

Inhibition of Human Immunodeficiency Virus Replication by Cell Membrane-Crossing Oligomers

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Although rapidly becoming a valuable tool for gene silencing, regulation or editing *in vitro*, the direct transfer of small interfering ribonucleic acids (siRNAs) into cells is still an unsolved problem for *in vivo* applications. For the first time, we show that specific modifications of antisense oligomers allow autonomous passage into cell lines and primary cells without further adjuvant or coupling to a cell-penetrating peptide. For this reason, we termed the specifically modified oligonucleotides “cell membrane-crossing oligomers” (CMCOs). CMCOs targeted to various conserved regions of human immunodeficiency virus (HIV)-1 were tested and compared with nontargeting CMCOs. Analyses of uninfected and infected cells incubated with labeled CMCOs revealed that the compounds were enriched in infected cells and some of the tested CMCOs exhibited a potent antiviral effect. Finally, the CMCOs did not exert any cytotoxicity and did not inhibit proliferation of the cells. *In vitro*, our CMCOs are promising candidates as biologically active anti-HIV reagents for future *in vivo* applications.

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INTRODUCTION

In *in vitro* experiments, antisense technology represents a simple method for the treatment of diseases as diverse as viral infections, cancer and inflammatory, metabolic or neurodegenerative diseases. There are efforts toward antisense drug discovery using cellular ribonucleic acids (RNAs) as molecular targets, but the effective delivery of these oligos *in vivo* is problematic. Among existing antisense strategies are peptide nucleic acids (PNAs), which were discovered in the

context of gene targeting and gene therapeutic drugs. PNAs contain a charge-neutral pseudo-peptide backbone (1,2) that confers high chemical stability and resistance against degradation by diverse nucleases. Furthermore, PNAs were not degraded by endogenous proteases and peptidases during 2-h incubations in human serum, bacterial cell extracts or mouse ascites (3,4).

The effective delivery of inhibitory antisense reagents by complex formation of the small interfering RNAs (siRNAs)/

PNAs, or other antisense technologies (for example, locked nucleic acids, methoxyethyl-RNA oligomers, phosphorothioates), with cationic lipids, or intracellular transfer by electroporation or microinjection, incurs no major problems *in vitro* (5,6), but *in vivo*, these methods cannot be applied or are of limited use in their target tissues. Recently, Stein *et al.* (7) demonstrated that modified locked nucleic acids can be efficiently delivered not only in adherent cells but also in suspension cultures without using a transfection reagent. This process was designated as “gymnosis,” because the oligomers were delivered “naked” (*gymnos* in Greek) without any conjugates or transfectants into the cells and takes advantage of normal cell growth properties to uptake the oligonucleotide. Beside locked nucleic acids, a number of delivery strategies for siRNAs have been developed to overcome multiple extracellular and intracellular barriers *in vivo*. Among these, siRNA conjugates with cholesterol (8), aptamers (9), chemically modified siRNAs (10), siRNAs delivered

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by viral or nonviral vectors (11–13) or targeted siRNA delivery (14) were tested *in vivo*. Additionally, Turner *et al.* (15) studied different types of cell-penetrating peptide (CPP)/PNA conjugates (16) and found that, despite the human immunodeficiency virus (HIV) inhibitory activity of their constructs, improvement of their membrane penetration and endosomal release properties was necessary.

The inhibition of HIV-1 replication is well suited to RNA interference, since several stages of the viral life cycle and many viral genes can be targeted. In addition to viral targets, inhibitory anti-sense reagents can be directed against host proteins. Brass *et al.* (17) identified host proteins essential for HIV infection by a functional genomic screen that yielded future targets of inhibitory oligonucleotides. In our study, we tested several oligonucleotides against conserved regions of the HIV-1 genome that were specifically modified to allow autonomous passage into the cell without further adjuvant or coupling to a CPP via a linker. We used the two most promising oligonucleotide sequences for further analyses and found that the HIV-specific cell membrane-crossing oligomers (CMCOs) were enriched in infected cells, were stable against degradation over a prolonged period of time and were able to block infection. Our “self-transfecting” inhibitory CMCOs are promising candidates for biologically active anti-HIV reagents for future *in vivo* applications.

