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(54) Title: VCP INHIBITOR FOR USE IN THE PREVENTION OF VIRUS INFECTION

(57) Abstract: The invention provides a VCP inhibitor for use in the prevention of infection of a cell by a virus.



VCP INHIBITOR FOR USE IN THE PREVENTION OF VIRUS INFECTION

The present invention relates to the role of VCP in mediating intracellular virus degradation in association with TRIM 21, and the use of VCP inhibitors to inhibit viral infection.

Antibodies and immune sera have long been used for the treatment of pathogenic infections.

5 For example, horse antiserum was used in the 1890s to treat tetanus and diphtheria. However, antisera are seen as foreign by the human immune system, which reacts by producing antibodies against them, especially on repeat doses. During most of the 20th C, the adverse effect of animal antibodies prompted the use of human antiserum from donors who had recovered from disease, typically for prophylaxis of respiratory and hepatitis B
10 infections. Following a reduction in the popularity of antibody therapy due to problems with toxicity, humanised and human antibodies have eliminated such concerns, and led to a return of such therapeutic approaches. See Casadevall et al., Nature Reviews Microbiology 2, 695-703 (September 2004), for a review. Diseases which have been targeted using antibody therapy include anthrax, whooping cough, tetanus, botulism, cryptococcosis,
15 cryptosporidiosis, enterovirus gastrointestinal-tract infections, group a streptococcal infections, necrotizing fasciitis, hepatitis B, measles, tuberculosis, meningitis, aplastic anaemia, rabies, respiratory syncytial virus (RSV) infection, pneumonia, shingles, chickenpox and pneumonia due to varicella zoster virus (VZV), and smallpox. Despite these developments, however, antibody therapy is considered only when no other suitable
20 therapies are available, requiring high doses of antibody and producing unpredictable results.

The effectiveness of antibodies against pathogens is understood to be at least partly dependent on the Fc portion of the antibody, which is responsible for mediating the effects of complement and of antibody-dependent cell-mediated cytotoxicity (ADCC). Therefore,
25 antibody fragments have not been generally proposed for antiviral therapy, despite their advantages of small size and lower cost of production.

The primary therapy for viral diseases remains vaccination, which is a prophylactic approach. It is believed that viral antigens, processed by antigen-presenting cells such as dendritic cells, are presented to the immune system and induce naïve T-cells to differentiate
30 into memory and effector T-cells. Memory T-cells are responsible for the more aggressive and immediate immune response to a secondary infection, mediating the benefits of vaccination. For a review, see Kaech et al., Nature Reviews Immunology, volume 2, April 2002, 251.

Another immunologically-based approach to the therapy of infectious disease is the use of cytokines, including inteferons. Interferon was first proposed for the treatment of cancer and multiple sclerosis, as well as viral infections. It has been licensed for the treatment of hepatitis C since 1998. Moreover, low dose oral or intranasal interferon is administered for the treatment of colds and flu, especially in Eastern Europe. However, the mechanism of its action is not known, since the doses used are believed to be lower than the doses at which an antiviral effect could be observed. O'Brien et al., J Gen Virol. 2009 Apr; 90 (Pt. 4):874-82, used interferon as an adjuvant to an adenovirus-delivered vaccine against Venezuelan equine encephalitis virus (VEEV); they observed a decrease in protection against the virus, but an increase in the immune response to the viral vector.

We have previously described an intracellular cytosolic protein called TRIM21 that is capable of binding to an invariant region of antibody molecules via its PRYSPRY domain. We found this activity to be structurally, thermodynamically and kinetically conserved across mammals. Hypotheses for the function of TRIM21 have been suggested, including its involvement in apoptosis and a role in directing of unfolded IgG made in B-cells to the proteasome. Our recently published International patent application WO2012010855 describes a new and surprising function of TRIM21; we have found that pathogens entering the cell bring in with them anti-pathogen immunoglobulins, and that TRIM21 binds to these immunoglobulins in the cytosol and directs them, and the attached pathogen to the proteasome for degradation. Thus, TRIM21 provides for an intracellular mechanism of immunity by which viruses and other pathogens are destroyed on entry into the cell. This process potentially neutralizes viral infection and has been termed antibody dependent intracellular neutralization (ADIN) (McEwan et al., 2011). ADIN is dependent upon the E3 ubiquitin ligase activity of TRIM21 and can be abrogated by chemical inhibition of the proteasome.

Whilst both proteasomal activity and ubiquitination are necessary for ADIN, the exact mechanism of virus degradation is poorly understood. Specifically, it is not clear how the proteasome can degrade a virion, a compact proteinaceous particle much larger than the proteasome itself. The 26S proteasome has a mass of ~2.5 MDa (Voges et al., 1999) and the pore through which substrates must pass to access the proteolytic chamber is no greater than 2 nm in diameter (Gallastegui and Groll, 2010; Xie, 2010). In contrast, human adenovirus (AdV) - a virus potentially neutralized by TRIM21 - has a diameter of approximately 100 nm and a mass of 150 MDa (Reddy et al., 2010). Although there are ATPases in the 19S regulatory subunit of the 26S proteasome that may unfold substrates and allow them to enter through the pore (Gallastegui and Groll, 2010; Voges et al., 1999), AdV virions are

much larger than any of the proteasome's known cellular substrates. Since ADIN has been shown to be independent of autophagy but dependent on proteasomal degradation (Mallery et al., 2010), we hypothesized that an additional energy-dependent step of AdV capsid disassembly and/or unfolding might precede proteasomal degradation of the virus.

5 In recent studies, the AAA ATPase valosin-containing protein (VCP, or p97) has been implicated in the proteasomal degradation of certain cytosolic substrates (Alexandru et al., 2008; Cao et al., 2003; Fu et al., 2003; Janiesch et al., 2007; Meyer et al., 2012). VCP is capable of dissociating proteins from large cellular structures such as the ER (Bays et al., 2001), the mitotic spindle (Janiesch et al., 2007), the nuclear envelope (Hetzer et al., 2001)
10 and chromatin (Ramadan et al., 2007). VCP forms a homo-hexameric barrel-shaped ring of about 16 nm in diameter (Zhang et al., 2000), a structure not dissimilar to the proteasome but without any associated protease activity. The N-domain of VCP binds directly to multi-ubiquitin chains (Dai and Li, 2001) and, although substrate ubiquitination does not seem to be a prerequisite for VCP interaction (Ye et al., 2001), a general role for VCP in unfolding
15 ubiquitin-fusion degradation (UFD) substrates prior to proteasomal degradation has been suggested (Beskow et al., 2009). Most recently it has been shown that VCP is recruited to stalled proteasomes and relieves their (experimentally induced) impairment (Isakov and Stanhill, 2011). It has been suggested that ATP hydrolysis-driven extraction of proteins from larger complexes or membranes as well as unfolding of proteins by VCP upon recruitment to
20 conjugated (poly-) ubiquitin may be the common mechanism underlying the diverse cellular roles of VCP (Meyer et al., 2012; Ramadan et al., 2007; Shcherbik and Haines, 2007).

WO2011069039 describes a number of hydrazine or diacyl hydrazine compounds which are inhibitors of P97/VCP. This document suggests that these compounds may be effective in treating viral infections by activating latent viral infection, and allowing co-administered
25 antiviral drugs to act against the infected cells, thereby treating the infection.

We have found that VCP inhibitors, different from those used in Wo2011069039, can be used to inhibit infection of cells which do not possess integrated viral DNA, and do not have a latent viral infection.

We demonstrate herein that VCP is an important cofactor for TRIM21-mediated degradation
30 of viral pathogens in the cell cytoplasm. Even more surprisingly, however, we also demonstrate that VCP is required for viral infection by certain viruses, including HIV. We

postulate that HIV has hijacked a life-essential, highly abundant, highly conserved cellular enzyme post-entry but pre-replication in order to productively infect cells.

Summary of the Invention

We have described a multifaceted role of VCP in viral infection. For certain viruses, VCP is required for viral infection and depletion of VCP inhibits viral infection. For other viruses, VCP is required for intracellular antibody-dependent, TRIM21-mediated virus degradation, and depletion of VCP results in increased levels of viral infection despite the presence of neutralizing antibody.

In a first aspect, there is provided a VCP inhibitor for use in the prevention of infection of a cell by a virus.

In embodiments, "infection" refers to the successful de novo infectious entry of a virus into a cell, which cell does not have an integrated copy of the DNA of the virus in question. In other words, it is provided herein that VCP inhibitors can act to prevent a virus from establishing a successful infection in a cell, which cell was not previously infected by the virus.

In one embodiment, therefore, the VCP inhibitors are administered to individuals who are at risk from viral infection but do not yet show signs of viral infection. The administration can be prophylactic, to prevent future viral infection, or therapeutic. Where is it therapeutic, the virus may be present in the organism of the individual but viral infection has not been established, and cannot proceed without the further infection of uninfected cells.

In embodiments, the individual does not have a latent viral infection.

In contrast to the inhibitors reported in WO2011069039, the inhibitors of the present application act to prevent viral uncoating during the infection process.

In one embodiment, the virus is a virus that enters the cell by a mechanism which results in the intact viral capsid being exposed to the cytoplasm.

In one embodiment, it is postulated that a feature of viral infection processes which results in involvement of VCP (for uncoating or other steps) is that the capsid of the virus is delivered to the cytosol, i.e. that the virus enters the cytosol in an encapsidated state. In contrast, viruses that do not deliver their capsids into the cytosol are potentially

independent of VCP for infection. An example of such a virus is rhinovirus, whose capsid remains within the endosome and only the viral nucleic acid is delivered to the cytosol.

In one embodiment, the invention provides a VCP inhibitor for use in the prevention of infection of a cell by a virus selected from a retrovirus and a herpesvirus. In one
5 embodiment the retrovirus is a lentivirus, such as a primate lentivirus.

The present inventors have found that VCP inhibitors are capable of preventing, reducing or inhibiting viral infection. In an advantageous embodiment, VCP inhibitors are used to treat infection by retroviruses or herpesviruses, particularly lentiviruses, such as primate
10 lentiviruses. Exemplary viruses include HIV1, HIV2, SIV, EIAV, MLV-B, MLV-N and HSV.

The effect is also seen on downregulation of VCP using VCP-specific siRNA, confirming the
15 general applicability of this finding to the inhibition of viral infection.

Exemplary inhibitors of VCP which inhibit viral uncoating and therefore viral infection include dibenzylquinazoline-2,4-diamine (DBeQ), Xanthahumol, Sorafenib and Syk inhibitor III (3,4-methylenedioxy-beta-nitrostyrene). Further compounds are described herein, and in Chou et
20 al., 2011 PNAS 108: 4834–4839.

The VCP inhibitor, in embodiments, may be an inhibitor of an adaptor protein which promotes the interaction between VCP and viruses in the viral infection cycle. Many VCP adaptor proteins are known, which mediate specificity of VCP activity within cells; examples
of adaptor proteins are described herein, as are methods to isolate VCP adaptor proteins.

According to a further aspect, there is provided a VCP inhibitor as set forth in the preceding
25 aspects of the invention, for use in inhibiting infection of a cell by a virus, wherein the intact virus capsid is exposed to the cytoplasm during the infection cycle.

Moreover, there is provided a method for inhibiting the infection of a cell by a virus comprising contacting the cell with an inhibitor of VCP. Further, there is provided a method
30 of inhibiting viral infection in a patient comprising administering a VCP inhibitor to a patient in need thereof, and monitoring viral infection levels in said patient. Advantageously, in the foregoing embodiments, the virus capsid is exposed to the cytoplasm during the infection cycle.

In embodiments, the patient is not demonstrating symptoms of viral infection and/or does not
35 suffer from a latent viral infection.

In embodiments, the VCP inhibitors are administered without other antiviral agents, and/or exert their antiviral activity independently of coadministered antiviral agents.

In one embodiment, the virus is selected from a retrovirus and a herpesvirus. The retrovirus may for example be a lentivirus, such as a primate lentivirus. For example, the virus may be
5 HIV1, HIV2, EIAV, MLV-B, MLV-N, SIV or HSV.

In a preferred embodiment, the VCP inhibitor inhibits the infection of HeLa cells by HIV1 or HIV2.

In a further preferred embodiment, the VCP inhibitor inhibits the infection of A549 cells by HSV.

10 According to a second aspect, there is provided an inhibitor of a VCP adaptor protein for use in prevention of infection of a cell by a virus.

In a further aspect of the invention, there is provided a method for inhibiting viral infection of a cell, comprising administering to the cell an inhibitor of VCP. In one embodiment, the method of the invention inhibits viral infection wherein the infection is by a virus that enters
15 the cell such that the intact viral capsid is exposed to the cytoplasm. In one embodiment, there is provided a method for treating a condition associated with a viral infection in a subject in need thereof by modulating the interaction of VCP and the virus, by administering a pharmaceutical composition capable of modulating interaction of VCP and virus in an amount sufficient to modulate the viral infection.

20 In a still further aspect, there is provided a method for screening for an inhibitor of viral infectivity, comprising the steps of: providing a cell which comprises a cell-based VCP-dependent ubiquitin proteasome degradation pathway, and analyzing the degradation of a VCP-dependent substrate in the presence of one or more candidate inhibitors. Suitable methods are set forth in WO2010/003908. VCP inhibitors, as shown herein, are effective
25 inhibitors of viral infection.

In another aspect, there is provided a method for identifying a compound capable of inhibiting viral infection, comprising contacting a VCP polypeptide with a viral capsid polypeptide in the presence of the compound, and determining the influence of the compound on the interaction between the VCP polypeptide and the viral capsid polypeptide.

As indicated above, we postulate that VCP can be involved in infection of cells by viruses in cases where the infection cycle results in the intact viral capsid being exposed to the cytoplasm. We postulate that VCP acts to facilitate viral uncoating, in the same manner as it acts in processing virions for proteasomal degradation in ADIN. Accordingly, agents which
5 influence the interaction between the viral capsid and VCP can disrupt viral infection.

In one aspect, the foregoing method is carried out in the presence of one or more VCP adaptor proteins. VCP is a very versatile protein, taking part in a wide range of processes through adapter proteins, which cause it to interact in different ways with different pathways. Accordingly, suitable adaptor proteins may be included in the assay, to detect compounds
10 which interact with the adaptor proteins, and therefore interact indirectly with VCP.

In one embodiment, there is provided a method for identifying a compound or compounds capable, directly or indirectly, of modulating the interaction of VCP and a virus and thereby the infectivity of HIV, comprising the steps of: incubating a compound or compounds to be tested with a VCP polypeptide and a viral capsid polypeptide, under conditions in which, but
15 for the presence of the compound or compounds to be tested, the interaction between VCP and the viral capsid induces a measurable chemical or biological effect;
determining the ability of the VCP polypeptide to interact, directly or indirectly, with the viral capsid polypeptide to induce the measurable chemical or biological effect in the presence of the compound or compounds to be tested ; and
20 selecting those compounds which modulate the interaction between VCP and the virus.

The chemical or biological effect can be, depending on the assay implementation, a readout of a suitable reporter gene system, or a functional assay such as viral infection.

The VCP inhibitors according to the present invention are suitable for drug development. Accordingly, there is provided a method for developing an anti-viral drug comprising the
25 steps of (a) identifying one or more compounds which demonstrate anti-infection activity; (b) screening said compounds and selecting one or more compounds which affect the interaction of VCP and the virus; (c) determining the structure of the compound and using structure-guided mutagenesis to prepare variants of the compound with improved activity.

VCP inhibitors are especially useful in the treatment of HIV infection. HIV is susceptible to
30 treatment with cocktails of drugs, which attach the virus at different points of its life cycle; accordingly, the invention provides a drug cocktail comprising two or more drugs for use in

the treatment or prevention of an HIV infection, wherein at least one of said drugs is indicated for the disruption of the interaction between VCP and a viral capsid protein.

For example, the drug cocktail further comprises one or more anti-HIV drugs selected from the group consisting of efavirenz, emtricitabine, tenofovir, disoproxil fumarate, rilpivirine, lamivudine, zidovudine, emtricitabine, azidothymidine (AZT), nevirapine, amprenavir, tipranavir, indinavir, saquinavir mesylate, lopinavir, ritonavir, Fosamprenavir Calcium, darunavir, atazanavir sulfate, nelfinavir mesylate, raltegravir, maraviroc and enfuvirtide.

In one embodiment, one or more drugs used in the cocktail capable of disrupting of the interaction between VCP and a viral capsid component can be selected according to the preceding aspects of the invention. For example, the drug is DBE_Q.

Description of the Figures

Fig. 1 VCP is essential for TRIM21-mediated neutralization of AdV by antibody.

(A) Levels of infection of HeLa cells by AdV-GFP pre-incubated with (grey bars) or without (white bars) neutralizing polyclonal antibody (pAb) and

(B) Resulting neutralization of virus by antibody are displayed for a set of conditions including RNA interference against VCP and/or TRIM21 as well as IFN treatment. Error bars represent the standard error of the mean (SEM) calculated from three replicas.

(C) Protein levels in these conditions are visualized by western blot.

Fig. 2 Chemical inhibition of VCP abrogates ADIN and targets the same pathway as proteasome inhibition.

(A) Levels of infection by AdV-GFP preincubated with (grey bars) or without (white bars) neutralizing mAb 9C12 and

(B) Resulting neutralization of virus by antibody are displayed for HeLa cells treated with a titration of DBE_Q.

(C, D) Neutralization of virus by mAb 9C12 is shown for HeLa cells treated with DMSO, 10 μ M Eerl, 12 μ M DBE_Q, 2 μ M epoxomicin (Epox), or both DBE_Q and Epox. Error bars represent the SEM calculated from three replicas.

Fig. 3 VCP is required for degradation of virus and antibody in ADIN, but not for TRIM21-mediated degradation of cytosolic IgG Fc.

(A) Western blots of AdV hexon and mouse IgG levels in HeLa cells treated with DMSO, 10 μ M DBeQ or 10 μ M Eerl, infected with mAb 9C12-bound AdV and harvested at indicated time points post infection; n.i., non-infected control.

(B) Western blot of overexpressed mouse (mm) IgG Fc in HeLa cells with or without treatment with TRIM21 shRNA, DMSO or 2 μ M epoxomicin (Epo);

(C) Western blot of overexpressed mm IgG Fc in HeLa cells treated with DMSO, 10 μ M DBeQ or 2 μ M Epo.

(D) Neutralization proceeds with similar kinetics as degradation. Relative neutralization of AdV-GFP by mAb 9C12 is displayed for HeLa cells treated with DMSO (white bars) or 9 μ M DBeQ (grey bars) at several time points post infection (p.i.). Error bars represent the SEM calculated from three replicas.

Fig. 4 VCP is essential for efficient and potent ADIN.

Relative levels of infection of HeLa cells by AdV-GFP in several conditions as a function of concentration of neutralizing mAb 9C12 that the virus was pre-incubated with:

(A) open circles, negative control siRNA; squares, VCP siRNA; triangles, TRIM21 shRNA and negative control siRNA; diamonds, TRIM21 shRNA and VCP siRNA;

(B) open circles, DMSO; squares, 3 μ M DBeQ; triangles, 4.5 μ M DBeQ; diamonds, 6 μ M DBeQ; closed circles, 9 μ M DBeQ. Error bars represent the SEM calculated from three replicas.

(C) Proposed VCP-dependent mechanism of ADIN. In the cytoplasm, antibody-bound AdV is detected and engaged by TRIM21, resulting in ubiquitination of a component of this complex. The proteasome and VCP are recruited and mediate degradation of viral capsid and antibody, leading to neutralization. AdV in dark blue, antibody in yellow, TRIM21 in cyan, ubiquitin in green, proteasome in orange/brown/yellow (19S regulatory 23 particles omitted for simplicity), and VCP in red. AdV, proteasome and VCP are represented to scale.

Figure 5

A: infectivity of HIV-1 is reduced in the presence of VCP inhibitor DBeQ, in the presence or absence of restriction factor TRIM5alpha.

B: DBeQ inhibits HIV-1 infection in a dose-dependent manner.

5 C: Treating HeLa cells with a titration of DBeQ before infection with HIV1-GFP-VSV potently inhibits infection.

D: Partial depletion of VCP using siRNA reduces HIV-1 infection almost 3-fold.

Figure 6

A: Inhibition of VCP prevents HIV-1 infection at all MOI tested.

