Research article

Anti-HIV-1 activity of cellulose acetate phthalate: Synergy with soluble CD4 and induction of "dead-end" gp41 six-helix bundles

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Abstract

Background: Cellulose acetate phthalate (CAP), a promising candidate microbicide for prevention of sexual transmission of the human immunodeficiency virus type 1 (HIV-1) and other sexually transmitted disease (STD) pathogens, was shown to inactivate HIV-1 and to block the coreceptor binding site on the virus envelope glycoprotein gp120. It did not interfere with virus binding to CD4. Since CD4 is the primary cellular receptor for HIV-1, it was of interest to study CAP binding to HIV-1 complexes with soluble CD4 (sCD4) and its consequences, including changes in the conformation of the envelope glycoprotein gp41 within virus particles.

Methods: Enzyme-linked immunosorbent assays (ELISA) were used to study CAP binding to HIV-1-sCD4 complexes and to detect gp41 six-helix bundles accessible on virus particles using antibodies specific for the α-helical core domain of gp41.

Results: 1) Pretreatment of HIV-1 with sCD4 augments subsequent binding of CAP; 2) there is synergism between CAP and sCD4 for inhibition of HIV-1 infection; 3) treatment of HIV-1 with CAP induced the formation of gp41 six-helix bundles.

Conclusions: CAP and sCD4 bind to distinct sites on HIV-1 IIIB and BaL virions and their simultaneous binding has profound effects on virus structure and infectivity. The formation of gp41 six-helical bundles, induced by CAP, is known to render the virus incompetent for fusion with target cells thus preventing infection.

Background

Cellulose acetate phthalate (CAP) is a promising microbicide candidate for prevention of infection by sexually transmitted disease (STD) pathogens, including HIV-1 [1–7]. CAP inactivates HIV-1 and blocks the coreceptor binding site on the virus envelope glycoprotein gp120, while leaving the site for the primary cellular receptor CD4 accessible [8,9]. Soluble CD4 (sCD4) was shown to inhibit HIV-1 infection by two mechanisms: reversible blockage of virus binding to receptors, and irreversible inactivation of virus infectivity [10]. Since CAP and sCD4 bind to distinct domains on the HIV-1 envelope, it was of interest to determine whether or not these two ligands affect virus infectivity synergistically as do other combina-
ations of anti-HIV-1 drugs and sCD4 [11, 12]. Binding of sCD4 leads to conformational changes in gp120 [13–17]. Binding of gp120 to coreceptors CXCR4 and CCR5, respectively, triggers additional conformational changes in HIV-1 envelope glycoproteins [18, 19]. For these reasons it was of interest to determine whether a) pretreatment of HIV-1 with sCD4 would affect subsequent binding of CAP to virus particles, and b) CAP binding to virus particles in the presence or absence of sCD4 would elicit conformational changes which could affect HIV-1 infectivity. Such studies were expected to elucidate further the mechanisms involved in the antiviral/virucidal activity of CAP and to contribute to the potential development of microbicides combining two or more anti-HIV-1 compounds with distinct target sites.