MATERIALS AND METHODS

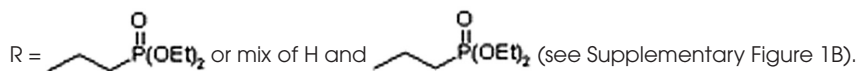
CMCO Design

The CMCOs were developed and produced by ugi chem GmbH (Innsbruck, Austria) and are accurately described in the patent WO 2008/009470 A1 by Lindhorst *et al.* (18). Shortly, the novel compounds contain PNA units substituted with phosphonic acid ester functions or phosphonic acid functions and exhibit at least one chiral center. The structure of the different modifications is shown in Figures 1A and B. If a monomer unit carries a phosphonic ester side chain, the

Table 1. Characterization (identifier, modifications, retroviral genomic area, sequences and molecular weight) of CMCOs used in this study.

Identifier	Area (HIV genome)	Sequence
CMCO1, type A	<i>gag</i>	FLU/Rho-Lys(Me3)- <u>TCGCTGCCAAAGAGT</u> -Gly-NH ₂
CMCO1, type B	<i>gag</i>	FLU/Rho-Lys(Me3)- <u>TCGCTGCCAAAGAGT</u> -Gly-NH ₂
nsCMCO, type B	GFP	FLU/Rho-Lys(Me3)- <u>AGCTCCICGCCITGC</u> -Gly-NH ₂
fvCMCO, type B	FV (nonspecific)	FLU/Rho-Lys(Me3)- <u>ATCGTCTAAGTTCAG</u> -Gly-NH ₂
CMCO1, type B	<i>gag</i>	Ac-Lys(Me3)- <u>TCGCTGCCAAAGAGT</u> -Gly-NH ₂
CMCO1, type B2	<i>gag</i>	Ac-(Lys(Me3)) ₄ - <u>TCGCTGCCAAAGAGT</u> -Gly-NH ₂
CMCO2, type B	<i>gag</i>	Ac-Lys(Me3)- <u>IATACITCGGGCTG</u> -Gly-NH ₂
nsCMCO, type B	GFP	Ac-Lys(Me3)- <u>AGCTCCICGCCITGC</u> -Gly-NH ₂
fvCMCO, type B	FV (nonspecific)	Ac-Lys(Me3)- <u>ATCGTCTAAGTTCAG</u> -Gly-NH ₂

FLU, fluorescein; Rho, Lissamine. Underlined text indicates nonmodified nucleosides (see Supplementary Figure 1B). Bold text indicates modified nucleosides.



The TCGCTGCCAAAGAGT sequence mainly used here (CMCO1) was modified by one base, as published by Sei *et al.* (37) (TCGTTGCCAAAGAGT) because of a base exchange of our NL4-3 strain at this position. Type A- and type B-modified CMCOs were tested in the first experiments (CMCO1). Because of the higher uptake and inhibitory effects, the study was continued only with type B CMCOs (CMCO1, CMCO2, fvCMCO and nsCMCO). Fluorescently labeled probes were used for FACS and confocal microscopic analysis (gray, CMCO1, type A and B, nsCMCO and fvCMCO), while infection, proliferation and cytotoxicity assays were performed using unlabeled probes (white, CMCO1, type B and B2, CMCO2, nsCMCO and fvCMCO).

stereochemistry is represented by R (according to Cahn-Ingold-Prelog priority rules). E represents a hydrogen atom, a substituted or unsubstituted phenyl rest, a substituted or unsubstituted heterocyclic rest, a nucleobase or a DNA intercalator. K represents an -NH₂ function and L an -OH function, an -NH₂ function, an -NH-(C1-C5)alkyl function, an amino acid, amino acid amide, peptide or peptide amid unit. The exact modifications at L are demonstrated in Table 1. For fluorescence-activated cell sorter (FACS) analyses and confocal microscopy, the CMCOs were labeled with either fluorescein or Lissamine™ (see Table 1, highlighted in gray). Prior cleavage from resin in the N-terminal protection group was removed, and the resulting terminal amine was capped with a 10-fold excess of sulforhodamine B acid chloride or fluorescein-5(6)-isothiocyanate, respectively. As the negative control, a nontargeting fluorescently labeled CMCO was used (see Table 1, nonspecific CMCO [nsCMCO]). Additionally, the experi-