10 B: Treatment with DBeQ inhibits HIV1-GFP-VSV infection of MDM with similar efficiency as observed in CRFK and HeLa cells.

C: HIV1-GFP virus pseudotyped with the naturally occurring HIV envelope SF162 was tested on TZM-bl cells for susceptibility to VCP inhibition. As for VSV G-pseudotyped HIV-1, DBeQ inhibited infection by SF162-pseudotyped virus in a dose-dependent fashion.

15 **Figure 7**

Treatment with DBeQ potently inhibited infection by the retroviruses:

A: HIV-2; B: SIV; C: MLV-B; D: MLV-N; E: EIAV; F: HSV.

Figure 8

20 Infection of cells by virus was not affected by inhibition of VCP for A: VSV G-pseudotyped feline immunodeficiency virus (FIV-GFP-VSV) on CRFK cells; B: RSV and C: hADV on HeLa cells.

Figure 9

25 A: Inhibition of HSV1-GFP with Xanthanol. A549 cells were pre-treated with Xan for 2 h and infected with HSV1-GFP. After 1 h incubation at 37 °C, the virus containing medium was removed and the cells were washed and incubated in drug containing medium. Samples

were harvested at 6 hpi and GFP expression was analysed by FACS. Mean and standard error were calculated in Excel (n=3) and shown.

B: The effect of Eeyarestatin 1 (e1) on HSV-1 (KOS) infection and replication in A549 and HeLa cells. Cells were treated with Eeyarestatin 1 for 3.5 h before infection with KOS at 6 PFU/cell. After 1 h incubation at 37 °C, the virus inoculants were replaced by fresh medium containing Eeyarestatin 1 at the indicated concentrations. Samples were harvested at 24 hpi and GFP expression was analysed by FACS.

C: HSV-1 GFP C12 virus was added to A549 cells in the presence of Syk inhibitor III (skIII) and the relative infection levels determined by FACS analysis 6 hours post-infection.

10 Figure 10

Inhibition of MHV68 replication by DBeQ. A549 cells were pre-treated with DBeQ for 2 h and infected with MHV68-GFP at 3 PFU/cell. After 1 h incubation at 37 °C, the virus containing medium was removed and the cells were washed and incubated in drug containing medium. Samples were harvested at 24 hpi and GFP expression was analysed by FACS. Relative infectivity was calculated by normalising samples to DMSO treated controls (set as 100%). Mean and standard error were calculated in Excel (n=3) and shown.

Figure 11

VCP is required for an early post-entry event in HIV-1 infection and associates with HIV-1 capsids.

- 20 (a) Immunoblot of the soluble cytoplasmic fraction of HeLa cells 4 h p.i. with HIV1-GFP(VSV) $\pm$ 7.5 μ M DBeQ, or with HIV1-GFP(Δ Env). (b-e) Levels of indicated viral DNA species 14 h p.i. of HeLa cells with HIV1-GFP(VSV) in conditions of DMSO, 8 μ M DBeQ or 50 μ M nevirapine (NVP) (b, c), transfection with ctrl or VCP siRNA (d) or with empty vector (e.v.), wild-type (VCP wt), K524A (VCP KA) or E305/578Q VCP (VCP EQEQ) (e). AU, arbitrary units; n.d., not detected. (f) Effect of 50 μ M DBeQ, 150 μ M XN, and 100 μ M NVP on HIV-1 RT activity *in vitro*. (g) Relative levels of HIV1-GFP(VSV) infection in HeLa cells following removal of DMSO or 7.5 μ M DBeQ at indicated times, as measured 48 h p.i. (h) CA concentration in fractions from a sucrose gradient after equilibrium sedimentation of HIV1-GFP(VSV). (i) Immunoblot analysis of an *in vitro* co-sedimentation experiment using HIV-1 cores $\pm$ HeLa cell lysate $\pm$ 60 μ M DBeQ. Mix, cores mixed with
- 25
- 30

lysate. (j) Immunoblot of a co-sedimentation experiment using lysate from HeLa cells infected with HIV1-GFP(VSV) or HIV1-GFP(Δ Env). Data are representative of three experiments and (b-g) displayed as means $\pm$ s.e.m. of three biological replicates. Two-tailed unpaired *t*-tests comparing inhibitor with DMSO control data: ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$.

Figure 12

VCP is required for an early post-entry event in HIV-1 infection.

Levels of early and late HIV-1 reverse transcription products (a, b), 2-LTR circles (c) and integrated provirus (d) in HeLa cells treated with DMSO, 8 μ M DBeQ or 50 μ M nevirapine (NVP), measured 24 h p.i. with HIV1-GFP(VSV). AU, arbitrary units; n.d., not detected. Two-tailed unpaired *t*-tests comparing inhibitor with DMSO control data: ****, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$.

Figure 13

Known host protein interactions with capsid do not impact on VCP dependence of HIV-1 infection.

(a) Effect of DBeQ on infection of HeLa cells by wild-type (wt) HIV1-GFP(VSV), CA mutant N74D (unable to bind CPSF616) or P90A (attenuated binding to cyclophilin A), normalized to the DMSO control. (b) DBeQ titration against HIV1-GFP(VSV) infection of HeLa cells in the presence of inhibitor PF3450074 (PF-74, blocks the CPSF6 binding site: Blair, *et al. PLoS Pathog* **6**, e1001220, doi: 10.1371/journal.ppat.1001220 (2010); Price, A.J. *et al. PLoS Pathog* **8**, e1002896, doi:10.1371/journal.ppat.1002896 (2012)) or DMSO. (c) Titration of DBeQ against HIV1-GFP(VSV) infection of feline CrFK cells devoid of functional TRIM5 α 25, normalized to DMSO. (d) Levels of HIV1-GFP(VSV) infection of CrFK cells stably transduced with empty vector (e.v.) or human (hu) or rhesus macaque (rh) TRIM5 α (T5 α) when treated with DMSO or 10 μ M DBeQ. Data are representative of three experiments and displayed as means $\pm$ s.e.m. of three biological replicates.

Figure 14**VCP promotes HIV-1 uncoating.**

(a) Immunoblot of input, supernatant (Sup) and pellet isolated in a fate of capsid (FOC) experiment 4 h p.i. of HeLa cells with HIV1-GFP(VSV) $\pm$ 7.5 μ M DBEq. (b, c) Relative quantity of CA pelleted in FOC experiments in conditions of DMSO, 7.5 μ M DBEq or 50 μ M NVP (b), or ctrl or VCP siRNA (c). AU, arbitrary units based on densitometry of immunoblots. (d) Fluorescence micrographs of HeLa cells treated with DMSO or 8 μ M DBEq, immunostained for CA 4 h p.i. with HIV1-GFP(VSV). (e-g) Number of CA foci per cell in conditions of DMSO or 8 μ M DBEq (e); ctrl or VCP siRNA (f); or empty vector (e.v.), wild-type (VCP wt), K524A (VCP KA) or E305/578Q VCP (VCP EQEQ) (g). (h) Relative levels of HIV1-GFP(VSV) infection in transfected HeLa cells. (i) Fluorescence micrographs of a HeLa cell infected with GFP-Vpr-containing HIV1-GFP(VSV) and immunostained for CA in *in situ* uncoating assays. Horizontal arrows, CA-positive cores; vertical arrows, CA-negative cores. (j, k) Percentage of intracellular GFP-Vpr-positive HIV-1 cores also positive for CA, as a function of time p.i., in conditions of DMSO or 8 μ M DBEq (j), or ctrl or VCP siRNA (k). Data are representative of three experiments and displayed as means $\pm$ s.e.m. of three biological replicates (b, c, h). Other sample sizes and quantification of microscopy data are described in the Methods. Two-tailed unpaired *t*-tests comparing inhibitor or transfection conditions with their respective controls (DMSO, ctrl siRNA, or e.v.): ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

Figure 15**Persistence of intracellular CA foci upon interference with VCP by siRNA or mutant overexpression.**

Fluorescence micrographs of HeLa cells immunostained for CA 4 h p.i. with HIV1-GFP(VSV) in conditions of transfection with ctrl or VCP siRNA (a); or transfection with empty vector (e.v.), wild-type (VCP wt), K524A (VCP KA) or E305/578Q VCP (VCP EQEQ) (b). Data are representative of three experiments.

Figure 16**Recombinant VCP does not impact on *in vitro* disassembly of HIV-1 capsids.**

(a) *In vitro* disassembly of HIV-1 capsids over a time course of incubation at 37° C in buffer containing ATP, supplemented with either BSA or recombinant VCP. (b) *In vitro* disassembly of HIV-1 capsids after incubation at 37° C for 45 min in buffer containing ATP, in the presence or absence of recombinant VCP. Data are displayed as the percentage of total CA that is no longer pelletable (disassembled). Data are representative of three experiments and displayed as means $\pm$ s.e.m. of technical duplicates (a) or two biological replicates (b).

Figure 17**10 Inhibition or depletion of VCP prevents dissociation of CA from HIV-1 cores during infection of human cells.**

Fluorescence micrographs of HeLa cells treated with DMSO or 8 μ M DBeQ (a), or transfected with ctrl or VCP siRNA (b), taken after cells were fixed 4 h p.i. with GFP- Vpr-incorporating HIV1-GFP(VSV) and immunostained for CA. Horizontal arrows, GFP-Vpr-positive HIV-1 cores also positive for CA; vertical arrows, GFP-Vpr- positive HIV-1 cores negative for CA. Data are representative of three experiments.

Figure 18

HeLa cells were plated at 1×10^5 cells per well. The next day, Sorafenib was added at indicated concentrations to wells. All conditions were normalised for solvent (DMSO) concentration. Cells were incubated for 1h incubation at 37°C then challenged with VSV pseudotyped HIV-1 GFP vector (HGV) at an moi of ~ 0.3 . GFP expressing cells were enumerated by FACS 48 h post infection.

Detailed description of the Invention

25 Unless otherwise stated, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. Methods, devices, and materials suitable for such uses are now described. All publications cited herein are incorporated

herein by reference in their entirety for the purpose of describing and disclosing the methodologies, reagents, and tools reported in the publications that might be used in connection with the invention.

The practice of the present invention employs, unless otherwise indicated, conventional
5 methods of chemistry, biochemistry, molecular biology, cell biology, genetics, immunology, virology and pharmacology, known to those of skill of the art. Such techniques are explained fully in the literature. See, e. g., Gennaro, A. R., ed. (1990) Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co.; Hardman, J. G., Limbird, L. E., and Gilman, A. G., eds. (2001) The Pharmacological Basis of Therapeutics, 10th ed., McGraw-Hill Co.;
10 Colowick, S. et al., eds., Methods In Enzymology, Academic Press, Inc.; Weir, D. M., and Blackwell, C. C., eds. (1986) Handbook of Experimental Immunology, Vols. I-IV, Blackwell Scientific Publications; Maniatis, T. et al., eds. (1989) Molecular Cloning: A Laboratory Manual, 2nd edition, Vols. I-III, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al., eds. (1999) Short Protocols in Molecular Biology, 4th edition, John Wiley & Sons; Ream et al., eds. (1998) Molecular Biology Techniques: An Intensive Laboratory Course, Academic Press; Newton, C. R., and Graham, A., eds. (1997) PCR (Introduction to Biotechniques Series), 2nd ed., Springer Verlag.

As used herein, a "VCP inhibitor" is a drug which is capable of inhibiting the activity of VCP which is required for viral infectivity. Inhibitors of VCP are known in the art and are being
20 discovered regularly, as VCP is a target for cancer therapy and other medical disciplines. Exemplary inhibitors include dibenzylquinazoline-2,4-diamine (DBeQ), Eeyarestatin I (Wang et al, 2010: PLoS ONE 5(11): e15479) and 2-Anilino-4-aryl-1,3-thiazole compounds (Bursavich et al., Bioorg Med Chem Lett. 2010 Mar 1;20(5):1677-9. Epub 2010 Jan 21). Methods for identifying VCP inhibitors are described in Chou et al., May 13, 2011, The
25 Journal of Biological Chemistry, 286, 16546. In some embodiments, a VCP inhibitor may be an inhibitor of a VCP adaptor protein which is involved in the role of VCP in viral infection.

VCP (p97 in mouse, TER94 in *Drosophila melanogaster*, and CDC48 in *S. cerevisiae*) is a highly conserved AAA+-ATPase that regulates a wide array of cellular processes. It is an 89 kDa protein composed of an N-terminal domain followed by tandem ATPase domains. VCP
30 functions as a homo-hexameric ring formed by the ATPase domains with the N-terminal domain oriented outward to permit interaction with adapter proteins. It is similar in structure to bacterial groEl chaperonin.

A catalytically dead mutant version of VCP, generated by mutations that impair both ATPase domains (E305Q/ E578Q), functions as dominant negative when expressed exogenously and has been extensively used to interrogate VCP function. VCP is essential to some aspects of ubiquitin-dependent proteasomal degradation including endoplasmic reticulum-associated degradation (ERAD), degradation of some cytosolic proteins by the ubiquitin-fusion degradation (UFD) pathway, and rapid degradation of nascent peptides during heat shock. VCP is also essential to some non-proteolytic aspects of ubiquitin signalling, including chromatin decondensation following mitosis, nuclear envelope formation and homotypic membrane fusion during biogenesis of the ER and Golgi apparatus.

- 10 An inhibitor may inhibit the activity of VCP, as measured in a chemical or biological reporter system. For example, the inhibitor may inhibit the activity of VCP by 20%, 30%, 40%, 50% or more. In embodiments, the inhibitor may inhibit the activity of VCP by up to 80, 90 or 100%.

The “prevention of infection” by a virus, as referred to herein, is the reduction of infection as measured by numbers of infected cells and/or the severity of infection of those cells which are infected. It does not imply that infection is completely prevented; extremely low levels of infection may result from the application of the present invention.

“Infection”, in the context of the present invention, is the ability of a virus to enter a cell and establish a viral infection. VCP inhibitors are shown to act to prevent the act of infection of the cell by a virus. Accordingly, references to prevention of infection are references to prevention of the establishment of an infection by a virus. The inhibition of viral replication, or treatment of existing viral infection except through the limitation of the spread of new infection, is not the subject of the present claims.

Advantageously, reduction in levels of infection of 50% or more may be observed. For example, levels of infection may be reduced by 60, 70, 80, 90%, or more.

- 25 A retrovirus is any RNA virus which reverse transcribes an RNA genome. Examples include lentiviruses, such as HIV, and gamma-retroviruses, such as MLV-B. A primate lentivirus is a lentivirus whose natural host is a primate, including but not limited to HIV1, HIV2 and SIV.

VCP adaptor proteins are proteins which interact with VCP in different biological pathways in which VCP plays an active role. Examples include p37, p47, Ufd1/Npl4, VCIP135, Derlin-1, and VIMP.

A "compound" which influences the interaction of VCP and a virus may be of almost any general description, including low molecular weight compounds, organic compounds which may be linear, cyclic, polycyclic or a combination thereof, peptides, polypeptides including antibodies, or proteins. In general, as used herein, "peptides", "polypeptides" and "proteins" are considered equivalent.

The terms "direct" and "indirect", as applied herein, refer to interactions between entities which either require, or do not require, an intermediary. An "indirect" action proceeds through an intermediary; for instance, interaction between VCP and some cellular pathways proceeds via a VCP adaptor protein.

Sequence homology (or identity) may moreover be determined using any suitable homology algorithm, using for example default parameters. Advantageously, the BLAST algorithm is employed, with parameters set to default values. The BLAST algorithm is described in detail at http://www.ncbi.nlm.nih.gov/BLAST/blast_help.html, which is incorporated herein by reference. The search parameters are defined as follows, and are advantageously set to the defined default parameters.

Advantageously, homology of nucleic acid sequences can be assessed using a suitable algorithm, such as BLAST. Preferred levels of homology, when assessed by BLAST, equate to sequences which match with an EXPECT value of at least about 7, preferably at least about 9 and most preferably 10 or more. The default threshold for EXPECT in BLAST searching is usually 10.

BLAST (Basic Local Alignment Search Tool) is the heuristic search algorithm employed by the programs blastp, blastn, blastx, tblastn, and tblastx; these programs ascribe significance to their findings using the statistical methods of Karlin and Altschul (see http://www.ncbi.nlm.nih.gov/BLAST/blast_help.html) with a few enhancements. The BLAST programs were tailored for sequence similarity searching, for example to identify homologues to a query sequence. For a discussion of basic issues in similarity searching of sequence databases, see Altschul et al. (1994) Nature Genetics 6: 119-129.

In one embodiment, sequence homology refers to percentage sequence identity, which can be assessed without the aid of an algorithm.

As used herein, a “VCP polypeptide”, or a “viral polypeptide”, is a polypeptide derived from VCP, or a virus, which maintains substantial sequence homology with the original sequence. Advantageously, such polypeptides have 90% or more sequence identity.

Several mechanistic hypotheses have been proposed to explain the role of VCP in proteasomal degradation of cellular substrates. It has been suggested that VCP may be required for initial unfolding of tightly folded substrates that lack an intrinsically unstructured region as initiation site for the proteasome (Beskow et al., 2009). The AdV capsid may qualify as such a substrate (Liu et al., 2010; Reddy et al., 2010). Alternatively, the direct but highly transient interaction between VCP and the proteasome (Besche et al., 2009) may be specifically stabilized when the proteasome faces a particularly challenging substrate or when it is otherwise impaired. The latter hypothesis was suggested in a recent study analysing the association of VCP with proteasomes in various stress conditions that induce the unfolded protein response (Isakov and Stanhill, 2011). We propose that in ADIN, VCP is specifically recruited during proteasomal degradation of a large virion. Our finding that proteasomal degradation of free IgG Fc does not depend on VCP supports this substrate-specific rather than constitutive role for VCP. During degradation of virus, VCP may mediate ATP hydrolysis-driven disassembly and/or partial unfolding of the AdV capsid, enabling the 19S regulatory particle to pass capsid components (and the associated antibody) into the 20S core particle for degradation (Fig. 4C, route a). However, our results do not rule out the alternative possibility that VCP is recruited to the ubiquitin-positive ADIN complex of AdV, antibody and TRIM21 prior to engagement of the proteasome in order to mediate processing upstream of degradation (Fig. 4C, route b). Future studies such as high-resolution time-resolved microscopy may help to clarify the mechanism in detail. A direct role for VCP in the physical destruction of virus is supported by our fate-of-capsid experiments, in which VCP inhibition by DBE-Q completely abrogates degradation of virus and antibody. Furthermore, we have made use of DBE-Q to demonstrate that it is the degradation of virus that drives neutralization. In time of addition experiments, we find that the kinetics of neutralization correlate closely with the kinetics of degradation. When virus degradation is blocked by VCP inhibition within the first few hours post infection, productive infection proceeds.

Surprisingly, we have found that although VCP is required for virus degradation mediated by TRIM21, which occurs in the presence of antiviral antibody, VCP is also required for the infectivity of certain viruses.

We postulate that the VCP pathway has been adopted by HIV, HSV and other viruses to uncoat themselves before transport of viral nucleic acid to the nucleus. Thus, contrary to the results obtained with ADIN and adenovirus, inhibition of VCP inhibits infection by HIV and other viruses which use expose intact viral capsids to the cytoplasm during infection.

5 Preparation of VCP polypeptides

The invention encompasses the production of VCP polypeptides for use in the assays as described herein. Preferably, VCP polypeptides are produced by recombinant DNA technology, by means of which a nucleic acid encoding a VCP polypeptide can be
10 incorporated into a vector for further manipulation. As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well within the skill of the artisan. Many vectors are available, and selection of appropriate vector will depend on the intended use of the vector, i. e. whether it is to be used for DNA amplification or for DNA
15 expression, the size of the DNA to be inserted into the vector, and the host cell to be transformed with the vector. The sequence of VCP is available on the Swiss Prot database under accession number P55072; GI: 6094447.

Each vector contains various components depending on its function (amplification of
20 DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: an origin of replication, one or more marker genes, an enhancer element, a promoter, a transcription termination sequence and a signal sequence.

25 Both expression and cloning vectors generally contain nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Typically in cloning vectors, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast and viruses.

30

The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2p plasmid origin is suitable for yeast, and various viral origins (e. g. SV 40, polyoma, adenovirus) are useful for cloning vectors in mammalian cells. Generally, the origin

of replication component is not needed for mammalian expression vectors unless these are used in mammalian cells competent for high level DNA replication, such as COS cells.