Methods

Reagents

The following monoclonal antibodies (mAbs) were used: NC-1, a mouse mAb raised against the gp41 six-helix bundle from HIV-1 IIIB [20]; and anti-p24 mAb (Immunodiagnostik, Inc., Woburn, MA). Rabbit antibodies against the gp41 six-helix bundle were prepared as described [21]. Rabbit antiserum against HIV-1 IIIB gp120 was prepared as described [22] and shown to cross-react with HIV-1 Bal (own unpublished data). Recombinant soluble CD4 (sCD4) was from Genentech Inc., South San Francisco, CA. Recombinant HIV-1 IIIB gp120, biotinylated gp120 and biotinylated sCD4 were from ImmunoDiagnostics, Inc., Woburn, MA. Purified recombinant protein A/G was from Pierce, Rockford, IL. Pelleted, 1000-fold concentrates of HIV-1 IIIB (6.8 × 10¹⁰ virus particles/ml) and Bal (2.47 × 10¹⁰ virus particles/ml) [23] were from Advanced Biotechnologies, Inc., Columbia, MD. Biotin labeled goat anti-mouse IgG and anti-rabbit IgG were from Roche Diagnostics Corporation, Indianapolis, IN. Chicken serum was from OEM Concepts, Toms River, NJ. Antiserum to phthalate was prepared by immunization of rabbits with phthalic anhydride treated rabbit serum albumin [24]. Horseradish peroxidase (HRP) labeled streptavidin was from Zymed, South San Francisco, CA. HRP was quantitated using a kit from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD. Biotin labeled goat anti-mouse IgG and anti-rabbit IgG were from Roche Diagnostics Corporation, Indianapolis, IN. Chicken serum was from OEM Concepts, Toms River, NJ. Antiserum to phthalate was prepared by immunization of rabbits with phthalic anhydride treated rabbit serum albumin [24]. Horseradish peroxidase (HRP) labeled streptavidin was from Zymed, South San Francisco, CA. HRP was quantitated using a kit from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD. Biotin labeled goat anti-mouse IgG and anti-rabbit IgG were from Roche Diagnostics Corporation, Indianapolis, IN. Chicken serum was from OEM Concepts, Toms River, NJ. Antiserum to phthalate was prepared by immunization of rabbits with phthalic anhydride treated rabbit serum albumin [24]. Horseradish peroxidase (HRP) labeled streptavidin was from Zymed, South San Francisco, CA. HRP was quantitated using a kit from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD. Biotin labeled goat anti-mouse IgG and anti-rabbit IgG were from Roche Diagnostics Corporation, Indianapolis, IN. Chicken serum was from OEM Concepts, Toms River, NJ. Antiserum to phthalate was prepared by immunization of rabbits with phthalic anhydride treated rabbit serum albumin [24]. Horseradish peroxidase (HRP) labeled streptavidin was from Zymed, South San Francisco, CA. HRP was quantitated using a kit from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD. Biotin labeled goat anti-mouse IgG and anti-rabbit IgG were from Roche Diagnostics Corporation, Indianapolis, IN. Chicken serum was from OEM Concepts, Toms River, NJ. Antiserum to phthalate was prepared by immunization of rabbits with phthalic anhydride treated rabbit serum albumin [24]. Horseradish peroxidase (HRP) labeled streptavidin was from Zymed, South San Francisco, CA. HRP was quantitated using a kit from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD. Biotin labeled goat anti-mouse IgG and anti-rabbit IgG were from Roche Diagnostics Corporation, Indianapolis, IN. Chicken serum was from OEM Concepts, Toms River, NJ. Antiserum to phthalate was prepared by immunization of rabbits with phthalic anhydride treated rabbit serum albumin [24]. Horseradish peroxidase (HRP) labeled streptavidin was from Zymed, South San Francisco, CA. HRP was quantitated using a kit from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD. Biotin labeled goat anti-mouse IgG and anti-rabbit IgG were from Roche Diagnostics Corporation, Indianapolis, IN. Chicken serum was from OEM Concepts, Toms River, NJ. Antiserum to phthalate was prepared by immunization of rabbits with phthalic anhydride treated rabbit serum albumin [24]. Horseradish peroxidase (HRP) labeled streptavidin was from Zymed, South San Francisco, CA. HRP was quantitated using a kit from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD. Biotin labeled goat anti-mouse IgG and anti-rabbit IgG were from Roche Diagnostics Corporation, Indianapolis, IN. Chicken serum was from OEM Concepts, Toms River, NJ. Antiserum to phthalate was prepared by immunization of rabbits with phthalic anhydride treated rabbit serum albumin [24]. Horseradish peroxidase (HRP) labeled streptavidin was from Zymed, South San Francisco, CA.
in 0.05 M acetate pH 6.0). For coating with antibodies, wells were first coated with protein A/G (1 µg/well) in 0.1 M Tris buffer, pH 8.8 for 2 h at 20°C, followed by mAb NC-1 or normal mouse IgG (= control wells) {1 µg/well; diluted in phosphate buffered saline (PBS)} for 1 h at 20°C. Coating with the N36/C34 peptide complexes (0.01 to 10 µM) was done under conditions described for protein A/G. Subsequently the wells were washed and post-coated with BSA and gelatin as described above, except that these proteins were dissolved in 0.14 M NaCl, 0.01 M Tris, pH 7.0 (TS). Chicken serum (10%) in PBS (Ch-PBS) was used instead in experiments with HIV-1 BaL to suppress binding of this virus to control wells. The wells were washed with TS and stored at 4°C overnight, binding of six-helix bundles was determined from subsequent binding of mAb NC-1, which was added at 1 µg/ml in PBS/1% BSA/1% gelatin (100 µl/well) for 1 h at 37°C. Subsequently the wells were washed three times with PBS/0.05% Tween 20 and biotin labeled anti-mouse IgG (100 µl/well; 125 ng/ml diluted in PBS containing 1% dry fat-free milk) was added. After incubation for 1 h at 37°C, the wells were washed six times with PBS/0.05% Tween 20. HRP was quantitated as described above.