ments were performed with a Friend murine leukemia virus (FV) sequence (see Table 1) to exclude off-target effects of the HIV-specific sequences. The CMCOs in this study were directed against HIV-1 *gag*, and the chosen sequences were aligned to HIV BLAST (<http://www.hiv.lanl.gov>; BLAST, Basic Local Alignment Search Tool). Depending on the methods performed, different CMCOs were used (fluorescein isothiocyanate [FITC]- or Lissamine-labeled CMCOs 1, type A or B, nsCMCO and FV CMCO [fvCMCO] for FACS analyses or fluorescence microscopy [see Table 1, highlighted in gray]; unlabeled CMCOs 1 and 2, type B, or CMCO 1, type B2, for infection assays; integration assays; and cytotoxicity testing). A nonspecific green fluorescent protein (GFP) or FV sequence was used as control in all experiments (nsCMCO, fvCMCO).

Cells

M8166 cells, a human T-lymphoblastoid cell line obtained from the National Insti-

tute for Biological Standards and Control (NIBSC) Center for AIDS Reagents, were cultured in RPMI 1640 (Gibco) supplemented with 2 mmol/L L-glutamine (Gibco) and 10% fetal calf serum (RPMI_c). M8166 cells were cloned from C8166 cells for increased susceptibility to simian immunodeficiency virus (SIV) or HIV syncytia formation due to higher expression of the main receptors of dual- or X4-tropic HIV, cluster of differentiation 4 (CD4) and chemokine (C-X-C motif) receptor 4 (CXCR4), on their surface. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of normal healthy donors (obtained from the local blood bank, Innsbruck, Austria) by centrifugation on a Ficoll-Hypaque density gradient (Pharmacia, Uppsala, Sweden). After several washing steps, the cells were cultured at 37°C/5% CO₂ in RPMI_c and stimulated with phytohaemagglutinin M (PHA-M) (2.5 µg/mL, Sigma) and interleukin (IL)-2 (20 U/mL, Peprotech, UK).

Virus Propagation and Purification

Primary isolate 93BR020 (subtype B/F₁R5X4-tropic) and the X4-tropic laboratory strain NL4-3 were obtained from the NIH AIDS Reagent Program (available through World Health Organization depositories). The virus was propagated in PHA- and IL-2-stimulated PBMCs. Virus supernatants were cleared by filtration through 0.22-µm pore-size filters and concentrated by ultracentrifugation at 20,000 rpm for 90 min at 4°C. The virus pellet was resuspended in RPMI without supplements and stored in small aliquots at -80°C to avoid multiple thawing. One aliquot was taken to determine the virus concentration by p24 enzyme-linked immunosorbent assay (ELISA) (20) and the tissue culture infectious dose 50 (TCID₅₀) of the viral stock. All experiments were performed with the laboratory strain NL4-3 at least three times; confocal analyses were performed with 93BR020.

Infection of M8166 and PBMCs

A total of 100 µL (1 × 10⁵) M8166 cells or PBMCs were preincubated for 24 h at 37°C/5% CO₂ with CMCO concentra-

tions ranging from 1.56125 µmol/L to 25 µmol/L or 2.5 nmol/L of the protease inhibitor indinavir (IDV). Thereafter, the cells were infected with 50 pg p24/mL (M8166 cells) or 500 pg p24/mL (PBMCs) of HIV. Cells were washed to remove unbound virus, resuspended in RPMI_c containing IL-2 (20 U/mL) and cultivated at 37°C/5% CO₂. Virus propagation was determined by p24 ELISA of supernatants on several days after infection. For real-time polymerase chain reaction (PCR) analyses, cells were lysed on d 7 and 14 after infection and DNA was isolated.

Flow Cytometry

Uninfected and infected M8166 cells or PBMCs (1 × 10⁶/mL) that were incubated with different concentrations of HIV- or nonspecific CMCOs (fluorescently labeled fvCMCO) for a further 24 h were washed with cold phosphate-buffered saline (PBS)/1% bovine serum albumin/0.1% NaN₃, fixed in PBS/4% paraformaldehyde and incubated overnight at 4°C. The analyses were performed using a FACScan (BD Biosciences, San Jose, CA) and CellQuest software (BD Biosciences).