Advantageously, an expression and cloning vector may contain a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, e. g. ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available from complex media.

As to a selective gene marker appropriate for yeast, any marker gene can be used which facilitates the selection for transformants due to the phenotypic expression of the marker gene. Suitable markers for yeast are, for example, those conferring resistance to antibiotics G418, hygromycin or bleomycin, or provide for prototrophy in an auxotrophic yeast mutant, for example the URA3, LEU2, LYS2, TRP1, or HIS3 gene.

Since the replication of vectors is conveniently done in *E. coli*, an *E. coli* genetic marker and an *E. coli* origin of replication are advantageously included. These can be obtained from *E. coli* plasmids, such as pBR322, Bluescript® vector or a pUC plasmid, e.g. pUC 18 or pUC 19, which contain both *E. coli* replication origin and *E. coli* genetic marker conferring resistance to antibiotics, such as ampicillin.

Suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up VCP nucleic acid, such as dihydrofolate reductase (DHFR, methotrexate resistance), thymidine kinase, or genes conferring resistance to G418 or hygromycin. The mammalian cell transformants are placed under selection pressure which only those transformants which have taken up and are expressing the marker are uniquely adapted to survive. In the case of a DHFR or glutamine synthase (GS) marker, selection pressure can be imposed by culturing the transformants under conditions in which the pressure is progressively increased, thereby leading to amplification (at its chromosomal integration site) of both the selection gene and the linked DNA that encodes VCP. Amplification is the process by which genes in greater demand for the production of a protein critical for growth, together with closely associated genes which may encode a desired

protein, are reiterated in tandem within the chromosomes of recombinant cells. Increased quantities of desired protein are usually synthesized from thus amplified DNA.

5 Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to VCP nucleic acid. Promoters suitable for use with prokaryotic hosts include, for example, the beta lactamase and lactose promoter systems, alkaline phosphatase, the tryptophan (trp) promoter system and hybrid promoters such as the tac promoter. Their nucleotide sequences have been published, thereby enabling the skilled worker operably to ligate them to DNA encoding VCP, using linkers or adaptors to
10 supply any required restriction sites. Promoters for use in bacterial systems will also generally contain a Shine-Delgarno sequence operably linked to the DNA encoding VCP.

Preferred expression vectors are bacterial expression vectors which comprise a promoter of a bacteriophage such as phage x or T7 which is capable of functioning in the bacteria. In one
15 of the most widely used expression systems, the nucleic acid encoding the fusion protein may be transcribed from the vector by T7 RNA polymerase (Studier et al, Methods in Enzymol. 185; 60-89, 1990). In the E. coli BL21(DE3) host strain, used in conjunction with pET vectors, the T7 RNA polymerase is produced from the lambda lysogen DE3 in the host bacterium, and its expression is under the control of the IPTG inducible lac UV5 promoter.
20 This system has been employed successfully for over production of many proteins. Alternatively the polymerase gene may be introduced on a lambda phage by infection with an int- phage such as the CE6 phage which is commercially available (Novagen, Madison, USA). other vectors include vectors containing the lambda PL promoter such as PLEX (Invitrogen, NL), vectors containing the trc promoters such as pTrcHisXpressTm (Invitrogen)
25 or pTrc99 (Pharmacia Biotech, SE), or vectors containing the tac promoter such as pKK223-3 (Pharmacia Biotech) or PMAL (new England Biolabs, MA, USA).

Moreover, the VCP gene according to the invention preferably includes a secretion sequence in order to facilitate secretion of the polypeptide from bacterial hosts, such that it
30 will be produced as a soluble native peptide rather than in an inclusion body.

The peptide may be recovered from the bacterial periplasmic space, or the culture medium, as appropriate.

35 Suitable promoting sequences for use with yeast hosts may be regulated or constitutive and

are preferably derived from a highly expressed yeast gene, especially a *Saccharomyces cerevisiae* gene. Thus, the promoter of the TRP 1 gene, the ADHI or ADHII gene, the acid phosphatase (PH05) gene, a promoter of the yeast mating pheromone genes coding for the α -factor or a promoter derived from a gene encoding a glycolytic enzyme such as the promoter of the enolase, glyceraldehyde-3-phosphate dehydrogenase (GAP), 3-phosphoglycerate kinase (PGK), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triose phosphate isomerase, phosphoglucose isomerase or glucokinase genes, the *S. cerevisiae* GAL 4 gene, the *S. pombe* nmt 1 gene or a promoter from the TATA binding protein (TBP) gene can be used. Furthermore, it is possible to use hybrid promoters comprising upstream activation sequences (UAS) of one yeast gene and downstream promoter elements including a functional TATA box of another yeast gene, for example a hybrid promoter including the UAS (s) of the yeast PH05 gene and downstream promoter elements including a functional TATA box of the yeast GAP gene (PH05-GAP hybrid promoter). A suitable constitutive PH05 promoter is e. g. a shortened acid phosphatase PH05 promoter devoid of the upstream regulatory elements (UAS) such as the PH05 (-173) promoter element starting at nucleotide -173 and ending at nucleotide -9 of the PH05 gene.

Transcription of a DNA encoding VCP by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are relatively orientation and position independent. Many enhancer sequences are known from mammalian genes (e. g. elastase and globin). However, typically one will employ an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270) and the CMV early promoter enhancer. The enhancer may be spliced into the vector at a position 5' or 3' to VCP DNA, but is preferably located at a site 5' from the promoter.

Eukaryotic expression vectors will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding VCP.

Construction of vectors according to the invention employs conventional ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required. If desired, analysis to confirm correct sequences in the

constructed plasmids is performed in a known fashion. Suitable methods for constructing expression vectors, preparing in vitro transcripts, introducing DNA into host cells, and performing analyses for assessing VCP expression and function are known to those skilled in the art. Gene presence, amplification and/or expression may be measured in a sample
5 directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA, dot blotting (DNA or RNA analysis), or in situ hybridization, using an appropriately labelled probe which may be based on a sequence provided herein. Those skilled in the art will readily envisage how these methods may be modified, if desired.

10 **VCP is a drug development target**

According to the present invention, VCP is used as a target to identify compounds, for example lead compounds for pharmaceuticals, which are capable of modulating the infectivity of HIV by modulating its interaction with nuclear transport factors.

15

Accordingly, the invention relates to an assay and provides a method for identifying a compound or compounds capable, directly or indirectly, of modulating the infectivity of a virus which enters the cell through endocytosis, comprising the steps of:

- (a) incubating VCP polypeptides with the compound or compounds to be assessed; and
20 (b) identifying those compounds which influence the binding of VCP to the virus.

VCP binding compounds

According to a first embodiment of this aspect invention, the assay is configured to detect
25 compounds which bind directly to VCP polypeptides.

Binding to VCP polypeptides may be assessed by any technique known to those skilled in the art.

30 Examples of suitable assays include the two hybrid assay system, which measures interactions in vivo, affinity chromatography assays, for example involving binding to polypeptides immobilized on a column, fluorescence assays in which binding of the compound (s) and VCP polypeptides is associated with a change in fluorescence of one or both partners in a binding pair, and the like. Preferred are assays performed in vivo in cells,
35 such as the two-hybrid assay.

In a preferred aspect of this embodiment, the invention provides a method for identifying a lead compound for a pharmaceutical useful in the treatment of disease involving viral infection, comprising incubating a compound or compounds to be tested with a VCP polypeptide, under conditions in which, but for the presence of the compound or compounds to be tested, VCP associates with the virus with a reference affinity; determining the binding affinity of VCP for the virus in the presence of the compound or compounds to be tested; and selecting those compounds which modulate the binding affinity of VCP for the virus with respect to the reference binding affinity.

Preferably, therefore, the assay according to the invention is calibrated in absence of the compound or compounds to be tested, or in the presence of a reference compound whose activity in interacting with VCP polypeptides is known or is otherwise desirable as a reference value. For example, in a two-hybrid system, a reference value may be obtained in the absence of any compound. Addition of a compound or compounds which increase the binding affinity of VCP for the virus increases the readout from the assay above the reference level, whilst addition of a compound or compounds which decrease this affinity results in a decrease of the assay readout below the reference level.

Compounds which modulate the functional VCP interaction with virus

In a second embodiment, the invention may be configured to detect functional interactions between a compound or compounds and VCP polypeptides. Such interactions can affect the ability of VCP to interact with nuclear transport factors such as TNPO3 or RanBP2, and therefore HIV infectivity.

Assays which detect modulation of the functional interaction between VCP and the virus are preferably cell-based assays. For example, they may be based on infection assays using cultured cells which are exposed to HIV virions in the presence or absence of the test compound(s).

In preferred embodiments, a nucleic acid encoding a VCP polypeptide is ligated into a vector, and introduced into suitable host cells to produce transformed cell lines that express the VCP polypeptides. The resulting cell lines can then be produced for reproducible qualitative and/or quantitative analysis of the effect (s) of potential compounds affecting VCP

polypeptides function. Thus VCP polypeptide-expressing cells may be employed for the identification of compounds, particularly low molecular weight compounds, which modulate the interaction between VCP and virus. Thus host cells expressing VCP polypeptides are useful for drug screening and it is a further object of the present invention to provide a method for identifying compounds which modulate the activity of VCP, said method comprising exposing cells containing heterologous DNA encoding VCP polypeptides, wherein said cells produce functional VCP, to at least one compound or mixture of compounds or signal whose ability to modulate the interaction of said VCP polypeptides is sought to be determined, and thereafter monitoring said cells for changes caused by said modulation. Such an assay enables the identification of modulators, such as agonists, antagonists and allosteric modulators, of the interaction between VCP and virus.

Cell-based screening assays can be designed by constructing cell lines in which the expression of a reporter protein, i.e. an easily assayable protein, such as beta galactosidase, chloramphenicol acetyltransferase (CAT) or luciferase, is dependent on the interaction between VCP and virus. For example, a reporter gene encoding one of the above polypeptides may be placed under the control of an enhancer which is activated by a factor assembled in a two-hybrid reaction between VCP and virus.

Alternative assay formats include assays which directly assess HIV infectivity in a biological system. Such systems are known in the art, and further described below.

Compounds which modulate the interaction between VCP and Virus.

As noted above, assays may be configured to detect binding between VCP and virus polypeptides, or the modulation of viral infectivity by disruption of the indirect interaction between VCP and the viral capsid.

Examples of compounds which are capable of modulating the interaction between VCP and virus include compounds which are inhibitors of VCP and/or VCP adaptor proteins.

VCP inhibitors include the compounds mentioned above, as well as the agents shown in Table 1 below, and agents which inhibit and/or disrupt VCP adaptor proteins.

VCP adaptor proteins are known in the art. For example, see Marsden et al., (2009) Int J Biochem Cell Biol., 41:2380-2388, especially Table 1 therein. Moreover, methods are known for identifying VCP adaptor proteins. For example Ritz et al. (2011) Nature Cell Biology DOI:1038.ncb2301 describe a method based on unbiased mass spectrometry, which they use to identify a complex between VCP and the UBXD1 cofactor.

Compounds

In a still further aspect, the invention relates to a compound or compounds identifiable by an assay method as defined in the previous aspect of the invention.

Accordingly, there is provided the use of a compound identifiable by an assay as described herein, for the modulation of the infectivity of HIV.

Compounds which influence the VCP/virus interaction may be of almost any general description, including low molecular weight compounds, including organic compounds which may be linear, cyclic, polycyclic or a combination thereof, peptides, polypeptides including antibodies, or proteins. In general, as used herein, "peptides", "polypeptides" and "proteins" are considered equivalent.

Certain VCP inhibitors are set forth in table 1. See also Yamanaka et al., (2012) BBA 1823:130-137.

Inhibitor	Potency of inhibition (IC50 on ATPase in vitro, EC50 on Ub- G76V in cell culture)	VCP domain targeted, or likely to be targeted	Proposed mechanism of inhibiting VCP	References and comments
2-anilino-4-aryl-1,3-thiazoles (several distinct compounds)	IC50 = 0.07 – 0.23 μ M EC50 = 0.09 – 1.2 μ M	D2 ATPase domain	Inhibition of ATPase activity. Reversibility?	Bursavich et al. 2010 Bioorganic & Medicinal Chemistry Letters (see table 4). By Myriad Pharmaceuticals, USA.
Syk inhibitor III: 3,4-methylenedioxy- β -nitrostyrene	IC50 = 1.7 μ M EC50 = 1.6 μ M	Cys522 in D2 ATPase domain	Inhibition of ATPase activity. Irreversible.	Chou and Deshaies 2011 Journal of Biological Chemistry.
DBEQ: N ² ,N ⁴ -dibenzylquinazoline-2,4-diamine	IC50 = 1.5 μ M EC50 = 2.6 μ M	D2 ATPase domain	ATP-competitive inhibition of ATPase activity. Reversible.	Chou et al. 2011 PNAS.
NMS-873	IC50 = 0.03 μ M EC50 information: likely to be around 0.4 – 0.7 μ M	Not published	Allosteric (new) mechanism suggested	Magnaghi et al. 2012: Identification of potent VCP/p97/CDC48 inhibitors (...). Proceedings: AACR Annual Meeting, Chicago, USA. Cancer Res. 2012:72, Issue 8, Suppl. 1. Also disclosed another Cys522-modifying inhibitor, NMS-859.
Eeyarestatin I	IC50 not measurable EC50 information: effective inhibition at 5 – 10 μ M	Likely D1 domain (D2 and N domains ruled out as binding sites)	Binding to D1 domain, possibly inducing conformational change and oligomerization of VCP. No effect on ATPase activity!	Wang et al. 2008 Journal of Biological Chemistry, and Wang et al. 2010 PLoS ONE.
Xanthohumol	IC50 not determined. EC50 information: effective inhibition around 30 μ M	N domain	Binding to N domain. Further details unknown.	Sasazawa et al. 2012 ACS Chemical Biology.

Table 1

Antibodies

Antibodies, as used herein, refers to complete antibodies or antibody fragments capable of binding to a selected target, and including Fv, ScFv, Fab' and F (ab')₂, monoclonal and polyclonal antibodies, engineered antibodies including chimeric, CDR-grafted and humanized antibodies, and artificially selected antibodies produced using phage display or alternative techniques. Small fragments, such as Fv and ScFv, possess advantageous properties for diagnostic and therapeutic applications on account of their small size and consequent superior tissue distribution.

The antibodies according to the invention are especially indicated for diagnostic and therapeutic applications. Accordingly, they may be altered antibodies comprising an effector protein such as a toxin or a label. Especially preferred are labels which allow the imaging of the distribution of the antibody in vivo. Such labels may be radioactive labels or radioopaque labels, such as metal particles, which are readily visualizable within the body of a patient. Moreover, they may be fluorescent labels or other labels which are visualizable on tissue samples removed from patients.

Recombinant DNA technology may be used to improve the antibodies of the invention. Thus, chimeric antibodies may be constructed in order to decrease the immunogenicity thereof in diagnostic or therapeutic applications. Moreover, immunogenicity may be minimized by humanizing the antibodies by CDR grafting [see European Patent Application 0 239 400 (Winter)] and, optionally, framework modification [see international patent application WO 90/07861 (Protein Design Labs)].

Antibodies according to the invention may be obtained from animal serum, or, in the case of monoclonal antibodies or fragments thereof, produced in cell culture.

Recombinant DNA technology may be used to produce the antibodies according to established procedure, in bacterial or preferably mammalian cell culture. The selected cell culture system preferably secretes the antibody product.

Therefore, the present invention includes a process for the production of an antibody according to the invention comprising culturing a host, e. g. *E. coli* or a mammalian cell, which has been transformed with a hybrid vector comprising an expression cassette

comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding said protein, and isolating said protein.

Multiplication of hybridoma cells or mammalian host cells in vitro is carried out in suitable culture media, which are the customary standard culture media, for example Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 medium, optionally replenished by a mammalian serum, e. g. fetal calf serum, or trace elements and growth sustaining supplements, e. g. feeder cells suspension culture, e. g. in an airlift reactor or in a continuous stirrer reactor, or immobilized or entrapped cell culture, e. g. in hollow fibers, microcapsules, on agarose microbeads or ceramic cartridges.

Large quantities of the desired antibodies can also be obtained by multiplying mammalian cells in vivo. For this purpose, hybridoma cells producing the desired antibodies are injected into histocompatible mammals to cause growth of antibody-producing tumors. Optionally, the animals are primed with a hydrocarbon, especially mineral oils such as pristane (tetramethylpentadecane), prior to the injection. After one to three weeks, the antibodies are isolated from the body fluids of those mammals. For example, hybridoma cells obtained by fusion of suitable myeloma cells with antibody-producing spleen cells from Balb/c mice, or transfected cells derived from hybridoma cell line Sp2/0 that produce the desired antibodies are injected intraperitoneally into Balb/c mice optionally pre-treated with pristane, and, after one to two weeks, ascitic fluid is taken from the animals.

The foregoing, and other, techniques are discussed in, for example, Kohler and Milstein, (1975) Nature 256: 495-497; US 4, 376, 110; Harlow and Lane, Antibodies: a Laboratory Manual, (1988) Cold Spring Harbor, incorporated herein by reference.

Techniques for the preparation of recombinant antibody molecules is described in the above references and also in, for example, EP 0623679; EP 0368684 and EP 0436597, which are incorporated herein by reference.

Antibodies and antibody fragments according to the invention are useful in targeting VCP polypeptides, and can inhibit binding between these molecules.

Peptides

Peptides according to the present invention are usefully derived from VCP or another polypeptide involved in the functional interaction between VCP and the mechanism of HIV infection.

Preferably, the peptides are derived from the domains in VCP which are responsible for VCP/virus interaction. For example, Thornberry et al., (1994) *Biochemistry* 33: 3934-3940 and Milligan et al., (1995) *Neuron* 15: 385-393 describe the use of modified tetrapeptides to inhibit ICE protease. In an analogous fashion, peptides derived from VCP or an interacting protein may be modified, for example with an aldehyde group, chloromethylketone, (acyloxy) methyl ketone or CH₂O-DCB group to inhibit the VCP/virus interaction.

In order to facilitate delivery of peptide compounds to cells, peptides may be modified in order to improve their ability to cross a cell membrane. For example, US 5, 149, 782 discloses the use of fusogenic peptides, ion-channel forming peptides, membrane peptides, long-chain fatty acids and other membrane blending agents to increase protein transport across the cell membrane. These and other methods are also described in WO 97/37016 and US 5, 108, 921, incorporated herein by reference.

Many compounds according to the present invention may be lead compounds useful for drug development. Useful lead compounds are especially antibodies and peptides, and particularly intracellular antibodies expressed within the cell in a gene therapy context, which may be used as models for the development of peptide or low molecular weight therapeutics. In a preferred aspect of the invention, lead compounds and VCP or other target peptide may be co-crystallized in order to facilitate the design of suitable low molecular weight compounds which mimic the interaction observed with the lead compound.

Crystallization involves the preparation of a crystallization buffer, for example by mixing a solution of the peptide or peptide complex with a "reservoir buffer", preferably in a 1:1 ratio, with a lower concentration of the precipitating agent necessary for crystal formation. For crystal formation, the concentration of the precipitating agent is increased, for example by addition of precipitating agent, for example by titration, or by allowing the concentration of precipitating agent to balance by diffusion between the crystallization buffer and a reservoir buffer. Under suitable conditions such diffusion of precipitating agent occurs along the gradient of precipitating agent, for example from the reservoir buffer having a higher

concentration of precipitating agent into the crystallization buffer having a lower concentration of precipitating agent. Diffusion may be achieved for example by vapor diffusion techniques allowing diffusion in the common gas phase. Known techniques are, for example, vapor diffusion methods, such as the "hanging drop" or the "sitting drop" method. In the vapor diffusion method a drop of crystallization buffer containing the protein is hanging above or sitting beside a much larger pool of reservoir buffer. Alternatively, the balancing of the precipitating agent can be achieved through a semipermeable membrane that separates the crystallization buffer from the reservoir buffer and prevents dilution of the protein into the reservoir buffer.

In the crystallization buffer the peptide or peptide/binding partner complex preferably has a concentration of up to 30 mg/ml, preferably from about 2 mg/ml to about 4 mg/ml.