To exclude the remote possibility that mAb NC-1 reacted with CAP, serial two-fold dilutions (0.25 to 8 µg/ml) of the mAb and of control mouse IgG, respectively (each at 16 µg/ml in PBS-BG) were added to CAP coated or CAP-gp120 coated wells for 1 h at 37°C. The wells were washed and bound IgG was quantitated as described above for the sandwich ELISA. CAP-gp120 wells were prepared by coating first with HIV-1 IIIB gp120 under conditions described above for protein A/G, except that the pH was 8.0 instead of 8.8. Subsequently, CAP was added to the wells as described above. In control experiments, serial dilutions (1/200 to 1/1,600) of rabbit anti-phthalate antise-
rum and of normal rabbit serum, respectively (each diluted 100-fold in PBS-BG) were added to the wells. Bound rabbit IgG was quantitated using biotinylated goat anti-rabbit IgG.

**Shedding of gp120 from virus particles**

Preparations of purified HIV-1 IIIB and BaL, respectively, were incubated for 5 min at 37°C in the presence or absence of CAP (final concentration 5 mg/ml). Control preparations were not exposed to 37°C. Virus particles and released gp120 were separated by centrifugation as described [28]. The virus containing pellets and supernatant fluids were assayed by an ELISA allowing gp120 determination in the presence of CAP. Wells of polystyrene plates were coated with protein A/G, followed by polyclonal rabbit anti-gp120 (diluted 500-fold) under conditions described above for virus capture assays. Serial twofold dilutions in PBS-BG of preparations containing gp120 were added to the wells. After 4 h at 20°C, the wells were washed and bound gp120 was detected by addition of biotinyl-sCD4 (1 µg) followed by HRP-streptavidin as described above. The amount of gp120 in the virus pellets and the supernatant fluids was calculated from calibration curves relating absorbance readings to gp120 dilutions. All determinations were done in triplicate.

**Molecular modeling: Docking of CAP on the gp41 core structure**

The acetylated and phthaloylated cellotetraose unit (CTAP) composed of four 1,4-linked β-D-glucose units, which is a representative part of CAP was created in Quanta 2000 [29] as described before [8]. CTAP was minimized by the steepest descent method followed by the adopted basis Newton-Raphson (ABNR) method. The energy difference of 0.05 Kcal/mol between two successive structures during both minimization steps was used as the termination criterion.