Immunofluorescence and Confocal Microscopy

M8166 cells or PBMCs (5 × 10⁴ cells/condition) were preincubated with different concentrations of directly labeled CMCOs at 37°C/5% CO₂ for 24 h before the addition of HIV-1 (500 ng p24/5 × 10⁴ cells) for 24 h. Cells were washed twice in PBS and left to adhere on poly-L-lysine-treated (Sigma-Aldrich, St. Louis, MO, USA) glass coverslips for 1 h at 37°C. Cells were then fixed for at least 1 h at room temperature or overnight at 4°C in 3% paraformaldehyde, permeabilized with 0.05% saponin (Sigma-Aldrich) and washed with PBS containing 0.2% bovine serum albumin (Sigma-Aldrich) and human IgG (20 µg/condition). Cells pulsed with FITC-labeled CMCOs and with or without HIV-1 were analyzed by confocal laser scanning microscopy in initial ex-

periments. In additional experiments, infected or uninfected cells incubated with Lissamine-labeled CMCOs were stained with a monoclonal antibody (mAb) against HIV-1-p24^{gag} (KC57-FITC; Beckman Coulter, Brea, CA, USA).

To determine the uptake mechanism, samples were incubated overnight at 4°C with 500 nmol/L or 25 µmol/L of Lissamine-labeled CMCO, type B; washed once; and immediately fixed. The fixed cells were left to adhere to glass coverslips, additionally stained with DRAQ5TM and analyzed by confocal microscopy. Confocal laser scanning microscopy was performed with an LSM 510 microscope (Zeiss, Göttingen, Germany).

Quantitative Analyses of HIV-1 DNA Dynamics by Real-Time PCR

Dynamics and integration efficiency of HIV-1 DNA in PBMCs incubated with different concentrations of CMCOs and HIV-1 were assessed by real-time PCR using primers and fluorescently labeled probes for strong-stop HIV-1 DNA, full-length HIV DNA or integrated forms of viral DNA as described by Suzuki *et al.* (21). The amount of integrated HIV-1 was determined by a nested PCR comprising an *Alu*-HIV-1 PCR as the first step, followed by a second PCR using a dilution of the first cDNA products and the strong-stop primers and probe set (21). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR with GAPDH-specific primers and probe served as a control to assess the relative quantity of the gene expression profile of the target genes (described below). PCRs using fluorescently labeled probes were performed using the 2xPCR kit from BioRad (Hercules, CA, USA). A total of 20 µL 2xPCR mix, 200 nmol/L of each of the primers, 100 nmol/L of the fluorescently labeled probe, 0.025 U of Sure Start TaqDNA polymerase and 5 µL of the isolated DNA were added in a total volume of 40 µL. Thermal cycling conditions for GAPDH, full-length HIV and minus-strong-stop HIV DNA (R/U5) consisted of 10 min at 95°C and 50 cycles

of 95°C for 15 s and 60°C for 30 s. The *Alu*-HIV-1 PCR was performed at 95°C for 10 min followed by 22 cycles of 30 s at 95°C, 30 s at 66°C, 10 min at 70°C and a final extension step for 10 min at 72°C. All real-time PCR runs were performed on an iCycler (BioRad).

Evaluation of HIV-1 DNA Replication Intermediates (Minus-Strong-Stop DNA, Integrated DNA)

The relative amount of minus-strong-stop, full-length and integrated HIV DNA replication intermediates were calculated with the Gene Expression Macro software (version 1.1) from BioRad. The calculations in this application allow using multiple reference genes. The calculations of the program were derived from the algorithms cited by Vandesompele *et al.* (22) and from the geNorm manual. The gene expression of the target genes (minus-strong-stop, full-length and integrated HIV DNA) was quantified by normalization to the reference gene GAPDH. Uninfected immature dendritic cells (iDCs) were used as control samples to verify the specific amplification of the different HIV-1 DNA forms.

Proliferation

The CellTiter 96[®] Non-Radioactive Cell Proliferation Assay was purchased from Promega, and the assay was performed according to the manufacturer's instructions using different amounts of untreated cells as the standard.

Annexin/7-Amino-Actinomycin D Staining

To assess whether the CMCOs used exerted cytotoxic effects, we double-stained CMCO-treated and untreated cells with annexin-V and 7-amino-actinomycin D (7-AAD) and analyzed the samples by FACS.