Formation of crystals can be achieved under various conditions which are essentially determined by the following parameters: pH, presence of salts and additives, precipitating agent, protein concentration and temperature. The pH may range from about 4.0 to 9.0. The concentration and type of buffer is rather unimportant, and therefore variable, e. g. in dependence with the desired pH. Suitable buffer systems include phosphate, acetate, citrate, Tris, MES and HEPES buffers. Useful salts and additives include e. g. chlorides, sulphates and other salts known to those skilled in the art. The buffer contains a precipitating agent selected from the group consisting of a water miscible organic solvent, preferably polyethylene glycol having a molecular weight of between 100 and 20000, preferentially between 4000 and 10000, or a suitable salt, such as a sulphates, particularly ammonium sulphate, a chloride, a citrate or a tartarate.

A crystal of a peptide or peptide/binding partner complex according to the invention may be chemically modified, e. g. by heavy atom derivatization. Briefly, such derivatization is achievable by soaking a crystal in a solution containing heavy metal atom salts, or organometallic compounds, e. g. lead chloride, gold thiomalate, thimerosal or uranyl acetate, which is capable of diffusing through the crystal and binding to the surface of the protein. The location (s) of the bound heavy metal atom (s) can be determined by X-ray diffraction analysis of the soaked crystal, which information may be used e. g. to construct a three-dimensional model of the peptide.

A three-dimensional model is obtainable, for example, from a heavy atom derivative of a

crystal and/or from all or part of the structural data provided by the crystallization. Preferably building of such model involves homology modeling and/or molecular replacement.

Computational software may also be used to predict the secondary structure of the peptide or peptide complex. The peptide sequence may be incorporated into the crystal structure. Structural incoherences, e. g. structural fragments around insertions/deletions can be modelled by screening a structural library for peptides of the desired length and with a suitable conformation. For prediction of the side chain conformation, a side chain rotamer library may be employed.

The final homology model is used to solve the crystal structure of the peptide by molecular replacement using suitable computer software. The homology model is positioned according to the results of molecular replacement, and subjected to further refinement comprising molecular dynamics calculations and modeling of the inhibitor used for crystallization into the electron density.

Other Compounds

In a preferred embodiment, the above assay is used to identify peptide but also non-peptide-based test compounds that can modulate VCP activity, as evidenced by HIV infectivity, or target polypeptide interactions. The test compounds of the present invention can be obtained using any of the numerous approaches involving combinatorial library methods known in the art, including: biological libraries, spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. These approaches are applicable to peptide, non-peptide oligomer, or small molecule libraries of compounds (Lam, K. S. (1997) *Anticancer Drug Des.* 12: 145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90: 6909; Erb et al. (1994) *Proc. Natl. Acad. Sci. USA* 91: 11422; Zuckermann et al. (1994). *J. Med. Chem.* 37: 2678; Cho et al. (1993) *Science* 261: 1303; Carrell et al. (1994) *Angew. Chem. Int. Ed. Engl.* 33: 2059; Carell et al. (1994) *Angew. Chem. Int. Ed. Engl.* 33: 2061; and in Gallop et al. (1994) *J. Med. Chem.* 3: 1233.

Libraries of compounds may be presented in solution (e. g., Houghten (1992) *Biotechniques* 13: 412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364: 555-556), bacteria (Ladner USP 5, 223, 409), spores (Ladner USP '409), plasmids (Cull et al. (1992) *Proc Natl Acad Sci USA* 89: 1865-1869) or on phage (Scott and Smith (1990) *Science* 249: 386-390); (Devlin (1990) *Science* 249: 404-406); (Cwirla et al. (1990) *Proc. Natl. Acad. Sci.* 87: 6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); Ladner (*supra*).

If desired, any of the compound libraries described herein may be divided into pre-selected libraries comprising compounds having, e. g., a given chemical structure, or a given activity, e. g., kinase inhibitory activity. Pre-selecting a compound library may further involve performing any art recognized molecular modelling in order to identify particular compounds or groups or combinations of compounds as likely to have a given activity, reactive site, or other desired chemical functionality. In one embodiment, modulators of VCP are pre-selected using molecular modelling designed to identify compounds having, or likely to have, activity on viral infectivity.

Suitable methods, as are known in the art, can be used to select particular moieties for interacting with a particular domain of VCP or target component. For example, visual inspection, particularly utilizing three-dimensional models, can be employed. Preferably, a computer modelling program, or software, is used to select one or more moieties which can interact with a particular domain. Suitable computer modeling programs include QUANTA (Molecular Simulations, Inc., Burlington, MA (1992)), SYBYL (Tripos Associates, Inc., St. Louis, MO (1992)), AMBER (Weiner et al., *J. Am. Chem. Soc.* 106: 765-784 (1984)) and CHARMM (Brooks et al., *J. Comp. Chem.* 4: 187-217 (1983)). Other programs which can be used to select interacting moieties include GRID (Oxford University, U. K.; Goodford et al., *J. Mod. Chem.* 28: 849-857 (1985)); MCSS (Molecular Simulations, Inc., Burlington, MA; Miranker, A. and M. Karplus, *Proteins: Structure, Function and Genetics* 11: 29-34 (1991)); AUTODOCK (Scripps Research Institute, La Jolla, CA; Goodsell et al., *Proteins: Structure, Function and Genetics*: 195-202 (1990)); and DOCK (University of California, San Francisco, CA; Kuntz et al., *J. Mol. Biol.* 161: 269-288 (1982)).

After potential interacting moieties have been selected, they can be attached to a scaffold which can present them in a suitable manner for interaction with the selected domains.

Suitable scaffolds and the spatial distribution of interacting moieties thereon can be determined visually, for example, using a physical or computer-generated three-dimensional model, or by using a suitable computer program, such as CAVEAT (University of California, Berkeley, CA; Bartlett et al., in "Molecular Recognition of in Chemical and Biological Problems", Special Pub., Royal Chemical Society 78: 182-196 (1989)); three-dimensional database systems, such as MACCS-3D (MDL Information Systems, San Leandro, CA (Martin, Y. C., J. Mod. Chem. 35: 2145-2154 (1992)); and HOOK (Molecular Simulations, Inc.). Other computer programs which can be used in the design and/or evaluation of potential VCP inhibitors include LUDI (Biosym Technologies, San Diego, CA; Bohm, H. J., J. Comp. Aid. Molec. Design: 61-78 (1992)), LEGEND (Molecular Simulations, Inc.; Nishibata et al., Tetrahedron 47: 8985 8990 (1991)), and LeapFrog (Tripos Associates, Inc.).

In addition, a variety of techniques for modeling protein-drug interactions are known in the art and can be used in the present method (Cohen et al., J. Med. Chem. 33: 883-894 (1994); Navia et al. Current Opinions in Structural Biology 2: 202-210 (1992); Baldwin et al., J. Mod. Chem. 32: 2510-2513 (1989); Appelt et al.; J. Mod. Chem. 34: 1925-1934 (1991); Ealick et al., Proc. Nat. Acad. Sci. USA 88: 11540-11544 (1991)).

Thus, a library of compounds, e. g., compounds that are protein based, carbohydrate based, lipid based, nucleic acid based, natural organic based, synthetically derived organic based, or antibody based compounds can be assembled and subjected, if desired, to a further preselection step involving any of the aforementioned modeling techniques. Suitable candidate compounds determined to be VCP modulators using these modeling techniques may then be selected from art recognized sources, e. g., commercial sources, or, alternatively, synthesized using art recognized techniques to contain the desired moiety predicted by the molecular modeling to have an activity, e. g., HIV inhibitory activity. These compounds may then be used to form e. g., a smaller or more targeted test library of compounds for screening using the assays described herein.

In one embodiment, an assay is a cell-based or cell-free assay in which either a cell that expresses, e. g., a VCP polypeptide or cell lysate/or purified protein comprising VCP is contacted with a test compound and the ability of the test compound to alter VCP activity, e.g., binding activity or HIV inhibition is measured.

Any of the cell-based assays can employ, for example, a cell of eukaryotic or prokaryotic origin. Determining the ability of the test compound to bind to a VCP polypeptide can be accomplished, for example, by coupling the test compound with a radioisotope or enzymatic label such that binding of the test compound to the polypeptide can be determined by detecting the labelled compound in a complex.

For example, test compounds can be labelled with ^{125}I , ^{35}S , ^{14}C , ^{33}P or ^3H , either directly or indirectly, and the radioisotope detected by direct counting of radioemission or by scintillation counting. Alternatively, test compounds can be enzymatically labelled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

It is also within the scope of this invention to determine the ability of a test compound to interact with a target polypeptide without the labelling of any of the interactants. For example, a microphysiometer can be used to detect the interaction of a test compound with VCP without the labelling of either the test compound or VCP (McConnell, H. M. et al. (1992) *Science* 257: 1906-1912). In yet another embodiment, an assay of the present invention is a cell-free assay in which, e. g., VCP is contacted with a test compound and the ability of the test compound to alter the interaction with virus is determined.

Determining the ability of the candidate compound to bind to either polypeptide can also be accomplished using a technology such as real-time Biomolecular Interaction Analysis (BIA) (Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63: 2338-2345 and Szabo et al. (1995) *Curr. Opin. Struct. Biol.* 5: 699-705). As used herein, "BIA" is a technology for studying bispecific interactions in real time, without labelling any of the interactants (e.g., BIAcore). Changes in the optical phenomenon surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be performed using purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound.

Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the in vitro system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with upstream or downstream elements. Accordingly, in an exemplary screening assay of the present invention, the compound of interest is contacted with the VCP polypeptide as set forth above. The efficacy of the test compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In another embodiment, various candidate compounds are tested and compared to a control compound with a known activity, e. g., an inhibitor having a known generic activity, or, alternatively, a specific activity, such that the specificity of the test compound may be determined.

In more than one embodiment of the above assay methods of the present invention, it may be desirable to immobilize the target polypeptide to facilitate separation of complexed from uncomplexed forms or accommodate automation of the assay. Binding of VCP to virus in the presence or absence of a test compound can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase/target polypeptide fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the test compound and incubated under conditions conducive to phosphorylation or complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, and the complex is measured either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of target polypeptide binding or phosphorylation activity can be determined using standard techniques. Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention.

In yet another aspect of the invention, VCP polypeptides can be used as "bait proteins" in a two-hybrid assay or three-hybrid assay (see, e. g., U. S. Patent No. 5, 283, 317; Zervos et al. (1993) Cell 72: 223-232; Madura et al. (1993) J. Biol. Chem. 268: 12046-12054; Bartel et al.

(1993) Biotechniques 14: 920-924; Iwabuchi et al. (1993) Oncogene 8: 1693-1696; and W094/10300), to identify other proteins or compounds, which bind to or interact with VCP.

This invention further pertains to novel agents identified by the above-described screening assays and to processes for producing such agents by use of these assays.

Accordingly, in one embodiment, the present invention includes a compound or agent obtainable by a method comprising the steps of any one of the aforementioned screening assays (e. g., cell-based assays or cell-free assays). For example, in one embodiment, the invention includes a compound or agent obtainable by any of the methods described herein.

Accordingly, it is within the scope of this invention to further use an agent, e.g., a VCP polypeptide or compound identified as described herein in an appropriate animal model. For example, an agent identified as described herein can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent.

Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. In addition, such an agent if deemed appropriate may be administered to a human subject.

The present invention also pertains to uses of novel agents identified by the above-described screening assays for diagnoses, prognoses, and treatments of any of the disorders described herein. Accordingly, it is within the scope of the present invention to use such agents in the design, formulation, synthesis, manufacture, and/or production of a drug or pharmaceutical composition for use in diagnosis, prognosis, or treatment of any of the disorders described herein.

4. Pharmaceutical Compositions

In a preferred embodiment, there is provided a pharmaceutical composition comprising a compound or compounds identifiable by an assay method as defined in the previous aspect of the invention.

A pharmaceutical composition according to the invention is a composition of matter comprising a compound or compounds capable of modulating the infectivity of HIV as an

active ingredient. Typically, the compound is in the form of any pharmaceutically acceptable salt, or e. g., where appropriate, an analog, free base form, tautomer, enantiomer racemate, or combination thereof. The active ingredients of a pharmaceutical composition comprising the active ingredient according to the invention are contemplated to exhibit excellent therapeutic activity, for example, in the treatment or prevention of HIV infection. For example, the invention encompasses any compound that can alter the binding of VCP to virus.

Dosage regimens may be adjusted to provide the optimum therapeutic response.

For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active ingredient may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intramuscular, subcutaneous, intranasal, intradermal or suppository routes or implanting (e. g. using slow release molecules).

Depending on the route of administration, the active ingredient may be required to be coated in a material to protect said ingredients from the action of enzymes, acids and other natural conditions which may inactivate said ingredient.

In order to administer the active ingredient by other than parenteral administration, it will be coated by, or administered with, a material to prevent its inactivation. For example, the active ingredient may be administered in an adjuvant, co-administered with enzyme inhibitors or in liposomes. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and nhexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin.

Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes.

The active ingredient may also be administered parenterally or intraperitoneally.

Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures

thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active ingredient in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredient is suitably protected as described above, it may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsules, or it may be compressed into tablets, or it

may be incorporated directly with the food of the diet. For oral therapeutic administration, the active ingredient may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. The amount of active ingredient in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following: a binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier.

Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active ingredient, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active ingredient may be incorporated into sustained-release preparations and formulations.

As used herein "pharmaceutically acceptable carrier and/or diluent" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The

specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such as active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired.

The principal active ingredients are compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

In a further aspect there is provided the active ingredient of the invention as hereinbefore defined for use in the treatment of disease either alone or in combination with art recognized compounds known to be suitable for treating the particular indication.

The invention is further described, for the purpose of illustration only, in the following examples.

Examples

Example 1

Presence of VCP is essential for intracellular neutralization

To determine whether VCP is involved in antibody-dependent intracellular neutralization of AdV, HeLa cells depleted of VCP, TRIM21 or both were infected with replication-deficient AdV type 5 encoding GFP (AdV-GFP) pre-incubated with or without a neutralizing polyclonal antibody (pAb). In cells transfected with control siRNA, pAb potently neutralized infection by 33-fold (Fig. 1A, B). In cells depleted of VCP, neutralization was substantially reduced, to ~6-fold (Fig. 1A, B). Cells depleted of TRIM21 by shRNA also displayed significantly reduced neutralization; however, depletion of TRIM21 in VCP knockdown cells did not relieve neutralization further than knockdown of VCP alone. The absence of an additive effect suggests that TRIM21 and VCP operate in the same pathway. Treating TRIM21 shRNA cells with the immunostimulatory cytokine interferon- α (IFN) 24 h prior to infection completely restored both TRIM21 levels (Fig. 1C) and neutralization (as seen previously (Mallery et al.,

2010; McEwan et al., 2012)), but this treatment had no effect in cells that were also depleted of VCP (Fig. 1A, B). This suggests that the presence of VCP is required for TRIM21-mediated neutralization of AdV by antibody.

VCP ATPase activity is required for ADIN

Next, the importance of VCP activity in ADIN was tested using specific chemical inhibitors. N2,N4-dibenzylquinazoline-2,4-diamine (DBeQ) was recently identified as a selective, potent, reversible and ATP-competitive inhibitor of VCP (Chou et al., 2011). DBeQ was titrated onto HeLa cells at concentrations around its IC₅₀ of 2.6 μ M (as measured by Chou et al. on VCP-dependent degradation of ubiquitinG76V-GFP, a UFD model substrate), then cells were infected with AdVGFP in the presence or absence of a neutralizing monoclonal antibody (mAb), 9C12. As previously shown (Mallery et al., 2010), pre-incubation of AdV with mAb 9C12 potently neutralized infection in HeLa cells (Fig. 2A). However, prior addition of DBeQ inhibited intracellular neutralization in a dose-dependent manner. Neutralization was almost completely abrogated above 9 μ M DBeQ, and higher concentrations of inhibitor had no further effect (Fig. 2B). This dose response curve closely correlates with that previously published for *in vitro* DBeQ inhibition of VCP ATPase activity (Chou et al., 2011). Importantly, in the absence of antibody, DBeQ had no effect on AdV infection (Fig. 2A).

Further validation of these results was obtained by treating cells with Eeyarestatin I (Eerl), an irreversible inhibitor of VCP (Wang et al., 2008; Wang et al., 2010). Similar to treatment with DBeQ or knockdown of VCP, treatment with 10 μ M Eerl prior to infection potently inhibited neutralization of AdV by mAb 9C12 (Fig. 2C). Taken together these data demonstrate that the ATPase activity of VCP is crucial for intracellular neutralization of AdV by antibody.

To test whether VCP functions independently of or on pathway with the proteasome, we compared DBeQ and the irreversible proteasome inhibitor epoxomicin. Neutralization of AdV by mAb 9C12 was abrogated by treatment with 12 μ M DBeQ or 2 μ M epoxomicin (Fig. 2D). Combined treatment with both inhibitors had a similar effect to epoxomicin alone and was only marginally stronger compared to DBeQ alone. The absence of significant additive effects indicates that VCP and the proteasome operate on the same pathway of neutralization.

VCP is required for proteasomal degradation of viral capsid and antibody in ADIN

VCP is involved in the proteasomal degradation of a specific set of cellular substrates (Beskow et al., 2009; Dai and Li, 2001; Meyer et al., 2012; Wojcik et al., 2006). To investigate whether VCP is required for viral capsid degradation during ADIN, we performed a fate-of-capsid assay. Previously, we have used this assay to show that intracellular neutralization of AdV is accompanied by rapid degradation of hexon, the major adenovirus capsid component, and associated antibody. Degradation is inhibited by depletion of TRIM21 and significantly slowed by chemical inhibition of the proteasome (Mallery et al., 2010). To determine the role of VCP in viral capsid degradation, HeLa cells treated with DMSO, 10 μ M DBeQ or 10 μ M Eerl were infected with AdV pre-incubated with mAb 9C12 and harvested at several time points between 1 h and 6 h post infection (p.i.). As previously observed (Mallery et al., 2010), both hexon and antibody were efficiently degraded within 2 h p.i. in DMSO-treated control cells (Fig. 3A, upper panel) to the extent where both were virtually undetectable after 2.5 h p.i.. Both VCP inhibitors – DBeQ and Eerl – potently inhibited degradation, with no detectable degradation of either hexon or antibody in DBeQ-treated cells during the entire 6 h period of the experiment (Fig. 3A, middle and lower panels). These data suggest that VCP has a crucial role during ADIN-mediated proteasomal degradation of the viral capsid.

The requirement for VCP in ADIN is substrate-specific

Recent reports have suggested that VCP may be required when the proteasome faces a particularly challenging substrate (Beskow et al., 2009) or when it becomes stalled by challenging amounts of substrate (Isakov and Stanhill, 2011). We therefore investigated whether VCP is constitutively required for TRIM21-mediated degradation or whether its role is substrate-dependent. In order to test if VCP is required for degradation of analogous but less challenging substrates than viral capsid, we established a model system based on overexpression of IgG Fc in HeLa cells. Given that TRIM21 binds to IgG with an affinity of ~ 0.6 nM and mediates ubiquitination of antibody-bound objects in the cytosol to target them for degradation irrespective of their identity (Mallery et al., 2010), it was expected that IgG Fc overexpressed in the cytosol would be degraded by the proteasome in a TRIM21-dependent fashion. Furthermore, IgG Fc could be considered the simplest substrate whose degradation would be mediated by TRIM21. Consistent with rapid degradation and turnover, relatively low levels of Fc were detected in Fc-overexpressing control cells. Importantly, levels of Fc were significantly increased when cells were depleted of TRIM21 by shRNA, treated with 2 μ M epoxomicin, or both (Fig. 3B). However, addition of 10 μ M DBeQ, which completely inhibited degradation of virus and antibody in the fate of- capsid experiment, failed to prevent

degradation of IgG Fc (Fig. 3C). This result suggests that the requirement of VCP is substrate-dependent and supports a hypothesis in which VCP is recruited to the proteasome upon processing of the viral capsid.