The docking simulations of CTAP were performed using the DockVision program [30] on the entire surface of the gp41 core structure (The X-ray crystal structure of the gp41 core, 1aik, was retrieved from the protein databank [http://www.rcsb.org]). A grid box (125 × 125 Å × 125 Å) was created to cover the entire gp41 core surface for CTAP to dock. The default forcefield (Research Potential Function) was used to perform 1000 Monte Carlo runs for docking simulations. Both CTAP and the gp41 core structure were kept rigid during docking. Intermolecular energy criteria were used to select the lowest energy docked CTAP.

**Electrostatic potential maps of gp120 and the gp120-CD4 complex**

Electrostatic potentials were calculated using a Poisson-Boltzmann solver included in the GRASP program [31]. All default parameters were used. The electrostatic potential maps are shown on the accessible surface of gp120 and the gp120-CD4 complex.
determined whether or not occupancy of CD4 binding sites within gp120 would affect subsequent interactions with CAP. To answer this question, the binding of gp120 and gp120-sCD4 complexes, respectively, to immobilized CAP was studied. Results shown in Fig. 1 indicated that sCD4 enhanced gp120-CAP binding. Maximum enhancement was observed at sCD4/gp120 weight ratios of ≥ 0.6 (Fig. 1 insert), corresponding to a molar ratio of ≥ 1.2 [32], in agreement with the observation that gp120-CAP4 complexes consist of one molecule each of CD4 and gp120 [16]. Biotinyl-sCD4 in the absence of gp120 did not bind to CAP. Similarly, pretreatment of HIV-1 with sCD4 resulted in subsequent increased binding of virus with CAP, the effect being much more pronounced with HIV-1 BaL in comparison with HIV-1 IIB (Fig. 2).

**Synergism between CAP and sCD4 in inhibiting HIV-1 infection**

Since HIV-1 can bind CAP and sCD4 simultaneously, and the binding of CAP is enhanced in the presence of sCD4, it was of interest to determine whether these two ligands act on HIV-1 cooperatively, resulting in synergism of their antiviral effects. This indeed was observed (Table 1 and 2, Fig. 3). CAP and sCD4 synergistically inhibited infection by HIV-1 IIB (Table 1) and HIV-1 BaL (Table 2). A similar synergism was observed for virucidal activity against HIV-1 IIB (Fig. 3) but only additive effects were found for HIV-1 BaL (in the absence of sCD4, ED50 for CAP = 1.49 ± 0.38 mg/ml; in the presence of sCD4 [100 µg/ml], which caused an 1.85-fold decrease of infectivity, ED50 = 1.39 ± 0.18 mg/ml for residual infectivity).

**Treatment of HIV-1 with CAP leads to induction of gp41 six-helix bundles**

Earlier studies [8], in which the binding of CAP treated and untreated virus with antibodies specific for distinct regions on the envelope glycoproteins gp120 and gp41 was studied, indicated that CAP had either no effect or caused decreased binding with antibodies against several peptides from gp120 but only against a single peptide, 557–586, from gp41 (see Discussion). It was intended to expand these studies to mAb NC-1, specific for the gp41 six-helix bundle [20]. Results of preliminary studies indicated that CAP did not interfere with the six-helix bundle formation from constituent peptides derived from near the N- and C-term of the gp41 ectodomain [20]; (own unpublished data). Surprisingly, and unlike with mAb 2F5 [8] specific for the C-terminal region of the gp41 ectodomain [33], CAP treatment enhanced the binding of both HIV-1 IIB and HIV-1 BaL to mAb NC-1, reacting with gp41 six-helix bundles from both HIV-1 IIB and BaL (Fig. 4), suggesting the formation of these structures within virus particles as a result of CAP treatment (Fig. 5). Treatment with both CAP and sCD4 further enhanced the expression of the six-helix bundles in the case of HIV-1 BaL