Statistical Analysis

Differences between triplicates in infection experiments were analyzed in the unpaired Student *t* test by the GraphPad prism software.

All supplementary materials are available online at www.molmed.org.

RESULTS

CMCOs Are Designed by Addition of Phosphonic Ester Residues

"Conventional" PNA molecules are poorly water soluble compared with DNA, and permeation of the cell membrane is a general problem for PNAs. This problem is solved by designing compounds according to the formula shown in Figure 1A. We hypothesized that phosphonic ester residues introduce highly amphiphilic properties to molecules, thereby leading to increased solubility in water or PBS as well as to an enhanced ability to penetrate through cellular membranes. Adding multiple phosphonic ester residues to a PNA backbone amplifies the latter property. In addition, the C-P linkage of a phosphonic ester group is chemically different from the C-O-P linkage of a phosphate group commonly found in biological systems. Therefore, an increased stability toward enzymatic degradation could be expected. PNA monomer building blocks are commercially available, and optically pure CMCO monomer building blocks (R-configuration according to Cahn-Ingold-Prelog priority rules) were synthesized according to previous reports (18,23,24). N²-Boc-N⁶,N⁶,N⁶-trimethyl-(L)-lysine iodide as a building block for Lys(Me₃) was prepared, as published by Chen and Benoiton (19). All CMCOs were synthesized on a fully automated solid-phase synthesizer (Multisynthes Syro) according to a protocol developed by Koch *et al.* (25). Whereas phosphonic ester side chains are synthesized to every nucleoside on type A CMCOs (see Table 1, bold), type B CMCOs are a mix of modified (see Table 1, bold) and nonmodified (see Table 1, underlined) nucleosides. The various modifications of the CMCOs are shown in Table 1 (see Table 1, type A, B or B2) and Figure 1B.

CMCOs Are Stable in Human Plasma

To verify the stability of CMCOs in plasma, they were incubated at different

concentrations (5–50 µg/mL) in human plasma or Tris buffer at 37°C for 10 min to 48 h by CIT Safety and Health Research Laboratories, France. This protocol allowed determination of the relative stability of the molecules in normal human plasma. High-performance liquid chromatography analyses of samples taken at various time points (0 h, 10 min, 20 min, 1 h, 4 h, 24 h and 48 h) demonstrated that the CMCOs remained intact over time, since similar values were obtained for CMCOs incubated in plasma or buffer as shown for CMCO1, type B (0–24 h) (Figure 2A). Additionally, the stability and functionality of the CMCOs was confirmed over a period of 2 wks using infection and integration experiments (see below, Figures 3A, B).

Enrichment of HIV-Specific CMCOs in Infected M8166 Cells and PBMCs

Following confirmation of the stability of the modified oligonucleotides in human plasma, we next investigated whether the modified, HIV-specific oligonucleotides accumulated in or on HIV-infected M8166 cells or stimulated PBMCs compared with uninfected cells. To this end, we applied different concentrations (0.8–100 µmol/L) of a nonspecific fvCMCO or HIV-specific CMCOs carrying two different modifications (type A and B) to cells for various time points (1–24 h). The sequence of the fvCMCO was shown not to be complementary to any target within the cell or the virus, as analyzed by BLAST. No treatment for cellular uptake of the CMCOs was used.

FACS analyses after 24-h incubation with the CMCOs revealed no differences in the mean fluorescence intensities between uninfected and HIV-infected cells when using the fvCMCO (Figure 2B, violet and blue triangles).

Conversely, type A (see Figure 2B, circles, dark red) and type B (see Figure 2B, squares, dark green) HIV-specific CMCOs accumulated to greater extents in or on infected cells (see Figure 2B, CMCO1) compared with uninfected cells (see Figure 2B, red circles and green squares) and