Mechanism of ADIN: Neutralization is intimately linked to degradation

The results of the fate-of-capsid experiments shown here and in our previous study (Mallery et al., 2010) suggest that ADIN prevents infection by catalysing (premature) degradation of antibody-bound viral capsid. To test this hypothesis, we investigated whether degradation and neutralization proceed with similar kinetics. A time-of-addition assay was performed in which HeLa cells were synchronously infected with AdV-GFP by cold attachment, in the presence or absence of mAb 9C12, and treated with either DMSO or 9 μ M DBE-Q at a range of time points post infection. Addition of DBE-Q concurrent with infection inhibited ADIN as potently as previously observed for pre-treatment at the same concentration (Fig. 3D and 2B). However, the effect of DBE-Q quickly diminished when the inhibitor was added at later time points p.i. (Fig. 3D). The majority of neutralization occurred within the first 2.5 h p.i., as evidenced by the reduced effect of DBE-Q when added after this time point. Thus the majority of neutralization coincides with degradation of the majority of viral capsid observed in the fate-of-capsid assay (Fig. 3A). These findings support our model wherein proteasomal degradation, enabled by VCP, is the key effector mechanism of neutralization in ADIN. *Both efficiency and potency of ADIN depend on VCP* Finally, we investigated the consequences of VCP perturbation on the efficiency of antibody neutralization and the level of persistent infection. AdV-GFP was preincubated with varying concentrations of mAb 9C12 before infection of HeLa cells that were subjected to RNA interference or to treatment with DBE-Q. Both depletion of VCP by siRNA and depletion of TRIM21 by shRNA significantly reduced the initial gradient of neutralization as a function of antibody concentration (Fig. 4A), as did treatment with increasing concentrations of DBE-Q (Fig. 4B). At high concentrations of mAb 9C12, where neutralization is maximal and further addition of antibody does not result in further neutralization (a phenomenon referred to as the persistent fraction of non-neutralized virus), depletion of VCP or TRIM21 led to a significant increase in the level of infection at maximum antibody neutralization (Fig. 4A). Depleting TRIM21 knockdown cells of VCP did not greatly increase infection levels further, in agreement with these factors being on pathway. Furthermore, the level of persistent infection increased in a dose-dependent manner with increasing DBE-Q concentration (Fig. 4B). These results show that without functioning VCP, a high proportion of cells become infected even at saturating levels of neutralizing antibody 9C12. It is therefore noteworthy that VCP is a very abundant protein

(Peters et al., 1990; Pleasure et al., 1993) suggesting that even though it is not IFN-inducible, it is unlikely to becoming rate limiting even at high multiplicities of infection.

Example 2

VCP inhibitors prevent infection by retroviruses and HSV

Materials and Methods

Cell culture and production of viruses

All cell lines were maintained as described previously (Mallery, D. L. *et al. Proc Natl Acad Sci U S A* **107**, 19985-19990, doi:10.1073/pnas.1014074107 (2010)). To deplete VCP, HeLa cells were transfected with siRNA oligonucleotides VCPHSS111263, VCPHSS111264 and VCPHSS187663 (Invitrogen) against human VCP, or with Silencer Negative Control siRNA # 1 (Ambion). For differentiation into monocyte-derived macrophages, THP-1 cells were stimulated with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) for 7 d prior to infection. Replication-deficient, E1-deleted GFP-expressing human adenovirus 5 (de Martin, R et al., *Gene Ther* **4**, 493-495, doi:10.1038/sj.gt.3300408 (1997)) was prepared by CsCl centrifugation. Lentiviruses and retroviruses were produced by transient transfection of 293T cells with plasmids encoding appropriate gag/pol, a GFP reporter gene and an envelope. Respiratory syncytial virus (RSV) GFP-expressing molecular clone rgRSV(224) (Hallak, L. K., et al. *J Virol* **74**, 10508-10513 (2000)) was provided by Dr Mark Peeples, Ohio State University and was expanded in HeLa cells.

Infection experiments

When used, siRNA was transfected into cells 72 h prior to infection, and VCP inhibitor DBeQ (or DMSO, as solvent control) was added to cells by medium exchange 30-60 min. before infection. Infection with retroviruses and lentiviruses were carried out in presence of 5 µg/ml polybrene (Santa Cruz Biotechnology). Unless indicated otherwise, cells were infected with virus at a multiplicity of infection (MOI) of 0.1 to 0.4. After incubation at 37 °C for 12 to 48 h, cells were harvested by trypsinization for either analysis on a BD LSRII Flow Cytometer (BD Biosciences) or quantitative PCR assays. Flow cytometry data analysis was carried out in FlowJo (Tree Star).

Quantitative PCR

For qPCR analysis, DNA was prepared in biological triplicates from frozen cell pellets using DNEasy Blood & Tissue Kit (Qiagen). Four separate TaqMan (Applied Biosystems) qPCR runs were performed in technical duplicates: HIV early RT product (GFP, primers and probe as in McEwan, W. A. *et al. J Virol*, doi:10.1128/JVI.00728-12 (2012)), HIV late RT product (primers and probe as for “total viral DNA” in Apolonia *et al.* 2007 *Mol Ther*) and 18S eukaryotic rRNA (Invitrogen assay Hs99999901_s1, detecting genomic DNA) were analyzed alongside their respective plasmid standard dilutions (pWPI for GFP/early RT product, pcRV-gag/pol for late RT product, cellular genomic DNA for 18S rRNA) for absolute quantification. In a separate run, HIV 2-LTR circles (primers and probe as for “2LTR circles” in Apolonia *et al.* 2007 *Mol Ther*) were analyzed alongside 18S on the same plate for relative quantification by the $\Delta\Delta C_t$ method.

VCP is essential for HIV-1 infection

To determine whether VCP is involved in either restriction of HIV-1 by TRIM5 α or in HIV-1 infection itself, we made use of feline CRFK cells that express only an inactive truncation of TRIM5. These cells were stably transduced to express human or rhesus macaque TRIM5 α , treated with the VCP inhibitor N²,N⁴-dibenzylquinazoline-2,4-diamine (DBeQ) (Chou, T. F. *et al. Proc Natl Acad Sci U S A* **108**, 4834-4839, doi:10.1073/pnas.1015312108 (2011)) or solvent DMSO, and infected with VSV G-pseudotyped HIV-1 (HIV1-GFP-VSV). Treatment with DBeQ did not rescue infection from restriction by TRIM5 α but potently inhibited infection of CRFK cells by HIV1-GFP-VSV (more than 90-fold) independently of presence or absence of restriction factor (Fig. 5A). Subjecting CRFK cells to a titration of VCP inhibitor before infection with HIV1-GFP-VSV confirmed that DBeQ inhibits HIV-1 infection in a dose-dependent manner (Fig. 5B). Next we sought to analyze HIV-1 infection of human cells. Treating HeLa cells with a titration of DBeQ before infection with HIV1-GFP-VSV potently inhibited infection (Fig. 5C), and the dose-response curve closely correlated with that previously published for *in vitro* inhibition of VCP ATPase activity (Chou *et al.*, 2011). Further evidence for a crucial role played by VCP in HIV-1 infection was obtained by reducing VCP levels in HeLa cells using siRNA. Partial depletion of VCP reduced HIV-1 infection almost 3-fold.

These findings prompted us to further investigate the requirement for VCP. First, HeLa cells treated with 10 μ M DBeQ or DMSO were infected with a titration of HIV1-GFP-VSV to

analyze the effect of VCP inhibition on infection at various multiplicities of infection (MOI). Inhibition of VCP prevented HIV-1 infection at all MOI tested (Fig. 6A). Second, non-dividing THP-1 monocyte-derived macrophages (MDM) were used to confirm that VCP is required for infection of cells that are a target of HIV *in vivo*. Treatment with DBeQ inhibited HIV1-GFP-VSV infection of MDM with similar efficiency as observed in CRFK and HeLa cells (Fig. 6B). Finally, an HIV1-GFP virus pseudotyped with the naturally occurring HIV envelope SF162 was tested on TZM-bl cells for susceptibility to VCP inhibition. As for VSV G-pseudotyped HIV-1, DBeQ inhibited infection by SF162-pseudotyped virus in a dose-dependent fashion. Taken together, these results demonstrate that both presence and ATPase activity of VCP are essential for efficient infection by HIV-1.

Primate lentiviruses, retroviruses and HSV rely on VCP

The striking dependence of HIV-1 infection on VCP raised the question of whether other, closely related or unrelated viruses likewise exploit the enzyme for infection. This was investigated by treating HeLa cells with a titration of DBeQ before infection with one of several VSV G-pseudotyped viruses. Treatment with DBeQ potently inhibited infection by the primate lentiviruses HIV-2 (Fig. 7A) and simian immunodeficiency virus (SIVmac, Fig. 7B), the gamma-retroviruses B-tropic and N-tropic murine leukemia virus (MLV-B and MLV-N, Fig. 7 C, D) and the lentivirus equine infectious anemia virus (EIAV, Fig. 7E). Similarly, inhibition of VCP using DBeQ abrogated infection of A549 cells by GFP-expressing HSV (Fig. 7F).

In contrast, infection of CRFK cells by VSV G-pseudotyped feline immunodeficiency virus (FIV-GFP-VSV) was not affected by inhibition of VCP (Fig. 8A). Infection of HeLa cells by respiratory syncytial virus (RSV, Fig. 8B) and human adenovirus (hAdV, Fig. 8C) were also found to be independent of VCP.

Example 3

Herpes Viruses depend on VCP for successful infection

In contrast to the findings for adenovirus, for which VCP acts as a TRIM21 cofactor for viral neutralisation mediated by antibodies, we have observed in the following experiments that certain viruses require VCP for viral infection. These viruses cannot uncoat during the viral infection process if VCP is absent or inhibited, and thus cellular infection is prevented.

In a first set of experiments, we tested the ability of HSV to infect cells in the presence or absence of inhibitors of HSV.

For HSV-1 we have shown that DBE (N2,N4-dibenzylquinazoline-2,4-diamine), Eer1 (Eeyarestatin) and Xanthahumol ((E)-1-[2,4-dihydroxy-6-methoxy-3-(3-methylbut-2-enyl)phenyl]-3-(4-hydroxyphenyl)prop-2-en-1-one) and Syk inhibitor III (skIII) are all effective inhibitors. These drugs target VCP in different ways; DBE targets the ATP binding pocket whilst Xanthahumol binds to the VCP N domain.

In one experiment, the effect of Eeyarestatin 1 (e1) on HSV-1 (KOS) infection and replication in A549 and HeLa cells was tested. Cells were treated with Eeyarestatin 1 for 3.5 h before infection with KOS at 6 PFU/cell. After 1 h incubation at 37 °C, the virus inoculants were replaced by fresh medium containing Eeyarestatin 1 at the indicated concentrations. Samples were harvested at 24 hpi and GFP expression analysed by FACS. Successful inhibition of HSV-1 infection is demonstrated in Figure 9b. Figure 9a illustrates inhibition of HSV by Xanthahumol, and Fig 9c by skIII.

HSV-1 is an alphaherpesvirus. Other herpesviruses include the gammaherpesviruses, such as EBV and KSHV. Viruses like EBV are an important human pathogen and are difficult to treat because antivirals like acyclovir do not work against them. We have shown that infection by the model gammaherpesvirus MHV68 is inhibited by DBE.

A549 cells were pre-treated with DBE for 2 h and infected with MHV68-GFP at 3 PFU/cell. After 1 h incubation at 37 °C, the virus containing medium was removed and the cells were washed and incubated in drug containing medium. Samples were harvested at 24 hpi and GFP expression was analysed by FACS. Relative infectivity was calculated by normalising samples to DMSO treated controls (set as 100%). See Figure 10. Mean and standard error were calculated in Excel (n=3) and shown.

Example 4

Characterizing the role of VCP in HIV-1 infection

In order to elucidate the role of VCP in HIV-1 infection, we examined at which stage the enzyme is required. The soluble cytoplasmic fraction of HeLa cells treated with DMSO or

DBeQ was probed for the HIV-1 capsid protein p24 (CA) 4 h post infection (p.i.) with HIV1-GFP(VSV). CA levels were identical in both conditions (Fig. 11a), demonstrating that entry is not affected by inhibition of VCP. Cells challenged with HIV-1 lacking an envelope gene (Δ Env) contained no detectable CA. Next, we analysed viral DNA intermediates (reverse transcription products, 2-LTR circles and integrated provirus) by quantitative PCR 14 and 24 h p.i. with HIV1-GFP(VSV). Production of these species was significantly diminished when VCP was inhibited with DBeQ (Fig. 11b, c, Fig. 12). Levels of viral DNA species were reduced to near or below the limit of detection when cells were treated with nevirapine (a non-nucleoside reverse transcriptase (RT) inhibitor), confirming their identity as products of *de novo* viral DNA synthesis. Similarly, levels of early reverse transcription products were reduced approximately 5-fold in cells depleted of VCP by siRNA (Fig. 11d) and significantly decreased in cells overexpressing the ATPase-deficient VCP mutants (DeLaBarre, et al., Mol Cell 22, 451-462, doi:10.1016/j.molcel.2006.03.036 (2006)) K524A or E305/578Q (Fig. 11e) relative to their respective control conditions. To investigate whether VCP inhibitors directly inhibit RT, DBeQ, XN or nevirapine was added to an *in vitro* HIV-1 RT assay. No inhibition of RT activity by either VCP inhibitor was observed at concentrations greater than those necessary for inhibition of HIV infection (Fig. 11f). These results implicate VCP in an early post-entry step in HIV-1 infection that is required for efficient reverse transcription.

In order to examine whether the process mediated by VCP must occur within a particular time window or whether infection stalled by VCP inhibition can be resumed at later time points, we performed an experiment wherein DBeQ was removed at several time points p.i. with HIV1-GFP(VSV). Infectivity was found to decline progressively with time of removal, following a first order exponential decay with a half-life of 4 h (Fig. 11g). This suggests that cytosolic virus particles deprived of the enzymatic activity of VCP rapidly lose infectivity; to remain fully infectious, HIV-1 particles require the action of VCP within a narrow, early time window concurrent with reverse transcription.

The physiological functions of VCP involve separation, disassembly and processing of complex protein substrates in cells (Meyer et al., 2012; Hauler et al., 2012). We therefore hypothesized that, during HIV infection, VCP may catalyse the disassembly of virus capsid (uncoating). To determine whether VCP associates with intact HIV-1 capsids, envelope-free but encapsidated HIV-1 cores were isolated by equilibrium sedimentation through a detergent layer (Kotov, 1999) (Fig. 11h), mixed with HeLa cell lysate, and subjected to ultracentrifugation in order to pellet intact capsids. Both CA and lysate-derived VCP were

detected in the pellet by immunoblot (Fig. 11i), suggesting that VCP binds to HIV-1 capsids *in vitro*. Addition of DBeQ did not prevent their association, and VCP from lysate was not detected in the pellet in the absence of HIV-1 cores. To assess whether VCP interacts with capsids in live cells, the soluble cytoplasmic fraction of HeLa cells was prepared 4 h after challenge with HIV1-GFP(VSV) or HIV1-GFP(Δ Env), and was probed for VCP and CA by immunoblot before (Input) and after ultracentrifugation (Pellet) (Stremlau, 2006). Cellular VCP was detected in the pellet specifically upon infection by HIV-1 (Fig. 11j), confirming that VCP binds to pelletable HIV-1 capsids inside cells. These interaction data suggest that cytosolic HIV-1 capsids may be substrates for VCP. Known interaction partners of capsid include the host factors cyclophilin A (Franke, 1994; Thali, 1994) and CPSF6 (Lee, 2010) as well as the restriction factor TRIM5 α (Stremlau, 2004). However, VCP did not act in a manner dependent on these virus-host interactions (Fig. 13).

To test whether VCP affects the stability of HIV-1 capsids during infection, we performed fate of capsid (FOC) experiments in which the amount of pelletable (intact) capsids in the cytoplasm of HeLa cells 4 h p.i. with HIV1-GFP(VSV) was quantified (Stremlau, 2006). Levels of CA in the pellet increased substantially upon treatment with DBeQ (Fig. 14a). Quantification of immunoblots by densitometry shows that inhibition of VCP caused an approximately 2-fold increase in pelletable CA (Fig. 14b). This increase was comparable to the capsid-stabilizing effect of RT inhibition by nevirapine (Fig. 14b), in line with previous findings (Yang, 2013). Similarly, depletion of VCP by siRNA prior to FOC experiments led to a 2-fold increase in pelletable CA (Fig. 14c). These results suggest that VCP destabilizes HIV-1 capsids in infected cells. To confirm these findings, the abundance of CA foci in the cytosol of infected HeLa cells was quantified by confocal immunofluorescence microscopy at the same time point. As observed in FOC experiments, catalytic inhibition or depletion of VCP significantly increased the persistence of HIV-1 capsids following infection (Fig. 14d-f, Fig. 15a). Next, HeLa cells were transfected with empty vector, wild-type VCP or the ATPase-deficient mutants K524A or E305/578Q before infection with HIV1-GFP(VSV). While overexpression of wild-type VCP did not significantly impact on the persistence of CA foci in the cytosol and led to a modest increase in the level of infection, overexpression of the catalytically impaired mutants resulted in a significant increase in the number of HIV-1 capsids remaining at 4 h p.i. (Fig. 14g, Extended Data Fig. 14b) and a corresponding reduction in the levels of infection (Fig. 14h). Taken together, these results suggest that VCP catalyses the disassembly of HIV-1 capsids during infection.

We attempted to reconstitute VCP catalysis of capsid disassembly *in vitro*. Encapsidated HIV-1 cores were incubated at 37° C in buffer containing ATP in the presence or absence of recombinant VCP. Following pelleting of intact capsids, the proportion of HIV-1 capsids disassembled was determined by ELISA (Forshey, 2002). The *in vitro* disassembly of capsids was found to proceed with similar kinetics in the presence and absence of VCP (Fig. 16a). A similar result was obtained in the laboratory that had originally established the assay (Fig. 16b). These data suggest that host cell-derived modifications such as ubiquitination or SUMOylation of HIV-1 capsids and/or cellular co-factors such as VCP adapter proteins (which are absent in the *in vitro* system) are required for VCP to exert its capsid-destabilizing effect.

To test whether VCP promotes the dissociation of CA from HIV-1 cores during infection of human cells, microscopy-based *in situ* uncoating assays (Yamashita, 2007; Hulme, 2011) were performed. Infection of HeLa cells with a virus incorporating GFP-tagged HIV-1 core protein Vpr, combined with immunofluorescent detection of CA, allows quantification of the fraction of HIV-1 cores that are CA-positive (Fig. 14i). In control conditions, CA is rapidly lost from GFP-Vpr-positive HIV-1 cores. However, chemical inhibition or siRNA-mediated depletion of VCP prevented the loss of CA from cores during the first 4 h p.i. (Fig. 14j, k, Fig. 17). Together, our data demonstrate that HIV-1 relies on cellular VCP to catalyse the disassembly of its capsid during infection. The discovery that HIV-1 uncoating is catalysed by host cell machinery identifies a novel group of potential targets for antiretroviral therapeutics.

Example 5

Role of VCP cofactors

The *in vitro* experiments reported in Example 4 suggest that cofactors of VCP may be important in mediating the effects on viral infection observed herein. To exemplify that inhibiting the activity of VCP co-factors inhibits viral infection we have used a kinase inhibitor (Sorafenib) that prevents VCP phosphorylation (Yi, 2012) and shown that this inhibits HIV-1 infection.

HeLa cells were plated at 1×10^5 cells per well. The next day, Sorafenib was added at indicated concentrations to wells (see Figure 18). All conditions were normalised for solvent

(DMSO) concentration. Cells were incubated for 1h incubation at 37°C then challenged with VSV pseudotyped HIV-1 GFP vector (HGV) at an moi of ~0.3. GFP expressing cells were enumerated by FACS 48 h post infection.

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Yi P, Higa A, Taouji S, Bexiga MG, Marza E and others. 2012. Sorafenib-mediated targeting of the AAA(+) ATPase p97/VCP leads to disruption of the secretory pathway, endoplasmic reticulum stress, and hepatocellular cancer cell death. *Mol Cancer Ther* 11:2610-20

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All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described aspects and embodiments of the

present invention will be apparent to those skilled in the art without departing from the scope of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in the art are intended to be within the scope of the following claims.