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**Results**

**Enhancement of CAP-HIV-1 binding by CD4**

Earlier studies [8] indicated that CAP binding to the envelope glycoprotein gp120 and to HIV-1 virus particles, respectively, did not interfere with their subsequent association with sCD4. Thus, it would be expected that CAP would not inhibit the attachment of HIV-1 to target cells and would block only subsequent steps obligatory for HIV-1 infection initiated by engagement of CXCR4 and CCR5 coreceptors, respectively. The latter event was shown to be blocked by CAP [8] and has been considered the basis for the virus inhibitory and virus inactivating properties of CAP at neutral pH. However, it has not been
trol peptide the tyrosines of which were not sulfated did not have this effect. Analogous experiments with HIV-1 IIIB were not performed since there are no published data concerning the biological properties of tyrosine sulfated peptides from the N-terminus of CXCR4.

In the experiments described above evidence for the formation of gp41 six-helix bundles was obtained from capture of CAP, CD4 and CCR5 S-peptide treated virus particles, respectively, onto wells coated with mAb NC-1. This assay is equivalent to an immunoprecipitation assay with solid phase mAb NC-1. In order to provide further evidence for the induction of the gp41 six-helix bundles by the distinct ligands binding to the HIV envelope, the newly formed structures in virus lysates were also quantitated by a sandwich ELISA [21]. The results not only unequivocally confirmed the induction of gp41 six-helix bundle structures by sCD4, CAP and the S-peptide from CCR5, but also provided evidence that these structures were undetectable in untreated virus particles (Fig. 8). CAP (0.078 to 10 mg/ml) in the absence of virus particles provided negative results in this assay. The CCR5 peptide lacking sulfated tyrosines did not induce the six-helix bundles. Thus, it seems likely that the detection of gp41 six-helix bundles in untreated HIV-1 using the virion capture assay was due to their spontaneous formation during prolonged incubation of HIV-1 in these tests. The six-helix bundles were also induced by heating (10 min at 60°C) HIV-1 virus particles (Fig. 8).

In summary, blocking by CAP of the coreceptor binding sites on the virus envelope glycoprotein gp120 within HIV-1 virus particles appears to induce conformational changes in gp41 leading to the formation of six-helix bundle structures.

**Shedding of gp120 from virus particles**

It was reported that treatment of HIV-1 gp120/gp41 envelope glycoprotein oligomers with sCD4 lead to shedding of gp120-sCD4 complexes concomitant with increased exposure of some cryptic epitopes on gp41 [13,34,35]. Therefore, it was of interest to determine whether or not shedding of gp120 from virus particles was required for the CAP induced exposure of binding sites for mAb NC-1 on gp41. Treatment of HIV-1 IIIB and BaL with CAP did not decrease the level of virus-associated gp120 in comparison with control virus preparations (Table 3). The results agree with the half life of 40 h for virus associated gp120 in the course of spontaneous gp120 shedding from HIV-1 HXB3 at 37°C [36] and the small sCD4 induced release of gp120 within 5 min at 37°C [37]. Thus gp120 shedding was not a prerequisite for the formation of gp41 six-helix bundles.

To exclude the remote possibility that mAb NC-1 reacted with CAP or protein-bound CAP, and that this, rather than binding to six-helix bundles, would lead to results shown in Fig. 5, the binding of the mAb to CAP coated wells was investigated. No significant binding of mAb NC-1 and of control mouse IgG, respectively, to wells coated by CAP directly or to CAP bound to HIV-1 IIIB gp120 was observed. On the other hand, anti-phthalate antibodies reacted with both forms of immobilized CAP (Fig. 6). These results support the conclusions from results in Fig. 5.