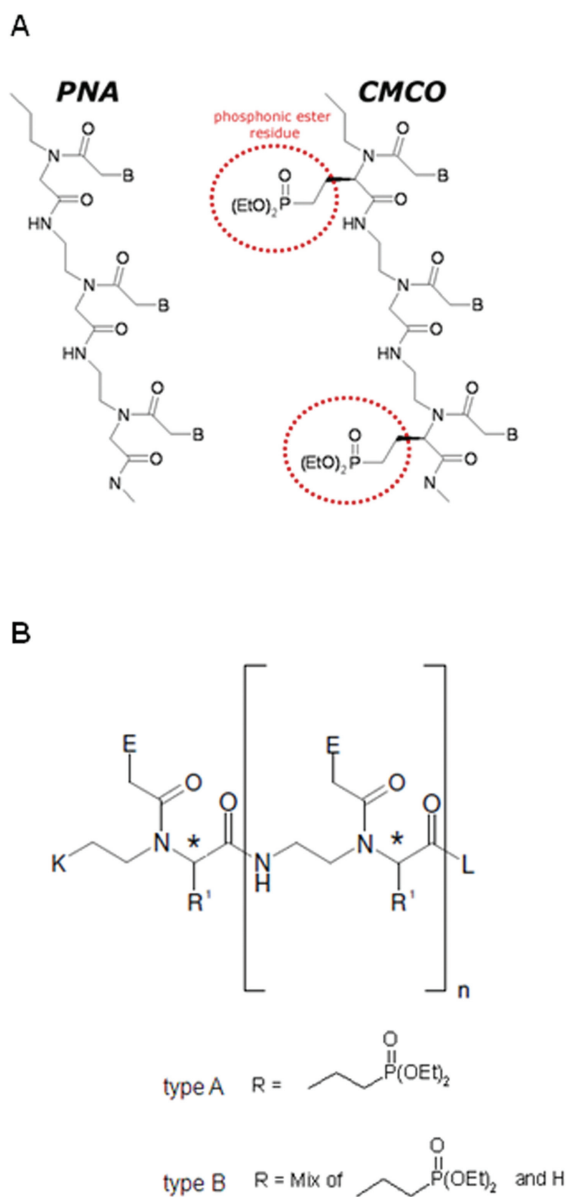


Figure 1. (A) Introducing phosphonic ester residues into a PNA backbone. Phosphonic ester residues $(\text{EtO})_2\text{-P=O}$, right, encircled red) were introduced into a PNA backbone (left) to improve the solubility of the PNAs and the penetration of the compounds through the cell membranes (CMCO). (B) Structure of type A and type B modifications of CMCOs. CMCOs are modified PNAs, where R represents a phosphonic acid (ester) residue in type A-modified oligos or a mix of hydrogen atoms and phosphonic acid (ester) functions in type B-modified oligos. These chains are attached to every base on type A CMCOs, whereas type B CMCOs are a mix of modified and nonmodified nucleosides (see Table 1). The exact modifications at L (-OH side) are demonstrated in Table 1.

compared with the non-HIV-specific FV control (see Figure 2B, violet and blue triangles).

Furthermore, the type B modification increased the cell accessibility of the

CMCO compared with the type A modification. Mean fluorescence intensities of uninfected cells were 70% to 80% lower than those of infected cells (see Figure 2B). The CMCO signal was detectable

from as early as 1 h after treatment and was still measurable 48 h after treatment, suggesting degradation of the CMCOs had not occurred (not shown).

Accumulation of HIV-Specific CMCOs Inside Cells

To exclude the possibility that the CMCOs only associate with the cell surface, confocal fluorescence microscopic analyses were performed. M8166 cells or PBMCs were incubated for 6- or 24-h time periods with various CMCO1 concentrations (100 nmol/L to 25 $\mu\text{mol/L}$). For initial experiments, we used type A- and type B-modified, fluorescently labeled, HIV-specific CMCO1s. We found that type B-modified CMCOs showed a vesicular appearance compared with the uniform distribution of type A-modified CMCOs within cells (Figure 4A, red CMCO, blue nucleus). Thus, a modification type-dependent effect on the intracellular distribution of the molecules was observed. These analyses could not identify the cellular compartment(s) in which the CMCOs became localized, and this is beyond the scope of this study. We performed analyses at low CMCO concentrations (100 nmol/L, Figure 4B, upper panel; 500 nmol/L, Figure 4B, lower panel) and incubated the cells for 6 h (see Figure 4B, upper panel) and 24 h (see Figure 4B, lower panel) with the reagents. These experiments demonstrated that the CMCOs are detectable and stable within HIV-infected cells at low concentrations (see Figure 4B, lower panel, Z-stack).