Claims

1. A VCP inhibitor for use in the prevention of infection of a cell by a virus.
2. A VCP inhibitor for use according to claim 1, wherein the VCP inhibitor inhibits VCP from catalysing viral uncoating within the cell.
3. A VCP inhibitor for use according to claim 1 or claim 2, wherein the intact viral capsid is exposed to the cytoplasm in the infection cycle.
4. A VCP inhibitor for use according to any preceding claim, wherein the virus is selected from a retrovirus and a herpesvirus.
5. A VCP inhibitor for use according to claim 4, wherein the retrovirus is a lentivirus.
6. A VCP inhibitor for use according to any preceding claim, wherein said VCP inhibitor inhibits infection by HIV1, HIV2, EIAV, MLV-B or MLV-N in HeLa cells.
7. A VCP inhibitor for use according to any one of claims 1 to 3, wherein said VCP inhibitor inhibits infection by HSV in A549 cells.
8. A VCP inhibitor for use according to any preceding claim, wherein the inhibitor is selected from dibenzylquinazoline-2,4-diamine (DBeQ), Xanthahumol, Sorafenib and Syk inhibitor III (3,4-methylenedioxy-beta-nitrostyrene).
9. An inhibitor of a VCP adaptor protein for use in prevention of infection of a cell by a virus as set forth in any on claims 1 to 7.
10. An inhibitor according to claim 9, wherein the VCP adaptor protein forms a complex with a viral coat protein in vivo.
11. A method for inhibiting viral infection of a cell, comprising administering to the cell an inhibitor of VCP, wherein the infection is by a virus that enters the cytosol whilst encapsidated.
12. A method for identifying a compound capable of inhibiting infection by virus that enters the cytosol whilst encapsidated, comprising contacting a VCP polypeptide

with a viral capsid polypeptide in the presence of the compound, and determining the influence of the compound on the interaction between the VCP polypeptide and the viral capsid polypeptide.

13. A method according to claim 11 or claim 12, which is carried out in the presence of one or more VCP adaptor proteins.

14. A method for identifying a compound or compounds capable, directly or indirectly, of modulating the interaction of VCP and a virus and thereby the infectivity of HIV, comprising the steps of: incubating a compound or compounds to be tested with a VCP polypeptide and a viral capsid polypeptide, under conditions in which, but for the presence of the compound or compounds to be tested, the interaction between VCP and the viral capsid induces a measurable chemical or biological effect;

determining the ability of the VCP polypeptide to interact, directly or indirectly, with the viral capsid polypeptide to induce the measurable chemical or biological effect in the presence of the compound or compounds to be tested; and selecting those compounds which modulate the interaction between VCP and the virus.

15. A method for treating a condition associated with a viral infection in a subject in need thereof by modulating the interaction of VCP and the virus, by administering a pharmaceutical composition capable of modulating interaction of VCP and virus in an amount sufficient to modulate the viral infection.

16. A method for developing an anti-viral drug comprising the steps of (a) identifying one or more compounds which demonstrate anti-infection activity; (b) screening said compounds and selecting one or more compounds which affect the interaction of VCP and the virus; (c) determining the structure of the compound and using structure-guided mutagenesis to prepare variants of the compound with improved activity.

17. A drug cocktail comprising two or more drugs for use in the treatment or prevention of an HIV infection, wherein at least one of said drugs is indicated for the disruption of the interaction between VCP and a viral capsid protein.

18. The drug cocktail for use according to claim 17, wherein the cocktail further comprises one or more anti-HIV drugs selected from the group consisting of efavirenz, emtricitabine, tenofovir, disoproxil fumarate, rilpivirine, lamivudine, zidovudine, emtricitabine, azidothymidine (AZT), nevirapine, amprenavir, tipranavir, indinavir, saquinavir mesylate, lopinavir, ritonavir, Fosamprenavir Calcium, darunavir, atazanavir sulfate, nelfinavir mesylate, raltegravir, maraviroc and enfuvirtide.

19. The drug cocktail for use according to any one of claims 17 or 18, wherein one or more drugs capable of disrupting the interaction between VCP and a viral capsid component can be selected according to any one of claims 10-14.

20. The drug cocktail for use according to claim 19, wherein the drug capable of disrupting of the interaction between VCP and a viral capsid component is DBEq.

Figure 1

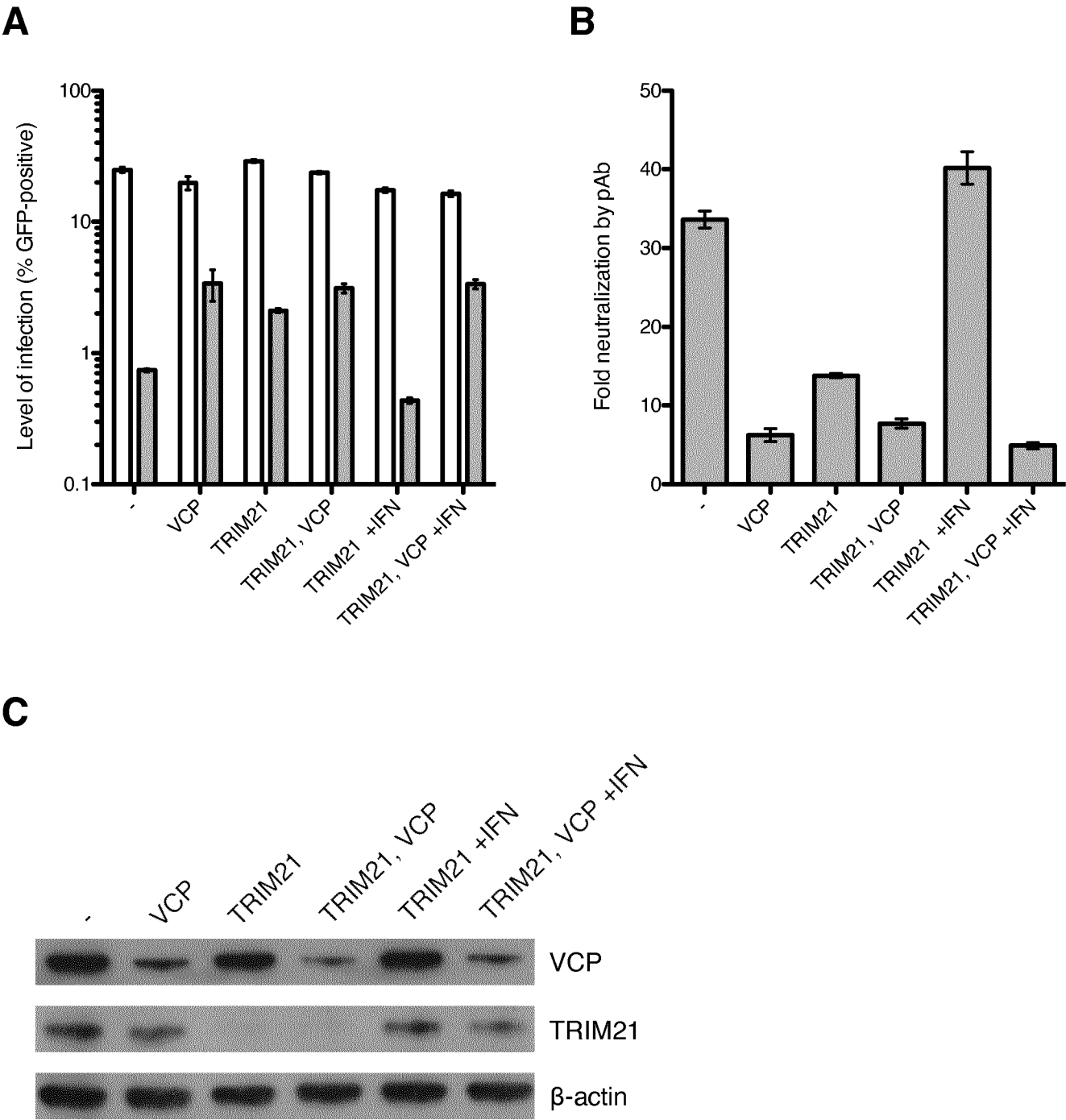


Figure 2

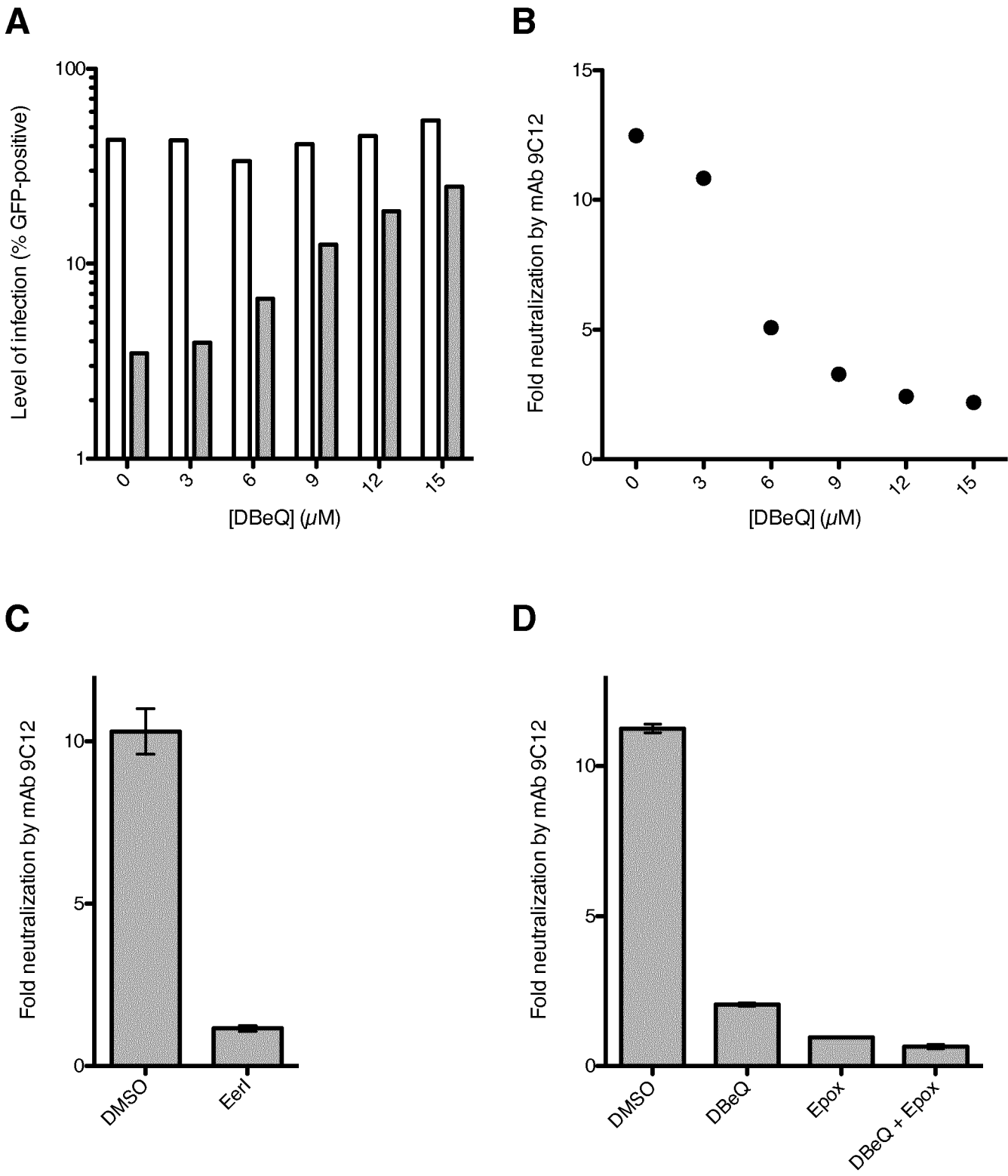


Figure 3

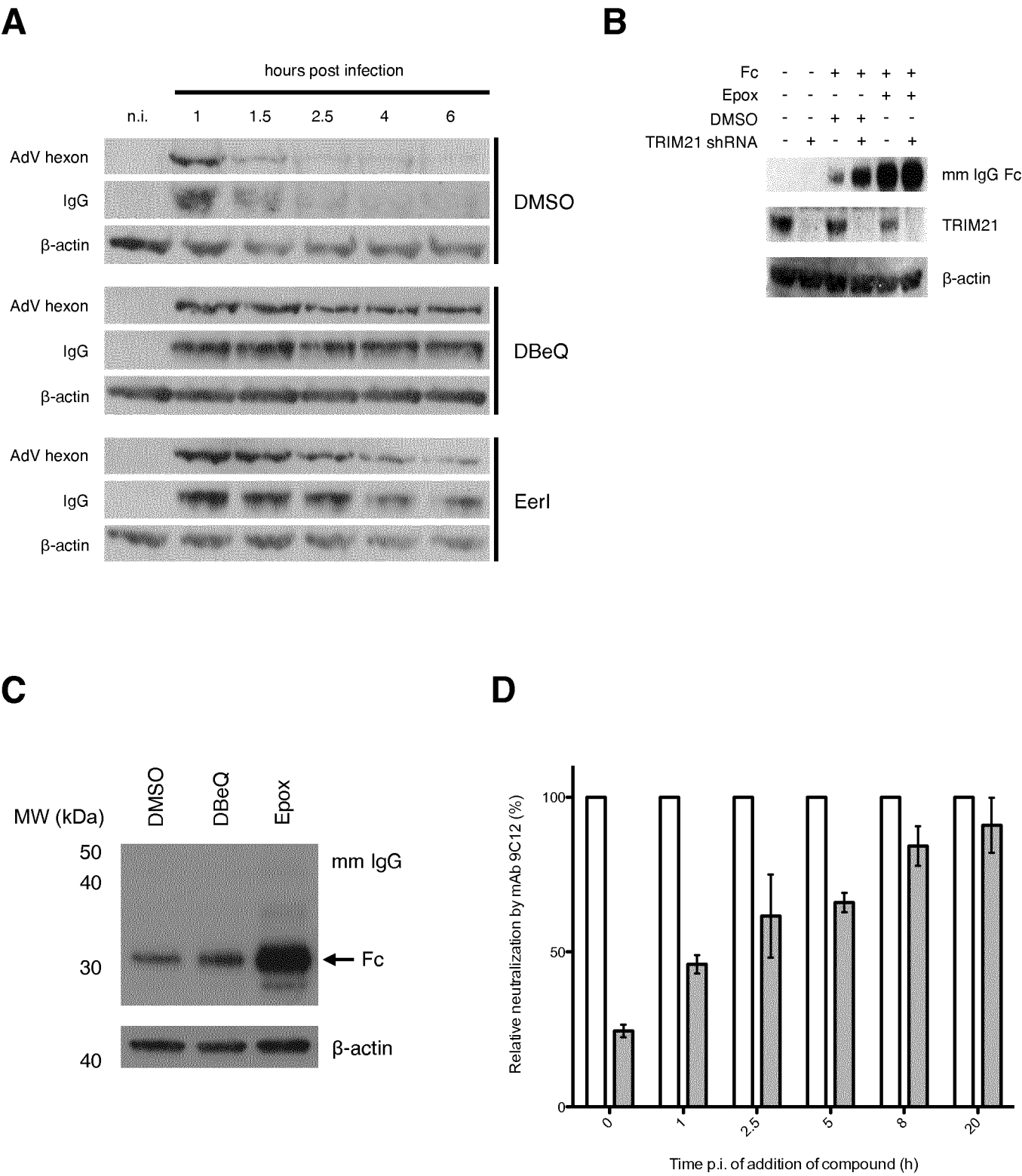
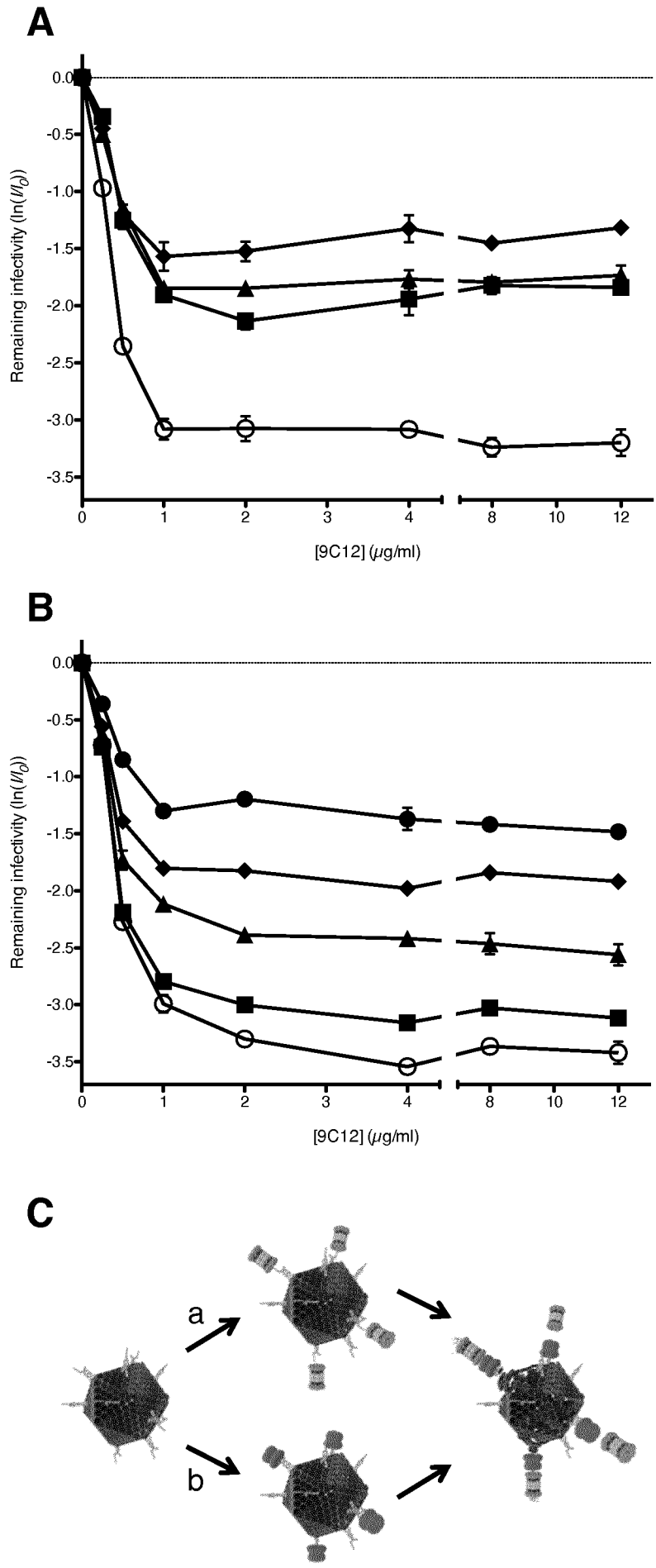
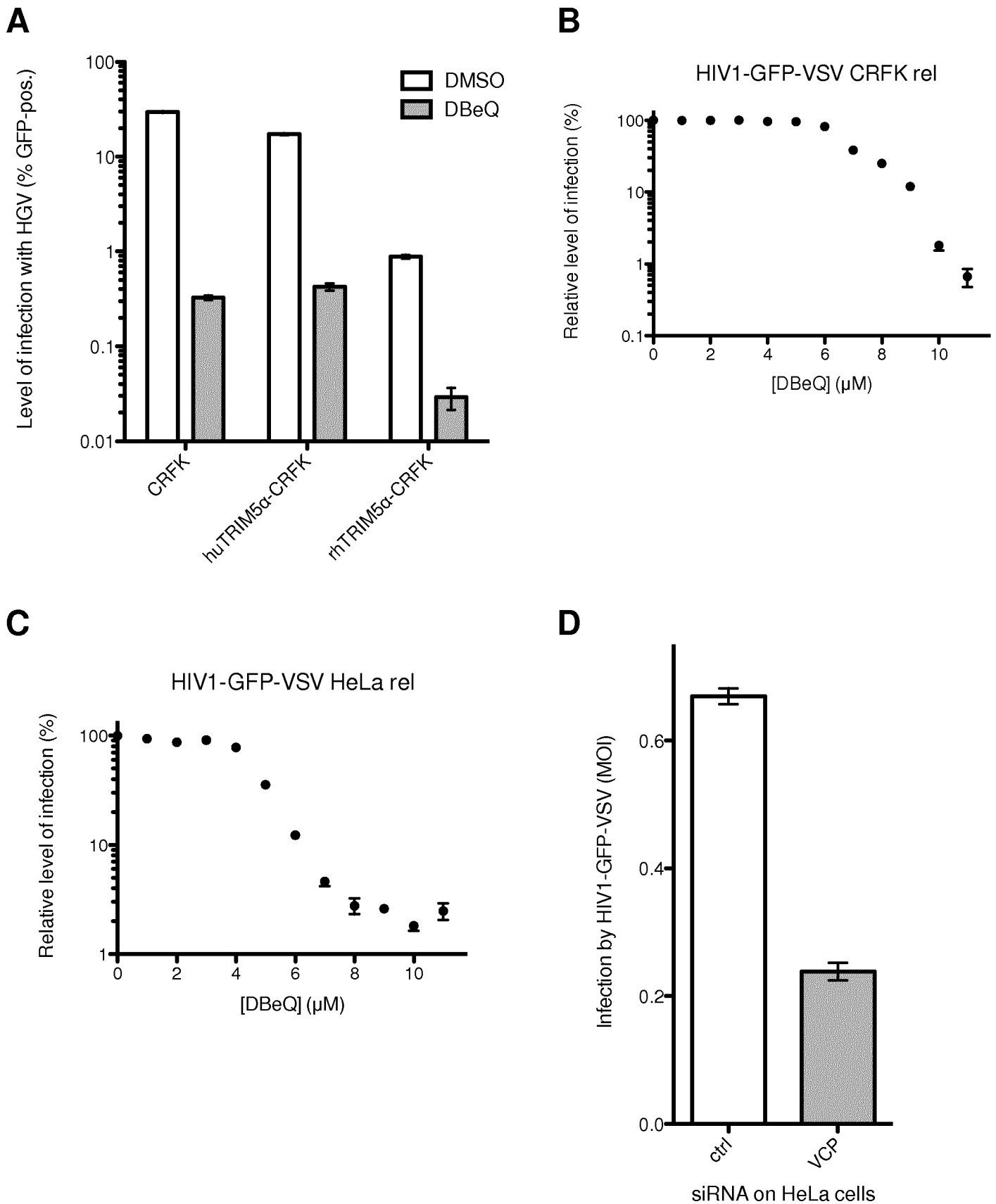