Engagement of the gp120 coreceptor binding site with a tyrosine sulfated peptide from the N-terminus of the coreceptor CCR5 [25], shown to inhibit infection by CCR5-dependent, but not CXCR4-dependent, HIV-1 isolates, was sufficient to increase the expression of the gp41 six-helix bundles in HIV-1 BaL virus particles (Fig. 7). A CCR5 control peptide the tyrosines of which were not sulfated did
Discussion

Earlier studies [8] indicated that CAP treated HIV-1 particles have their coreceptor, i.e. CXCR4 and CCR5, binding sites obstructed, while sites involved in association with CD4 appeared unaffected. This suggested that HIV-1 could bind CD4 and CAP at the same time, independently. Since association with CD4 induces conformational changes in the HIV-1 envelope glycoprotein gp120 [14,16,17], it was of interest to determine: (a) whether CD4 binding to HIV-1 would affect subsequent association of the virus with CAP and (b) the consequences of both CD4 and CAP binding to virus particles. First, it was found that pretreatment of gp120 with sCD4 enhanced subsequent binding with CAP (Fig. 1). This could be attributed to conformational changes in gp120, to the concealment of surface areas with the greatest negative charge on gp120 by CD4 [16], (Fig. 9), which could diminish the electrostatic attraction between gp120 and negatively charged CAP, or to additional CAP binding sites on the CD4 portion of gp120-sCD4 complexes. The binding of HIV-1 with CAP was similarly enhanced by pretreatment with sCD4 (Fig. 2), the effect being much more pronounced with the R5 virus, HIV-1 BaL, in comparison with the X4 virus, HIV-1 IIIB. These observations may be related to the recognized role of CD4 in inducing confor-

Table 1: Synergism between CAP and sCD4 in inhibiting infection by HIV-1 IIIB.

| % Inhibition | CI* | Concentrations for inhibition of infection |
|--------------|-----|------------------------------------------|
|              |     | CAP (µg/ml) | sCD4 (µg/ml) |
|              |     | Alone | Mix | Alone | Mix |
| 50           | 0.29 | 7.10  | 1.05 | 0.72  | 0.11 |
| 70           | 0.39 | 10.59 | 2.01 | 1.02  | 0.20 |
| 90           | 0.60 | 20.05 | 5.63 | 1.78  | 0.56 |
| 95           | 0.76 | 28.55 | 9.96 | 2.42  | 0.99 |

* CI = Combination index values

Table 2: Synergism between CAP and sCD4 in inhibiting infection by HIV-1 BaL.

| % Inhibition | CI* | Concentrations for inhibition of infection |
|--------------|-----|------------------------------------------|
|              |     | CAP (µg/ml) | sCD4 (µg/ml) |
|              |     | Alone | Mix | Alone | Mix |
| 50           | 0.40 | 3.24  | 1.10 | 9.05  | 0.55 |
| 70           | 0.43 | 4.43  | 1.68 | 15.60 | 0.84 |
| 90           | 0.49 | 7.31  | 3.24 | 37.12 | 1.62 |
| 95           | 0.52 | 9.64  | 4.67 | 59.98 | 2.34 |

* CI = Combination index values
mational changes in gp120 that contribute to the exposure of binding sites for CXCR4 and CCR5 [16,17,38]. Furthermore, it seemed important to determine whether simultaneous CD4 and enhanced CAP binding to HIV-1 would result in synergistic effects for inhibition of HIV-1 infection. Evidence for such synergism was indeed established (Table 1 and 2, Fig. 3).

Earlier studies [8], in which the binding of CAP treated and untreated virus to antibodies specific for distinct regions on the envelope glycoproteins gp120 and gp41 was studied, indicated that CAP caused decreased binding with antibodies against several peptides from gp120 but only with a single antibody against peptide 557–586 from gp41 (for numbering of amino acid residues see our earlier publication [39]). Thus, there are fewer binding sites for CAP on gp41 than on gp120. Interestingly, molecular docking studies revealed that phthalic and acetic acid anhydride modified cellotetratose, a subunit of CAP, docked to a single site on the gp41 core structure overlapping the peptide 557–586 (Fig. 10). This region is in the vicinity of the most prominent positively charged areas on the surface of the gp41 core which has an overall negative charge. Since CAP blocks coreceptor binding sites on gp120 [8] it was of interest to determine whether this blockade would lead to conformational changes in HIV-1 gp41, similar to those elicited by CD4 or coreceptor binding to gp120.

