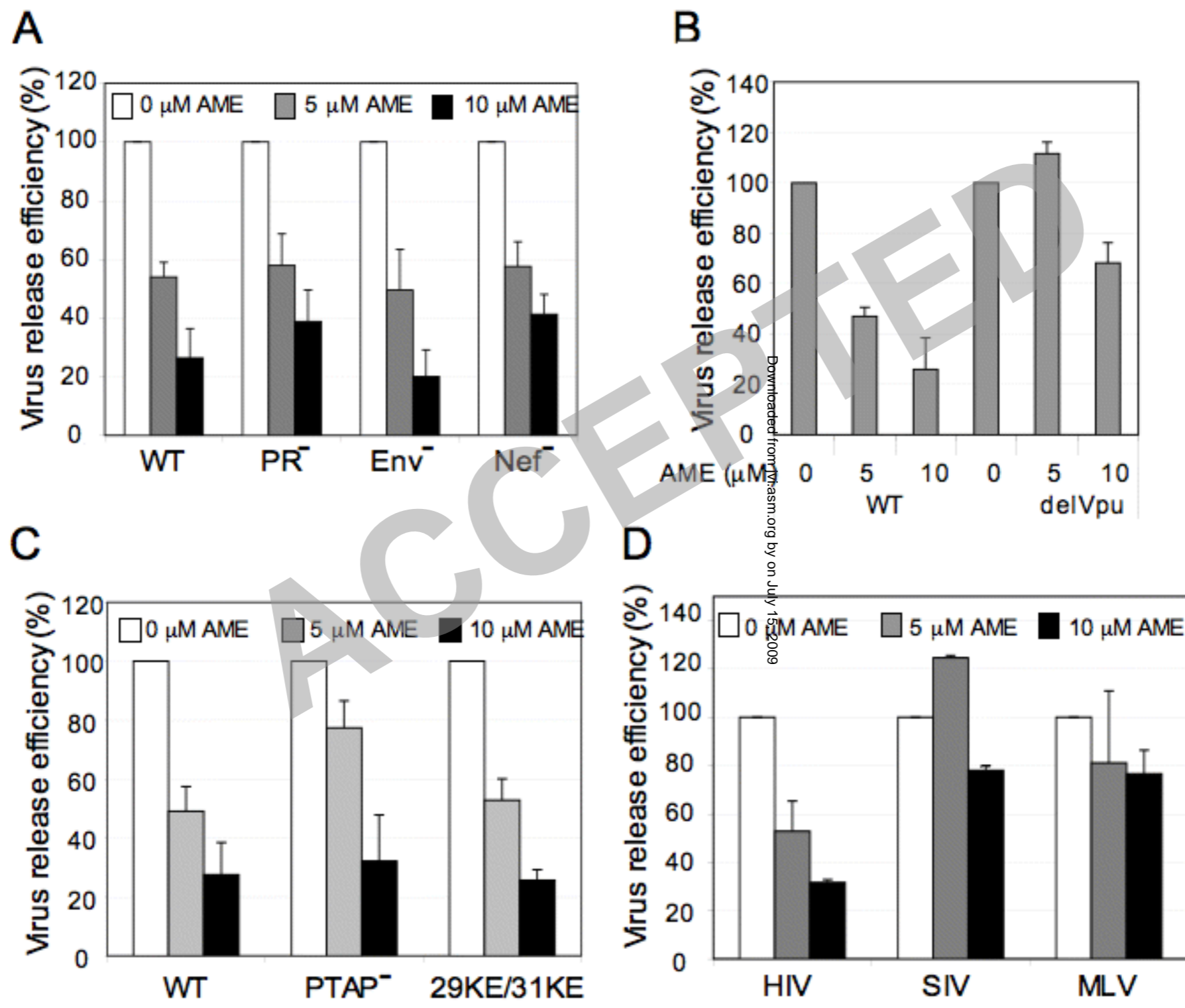


FIG. 4

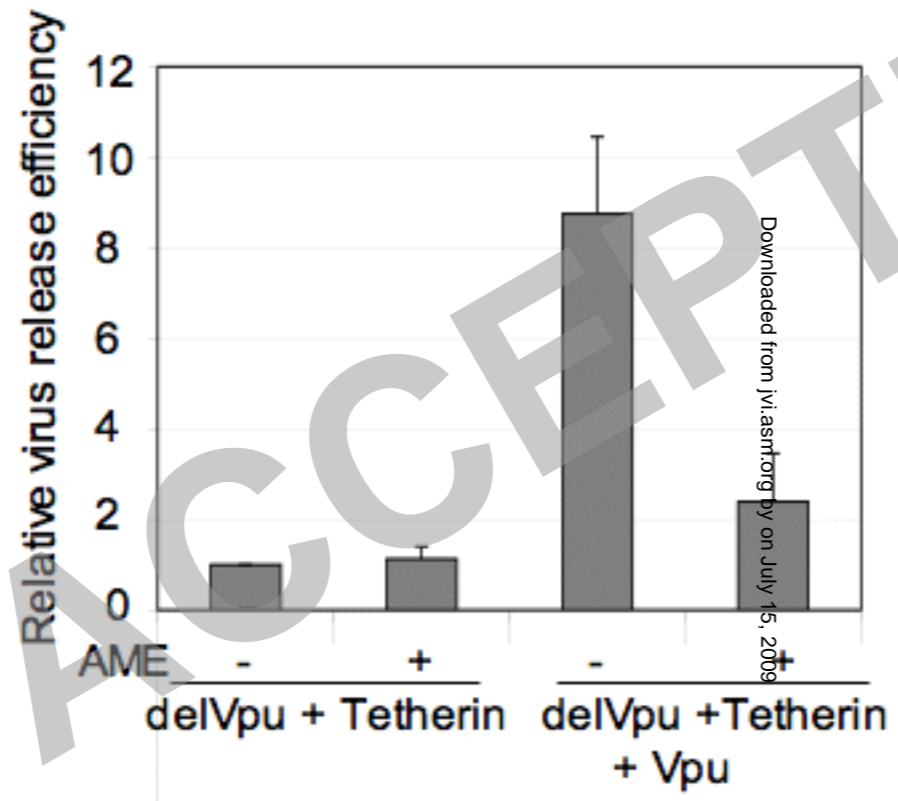


FIG. 5