Figure 4



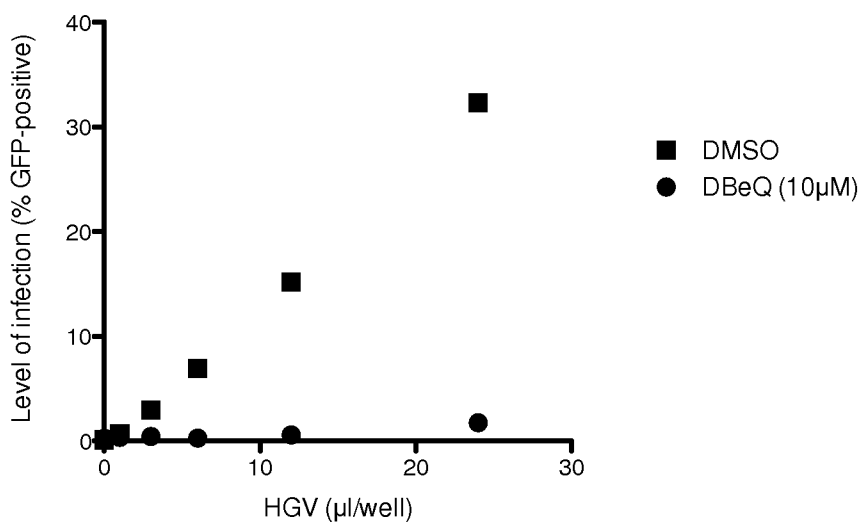
VCP is essential for HIV-1 infection

Figure 5

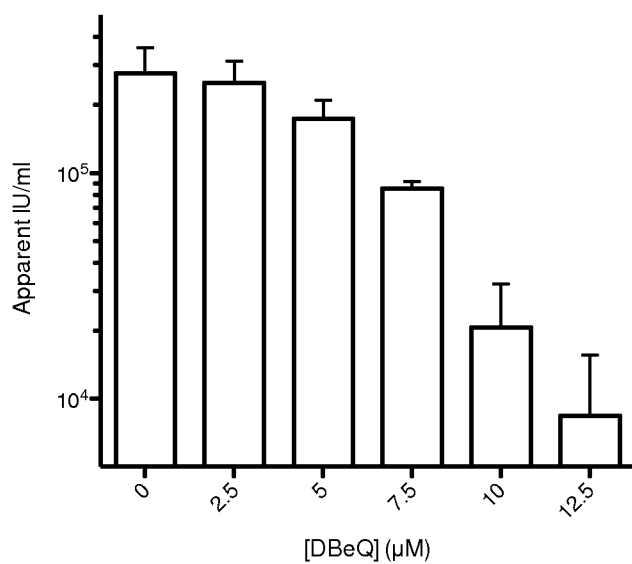


VCP is essential for HIV-1 infection: varying MOI, macrophages, and naturally occurring envelope

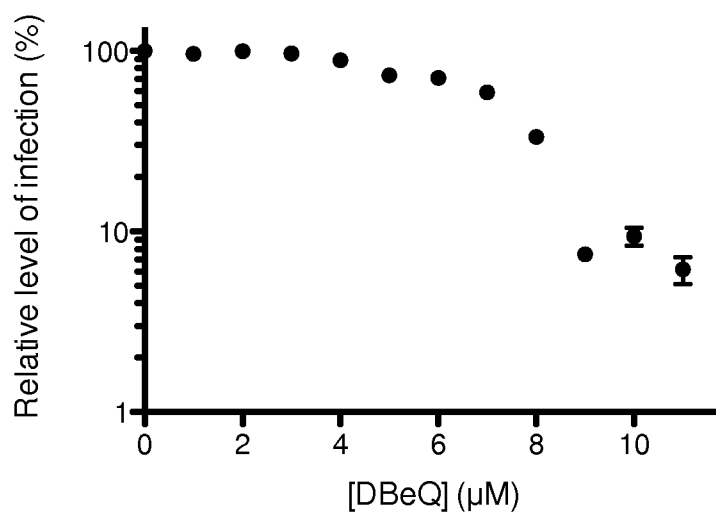
Figure 6

A10 μ M DBeQ vs. titration of HIV1-GFP-VSV**B**

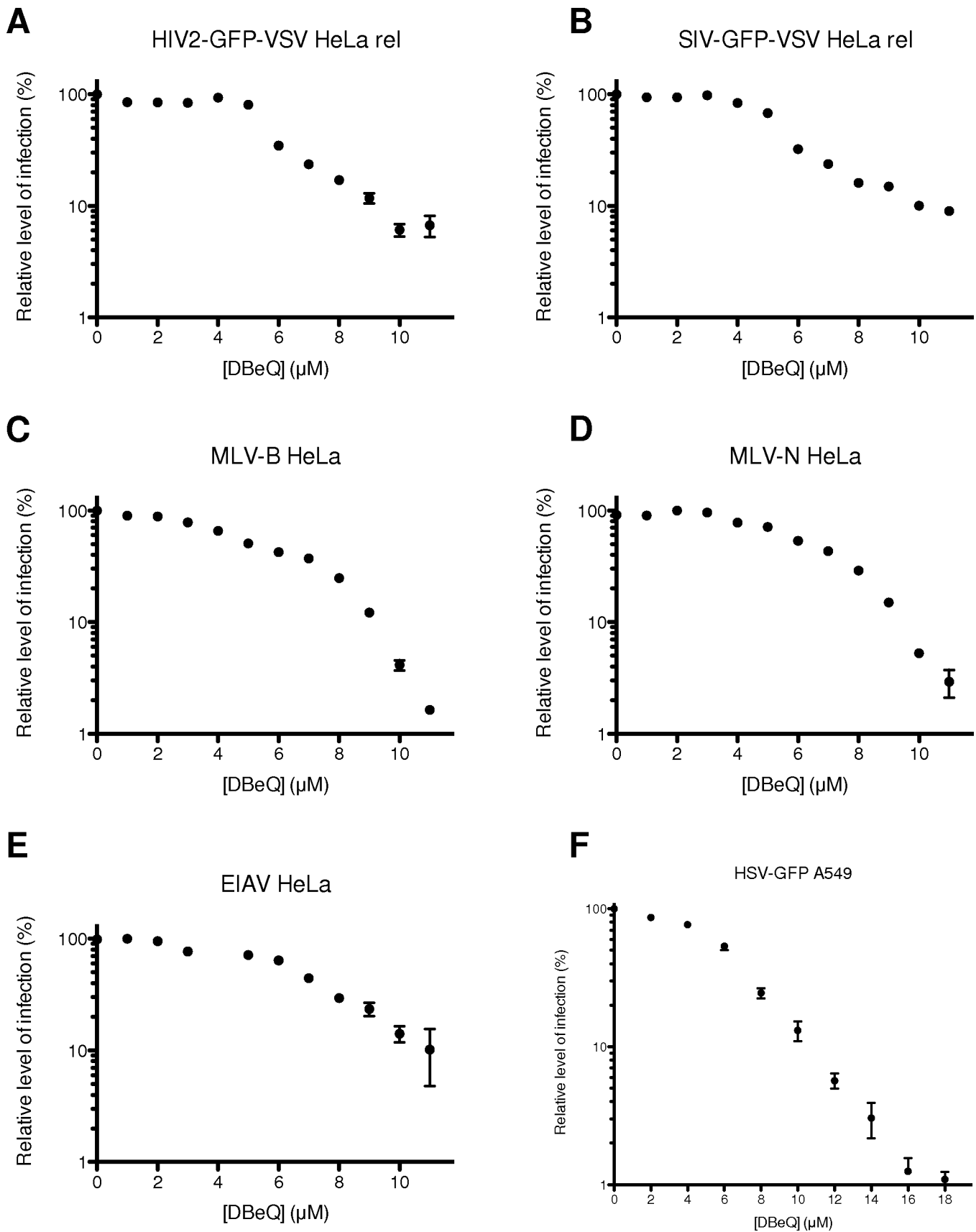
HIV1-GFP-VSV THP-1 MDM

**C**

HIV1-GFP-SF162 TZM-bl rel



Closely related and other virus(es) also rely on VCP Figure 7



FIV, RSV and hAdV do not require VCP

Figure 8

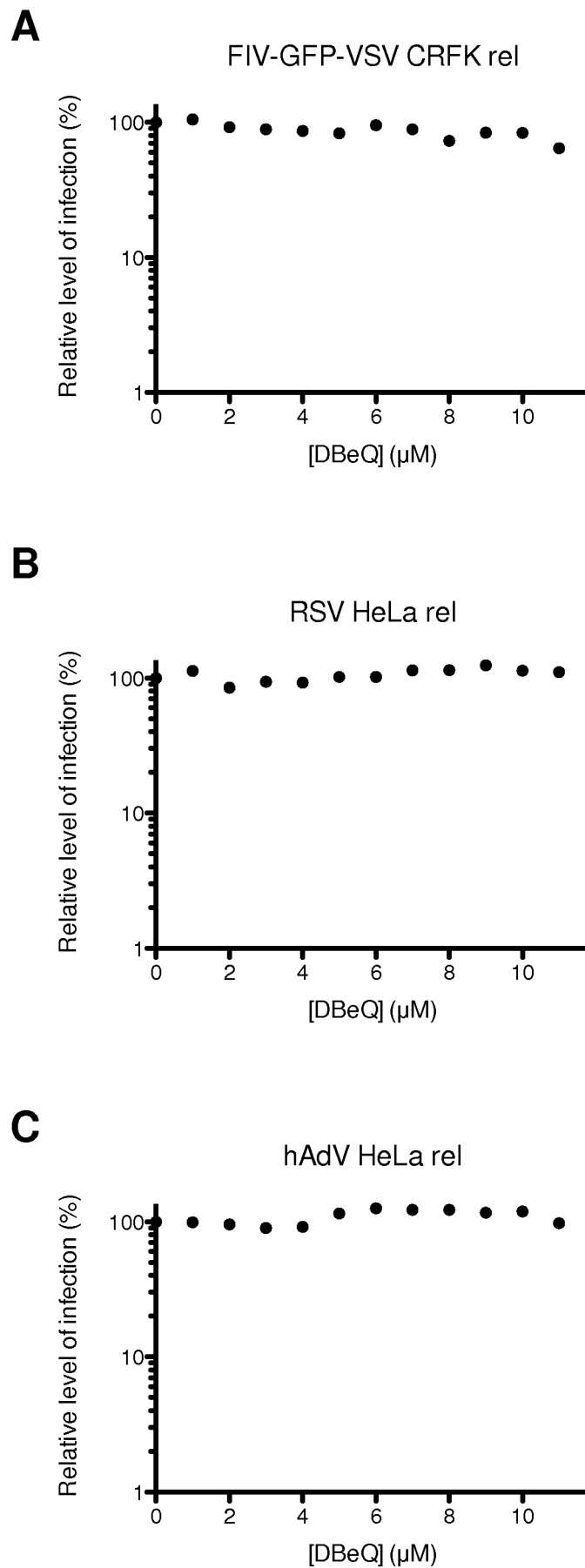
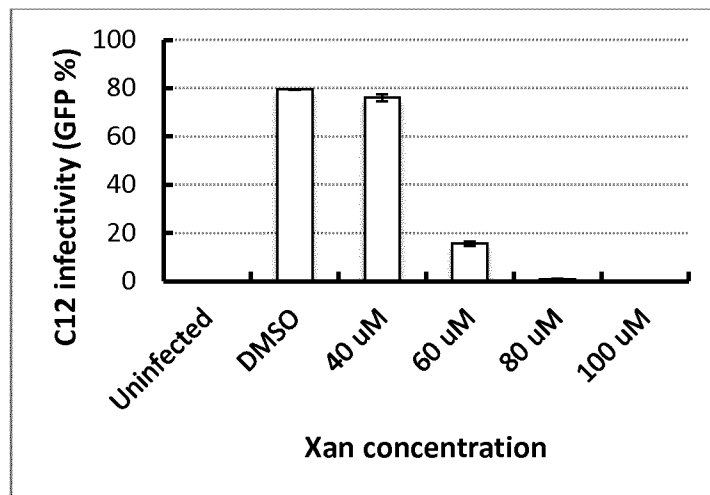