The occupancy of CD4 and coreceptor binding sites by their respective ligands elicits downstream conformational changes in the envelope glycoprotein gp41, rendering it competent for fusion between virus and target cell membranes [18,19,40–43]. Shedding of gp120 from virus particles is not required for subsequent membrane fusion events [44]. The induction of gp41 six-helix bundles, detectable by mAb NC-1, by CAP in the absence of gp120 shedding is consistent with this conclusion. The conformational changes lead to the formation of a coiled-coil in gp41, consisting of three NH2-terminal leucine/isoleucine zipper regions, each contributed by one of the three subunits of the envelope glycoprotein trimer. In the presence of target cell membranes, the NH2-terminal fusion peptide is displaced in the direction of the target cell membrane, into which it inserts. Thus, the HIV-1 envelope glycoprotein gp41 becomes an integral component of two membranes, the viral membrane and the cellular membrane. The outer surface of the coiled-coil contains grooves into which three heptad repeat regions from the C-terminal part of the gp41 ectodomain pack, resulting in a stable six-helix bundle [42,45–54]. The six-helix bundle structure can be detected by specific antibodies [14,20,43,55].

Results presented here indicate that purified HIV-1 particles do not contain detectable six-helix bundle structures.
Their expression is induced by CAP treatment of the virus (Fig. 5 and 8). Prior engagement of CD4 binding sites is not required for the induction of the six-helix bundles by CAP, but increases their expression (Fig. 5 and 8). The apparent cooperativity between CAP and sCD4 in induction of the gp41 six-helix bundle structures may possibly be related to the observed synergism between these two ligands for inhibition of HIV-1 infection (Table 1 and 2, Fig. 3). The hypothesis that engagement of coreceptor binding sites on gp120 by CAP leads to the expression of gp41 six-helix bundle structures is supported by the finding that a tyrosine sulfated S-peptide, but not the non-sulfated peptide, from the N-terminus of CCR5 [25,56,57] has an effect similar to that of CAP (Fig. 7). The helix-bundles were also induced by heating HIV-1 virus particles at 60°C, in agreement with the irreversible induction of the fusogenic conformation in influenza virus hemagglutinin by heat [58,59].

In summary, the results presented here suggest that treatment of HIV-1 with CAP leads to conformational changes in the envelope glycoproteins, ultimately resulting, in the absence of target cell membranes, in the formation of gp41 six-helix bundles. These structures are extremely stable and represent a terminal, functionally inactive viral constituent [54,60]; (Fig. 11), analogous to that of inactivated influenza virus hemagglutinin HA2 exposed to low pH in the absence of cell membranes [59,61–64].

**Conclusions**

Earlier studies describing the underlying molecular mechanisms involved in the HIV-1 inhibitory effect of the candidate microbicide CAP indicated that this compound remains bound to HIV-1, impairing virus infectivity by blockade of binding sites for cellular coreceptors CXCR4 and CCR5 [8]. Results reported here further extend these findings and show that: 1) there is synergism between sCD4 and CAP for inhibition of virus infectivity; 2) CAP

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**Table 3: Treatment of HIV-1 with CAP does not result in gp120 shedding from virus particles**

| Treatment | % gp120 associated with virus particles |
|-----------|----------------------------------------|
| HIV-1 IIIB | HIV-1 BaL                              |
| None      | 77.0 ± 2.8                             | 83.8 ± 6.9 |
| 5 min 37°C, No CAP | 84.6 ± 6.4 | 80.0 ± 0.5 |
| 5 min 37°C + CAP | 87.1 ± 2.4 | 88.1 ± 7.3 |