Figure 9

A



B

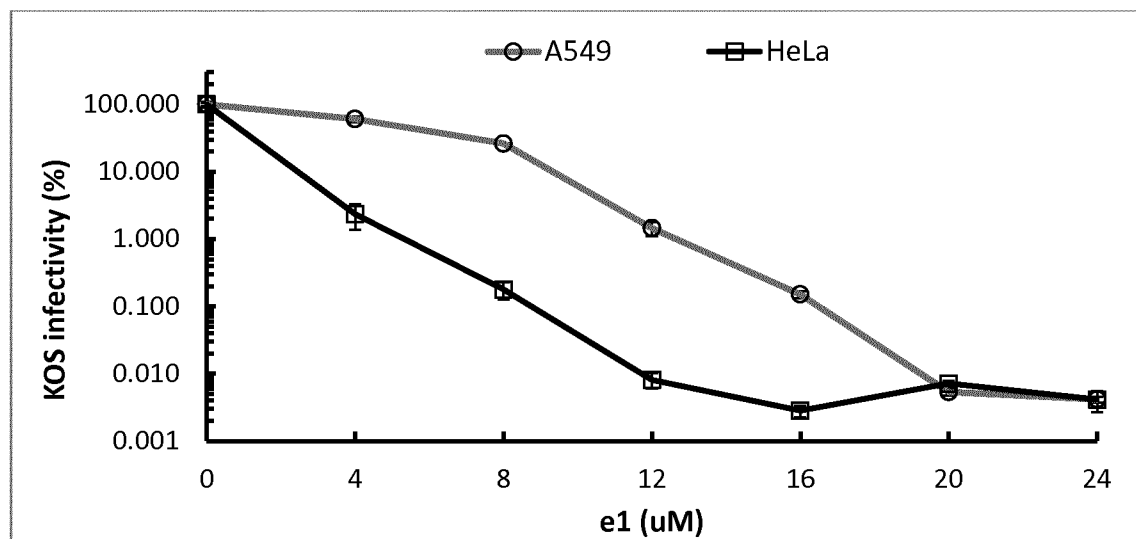


Figure 9 C

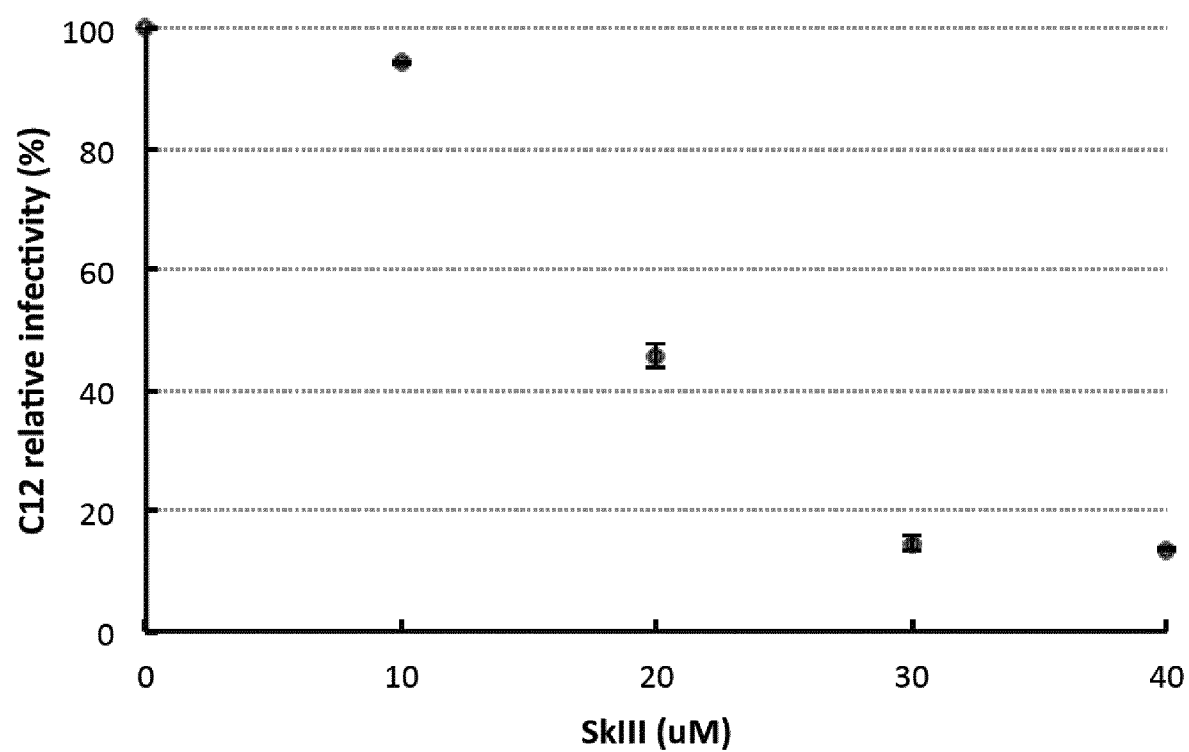


Figure 10

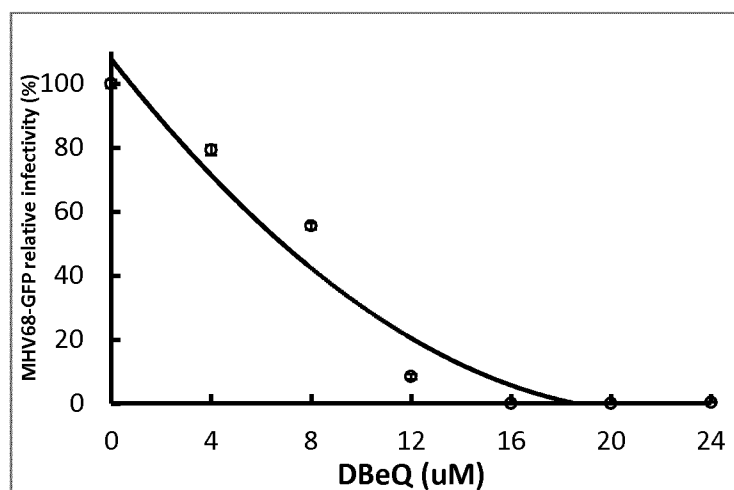


Figure 11

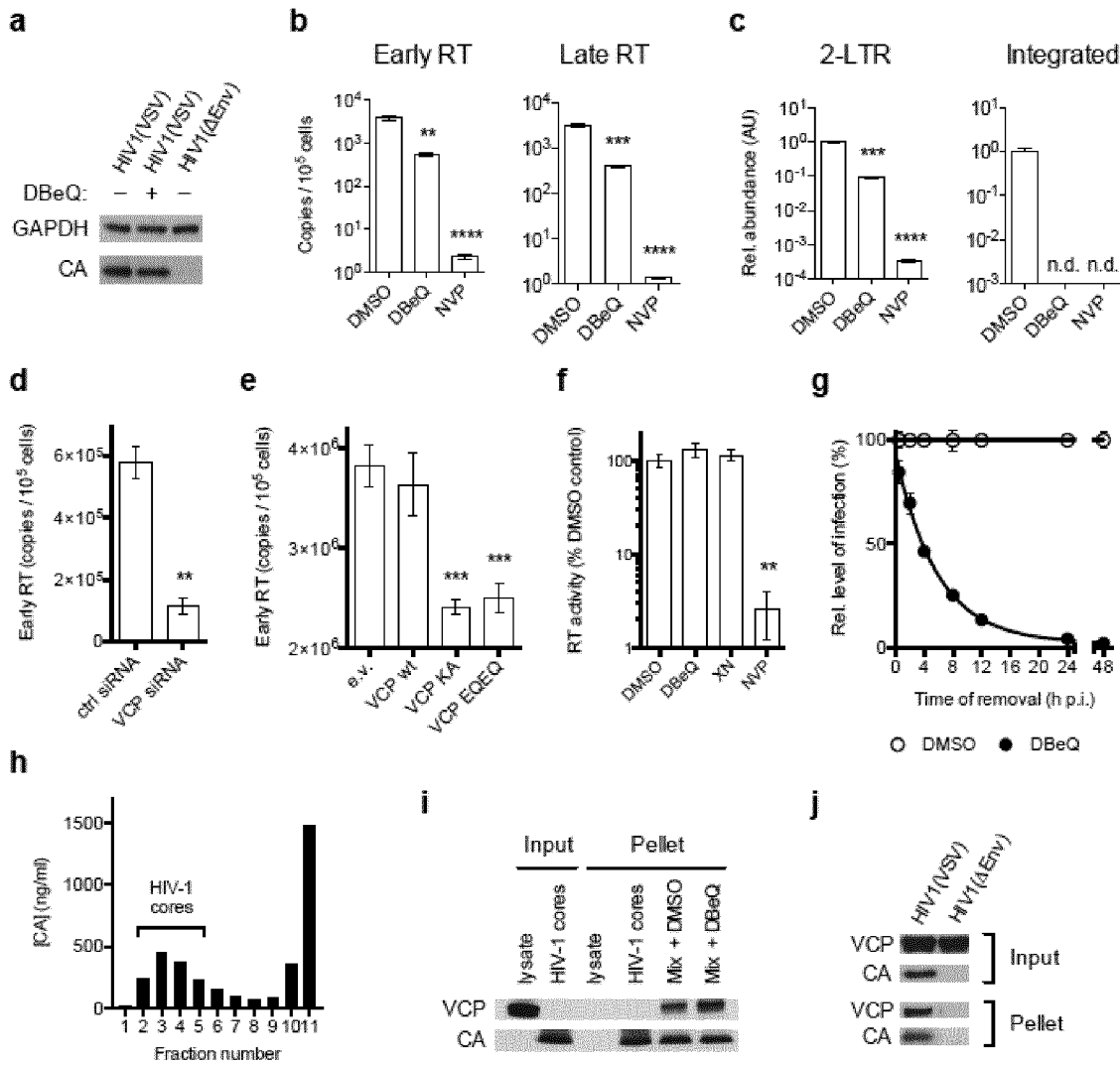


Figure 12

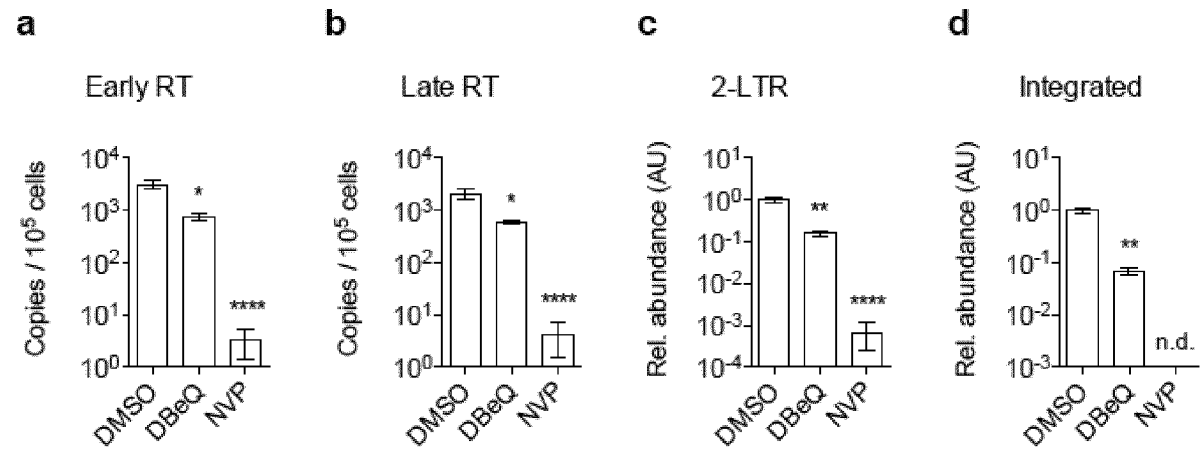


Figure 13

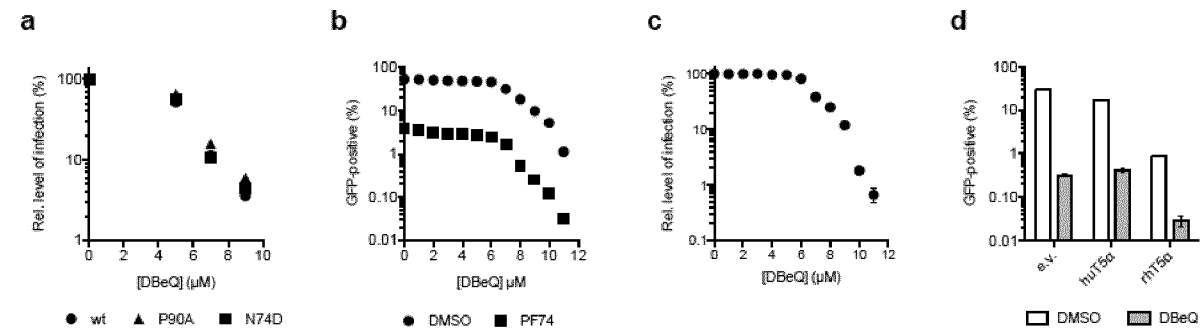


Figure 14

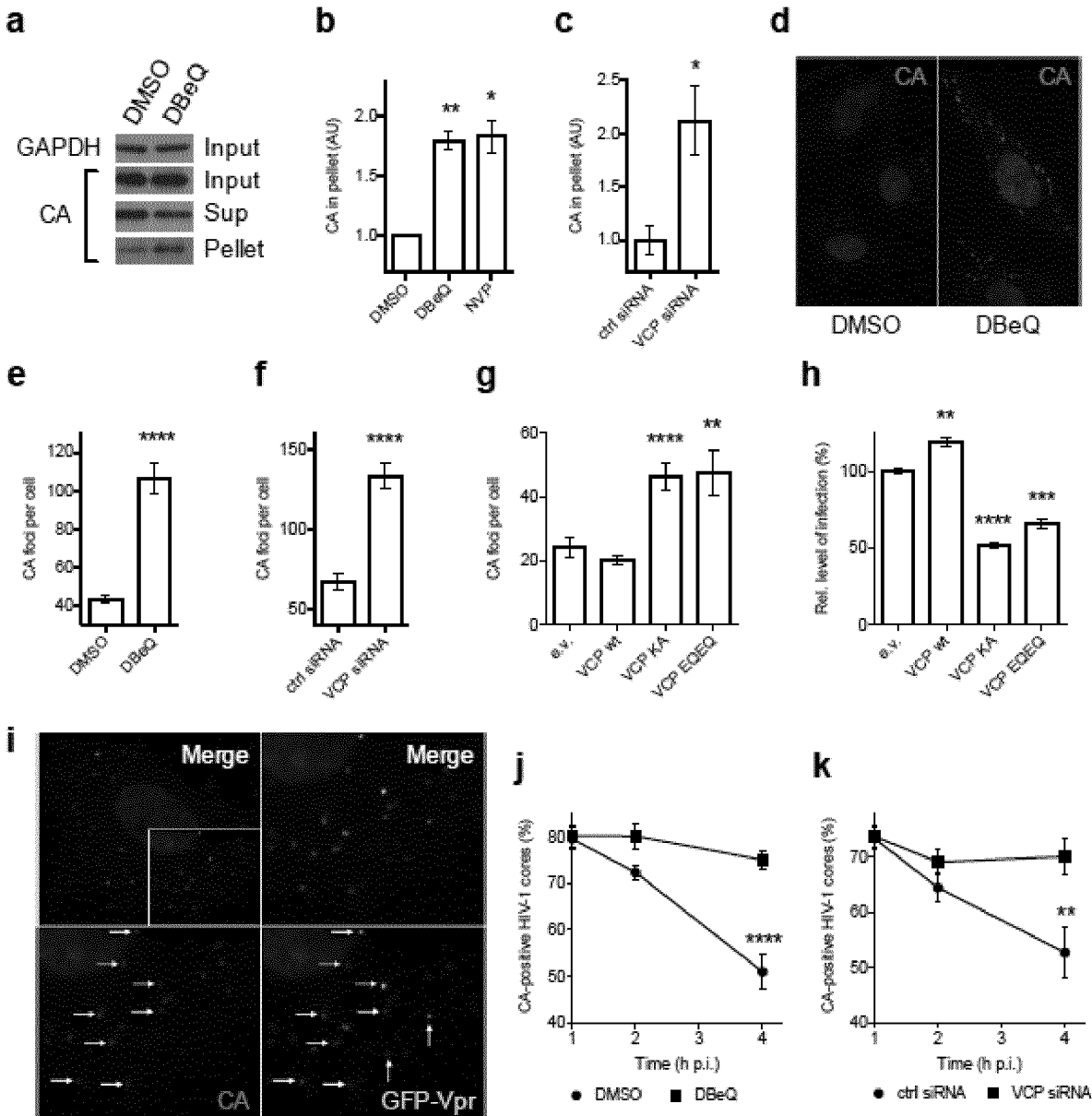


Figure 15

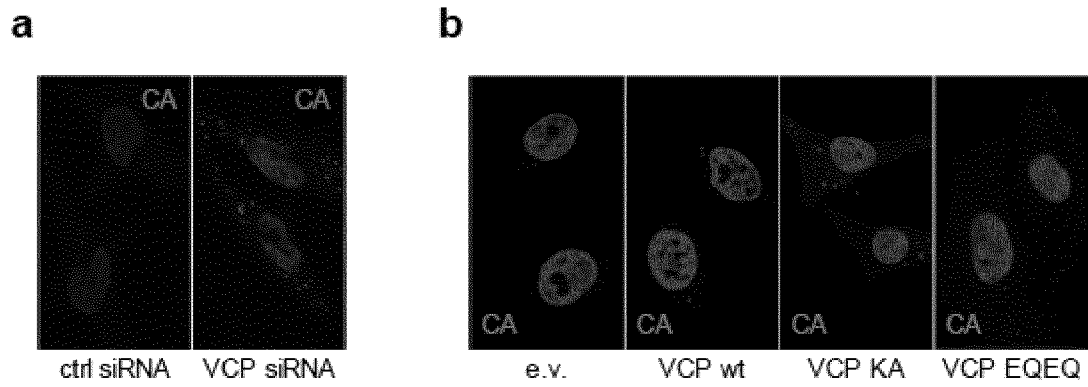


Figure 16

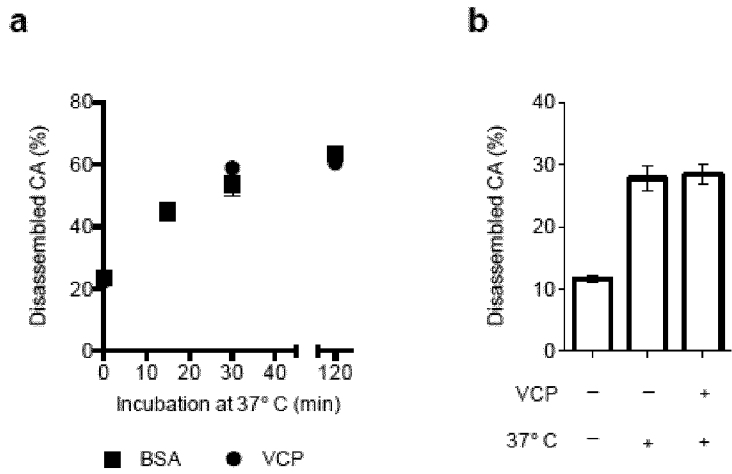


Figure 17

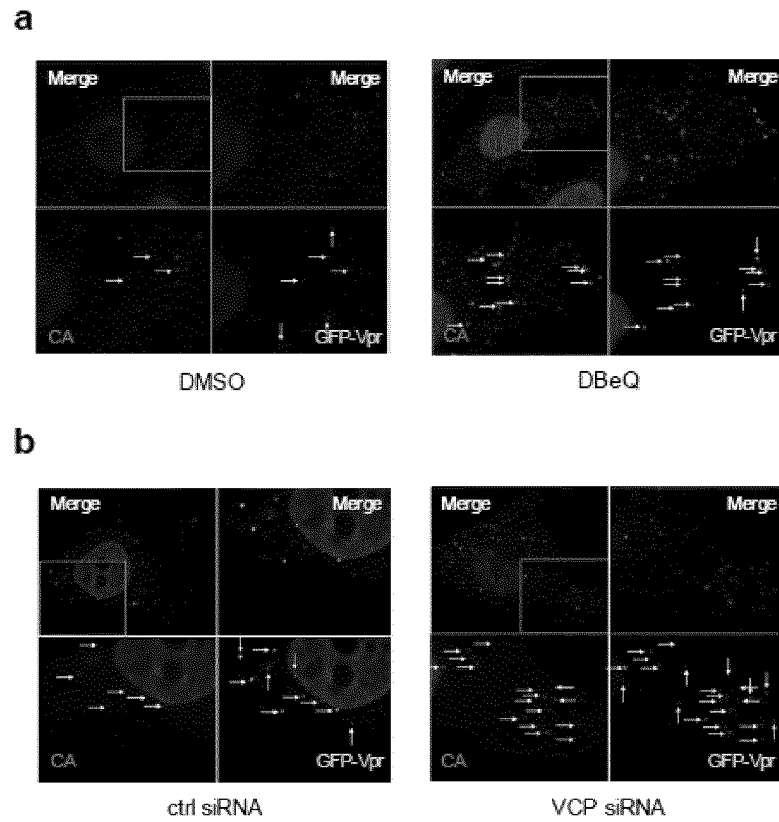
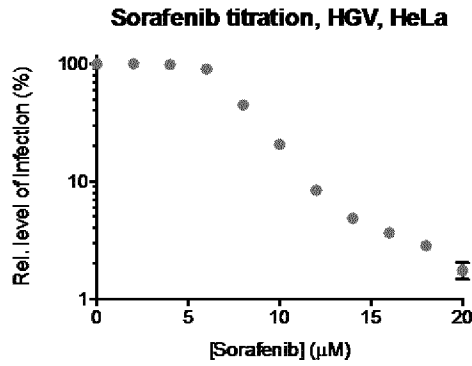


Figure 18



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/071563

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/517 A61K31/05 A61K31/4412 A61K31/36
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/069039 A1 (US HEALTH [US]; WUESTNER ADRIAN [US]; YE YIHONG [US]; WANG QIUYAN [US]) 9 June 2011 (2011-06-09) cited in the application paragraphs [0010], [0011], [0068] - [0070], [0121], [0126], [0131] - [0133] -----	1-7, 9-11,15, 17-20
X	US 2011/158942 A1 (WEBER OLAF [DE] ET AL) 30 June 2011 (2011-06-30) paragraphs [0037] - [0039], [0081] ----- -/-	1-11,15, 17-20



Further documents are listed in the continuation of Box C.



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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

9 December 2013

Date of mailing of the international search report

02/01/2014

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Engl, Brigitte

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/071563

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HIMMELSBACH K ET AL: "New aspects of an anti-tumour drug: sorafenib efficiently inhibits HCV replication", GUT, BRITISH MEDICAL ASSOCIATION, LONDON, UK, vol. 58, no. 12, 1 December 2009 (2009-12-01), pages 1644-1653, XP008136282, ISSN: 0017-5749, DOI: 10.1136/GUT.2009.182212 the whole document	1-11,15, 17-20
X	----- WANG Q ET AL: "Xanthohumol, a novel anti-HIV-1 agent purified from Hops Humulus lupulus", ANTIVIRAL RESEARCH, ELSEVIER BV, NL, vol. 64, no. 3, 1 December 2004 (2004-12-01), pages 189-194, XP004646261, ISSN: 0166-3542, DOI: 10.1016/J.ANTIVIRAL.2004.08.005 the whole document	1-11,15, 17-20
X	----- ZHANG N ET AL: "Inhibition of bovine viral diarrhea virus in vitro by xanthohumol: Comparisons with ribavirin and interferon-alpha and implications for the development of anti-hepatitis C virus agents", EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES, ELSEVIER, AMSTERDAM, NL, vol. 38, no. 4, 5 November 2009 (2009-11-05), pages 332-340, XP026691291, ISSN: 0928-0987, DOI: 10.1016/J.EJPS.2009.08.005 [retrieved on 2009-08-29] the whole document	1-11,15, 17-20
A	----- SASAZAWA YUKIKO ET AL: "Xanthohumol Impairs Autophagosome Maturation through Direct Inhibition of Valosin-Containing Protein", ACS CHEMICAL BIOLOGY, vol. 7, no. 5, May 2012 (2012-05), pages 892-900, XP002717546, DOI: 10.1021/CB200492H the whole document	1-20
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/071563

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	T.-F. CHOU ET AL: "Reversible inhibitor of p97, DBeQ, impairs both ubiquitin-dependent and autophagic protein clearance pathways", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 108, no. 12, 22 March 2011 (2011-03-22), pages 4834-4839, XP055021791, ISSN: 0027-8424, DOI: 10.1073/pnas.1015312108 the whole document -----	1-20
Y	BO-YAN JIAO ET AL: "Hepatitis B virus X protein enhances activation of nuclear factor kappa B through interaction with valosin-containing protein", ARCHIVES OF VIROLOGY ; OFFICIAL JOURNAL OF THE VIROLOGY DIVISION OF THE INTERNATIONAL UNION OF MICROBIOLOGICAL SOCIETIES, SPRINGER-VERLAG, VI, vol. 156, no. 11, 15 September 2011 (2011-09-15), pages 2015-2021, XP019971704, ISSN: 1432-8798, DOI: 10.1007/S00705-011-1099-4 the whole document -----	1-20
Y	WANG WEI-YA ET AL: "Prevention of platelet glycoprotein IIb/IIIa activation by 3,4-methylenedioxy-beta-nitrostyrene, a novel tyrosine kinase inhibitor", MOLECULAR PHARMACOLOGY, vol. 70, no. 4, October 2006 (2006-10), pages 1380-1389, XP002717547, ISSN: 0026-895X page 1385, right-hand column, paragraph 3 - page 1386, right-hand column, paragraph 1 -----	8
Y	CHU J J H ET AL: "c-Src protein kinase inhibitors block assembly and maturation of dengue virus", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES - PNAS, NATIONAL ACADEMY OF SCIENCES, US, vol. 104, no. 9, 27 February 2007 (2007-02-27), pages 3520-3525, XP002514803, ISSN: 0027-8424, DOI: 10.1073/PNAS.0611681104 the whole document -----	8

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2013/071563

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011069039 A1	09-06-2011	US 2012316198 A1 WO 2011069039 A1	13-12-2012 09-06-2011
US 2011158942 A1	30-06-2011	CA 2633417 A1 EP 1962829 A1 JP 2009524585 A US 2011158942 A1 US 2012009150 A1 WO 2007068383 A1	21-06-2007 03-09-2008 02-07-2009 30-06-2011 12-01-2012 21-06-2007