**Figure 8**

Induction of gp41 six-helix bundles by HIV-1 treatment with sCD4, CAP, CCR5 S-peptide and heat. HIV-1 IIIB and HIV-1 BaL, respectively, were treated with CAP and sCD4 as described in the legend for Fig. 5 or exposed to 60°C for 10 min. HIV-1 BaL was also treated with the S-peptide and a control non-sulfated peptide from CCR5 as described in the legend for Fig. 7. The treated virus preparations and untreated control virus were treated with lysis buffer (see Methods) for 30 min at 20°C. CAP in lysis buffer (0.078 to 10 mg/ml) was also tested; the results for a 5 mg/ml concentration are shown (identical results were obtained for all other concentrations). The lysates were tested by a sandwich ELISA for the gp41 six-helix bundle (see Methods). All experiments were done at least in triplicate.

**Figure 9**

Electrostatic potential maps of gp120 and the gp120-CD4 complex. Electrostatic potentials are shown for the solvent accessible surfaces. (A) The electrostatic potential map on gp120. Blue indicates electropositive areas whereas red represents electronegative areas. (B) After CD4 binds to gp120, the electropositive surface area (blue) increases markedly while the most negatively charged (red) area on gp120 (arrow) becomes blocked by sCD4.
binding to HIV-1 leads to conformational changes in viral envelope glycoproteins resulting in the expression of functionally inert six-helix bundle structures.

To the best of our knowledge, results reported here and earlier [8] represent the most detailed study on the mechanism of action of a polymeric anti-HIV-1 compound and offer new opportunities for microbicide research, including the design of combined microbicides with distinct target sites on HIV-1 and acting synergistically.

**List of abbreviations**

CAP, cellulose acetate phthalate; STD, sexually transmitted disease; ELISA, enzyme-linked immunosorbent assay; mAbs, monoclonal antibodies; FBS, fetal bovine serum; PEG 6000, polyethylene glycol 6000; HIV-1, human immunodeficiency virus type 1; BSA, bovine serum albumin; PBS, phosphate buffered saline; Ch-PBS, chicken serum (10%) in PBS; PBS-BG, 1% BSA/1% gelatin in PBS; HRP, horseradish peroxidase; sCD4, soluble CD4; TS, 0.14 M NaCl, 0.01 M Tris, pH 7.0; CTAP, cellotetraose acetate phthalate; pdb, Protein Data Bank; ED$_{50}$, effective dose for 50 % inhibition.

**Figure 10**

Docking of a cellotetraose acetate phthalate (CTAP) unit of CAP to the gp41 core structure. (A) Docking of CTAP on the gp41 core. The inner N-peptide coiled-coiled trimer is represented in green whereas the outer C-peptide helices are represented in yellow. Residues on the gp41 core interacting with CTAP (gray and red) are color-labeled. The brown residues are hydrophobic whereas blue residues are positively charged. One of the negatively charged groups from the phthalic acid moieties of CTAP docked near an R579 residue of the gp41 core. Two of the CTAP phenyl groups have hydrophobic contact with gp41 W571. The peptide segment 557–586 ([8,39], corresponding to residues 550–571 in the X-ray crystal structure) antibodies to which are prevented by CAP from binding to gp41, is indicated in light blue in one of the three inner helices. (B) Electrostatic potential surface of the gp41 core created by the GRASP [31] program. The CTAP molecule docked near a relatively electropositive site on gp41. Most of the surface is highly electronegative.
Competing interests
None declared

Authors' contributions
Author 1 ARN developed the concepts representing the basis of the manuscript and designed most experiments. Author 2 NS carried out most experiments and contributed to the development of experimental techniques. Author 3 SJ developed monoclonal antibody NC-1 and was involved in studies on the gp41 six helix bundles and on synergism between CAP and sCD4 for inhibition of HIV-1 infectivity. Author 4 YYL did most tissue culture work and infectivity assays. Author 5 AKD did all the molecular modeling studies.

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