Colocalization of Specific CMCOs and HIV in Infected Cells

Next, we investigated whether the CMCOs colocalize with infectious HIV particles in infected cells. PBMCs preincubated with Lissamine-labeled CMCO1, type B, for 24 h were incubated with the virus for another 24 h. Therefore, the experiments were performed in live cells, and after 24 h, fixing of the cells using paraformaldehyde was necessary because of the infectivity of the cells. The

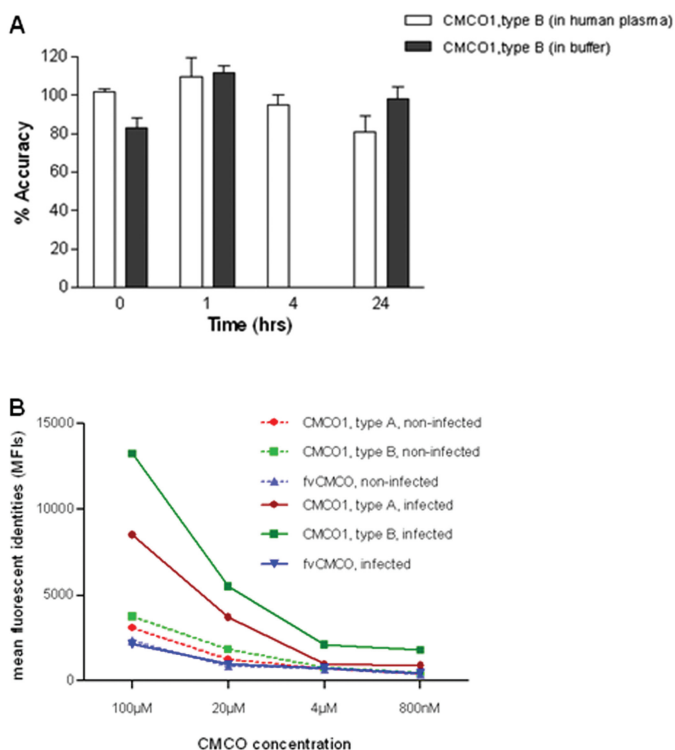


Figure 2. (A) CMCOs are stable in human plasma. The stability of the CMCOs was tested in human plasma at 37°C for 1, 4 and 24 h (A) and 48 h (not shown). Only a slight loss in stability was observed over time. In parallel experiments, the CMCOs were incubated for various times in Tris buffer as control (gray bars) and compared with plasma-incubated CMCOs (white bars). The recovery of the CMCOs is similar in plasma and buffer. A representative example out of three is given (CMCO1). (B) Type B-modified CMCOs accumulate to higher extents in HIV-infected cells compared with type A-modified and nsCMCOs. Uninfected and infected PBMCs were incubated with various concentrations (800 nmol/L to 100 μmol/L) of fluorescently labeled CMCO1, type A and B, or a non-specific fvCMCO, type B, and analyzed by FACS after 24 h. The mean fluorescence intensity values of uninfected and CMCO-treated PBMCs are shown. The graph shows noninfected and infected PBMCs treated with HIV-specific CMCO1, type A (circles, red and dark red) or type B (quadrants, green and dark green) and fvCMCO (triangles violet and blue). This experiment was repeated four times using PBMCs from different donors. Because of the donor-specific differences, a representative example is given.

HIV-1-specific CMCO1, type B (Figure 4C, red), colocalized with the virus (see Figure 4C, green, colocalization yellow) near the membrane, where virus assembly takes place. The exact compartment of colocalization has yet to be determined. A strong colocalization of HIV and CMCO was detectable at the membrane interface of an infected cell during contact with other cells, suggesting HIV and CMCO colocalization at “virological” synapses during viral transfer (Supplementary Figure 1).

CMCOs Traverse the Plasma Membrane by Diffusion or Non-Receptor-Mediated Endocytosis

To elucidate the mechanism by which the modified oligomers cross the cell membranes, we incubated primary cells with a type B-modified HIV-specific (Figure 4D) or non-specific fvCMCO (Figure 4E) overnight at 4°C. Subsequent to the incubation period, cells were fixed with 3% paraformaldehyde to ensure no internalization occurs during the adherence period of cells to the

slides. Cells were additionally stained with the nuclear dye DRAQ5 (see Figures 4D, E, blue) and the cell surface was located using a fluorescently labeled, antihuman MHC class I antibody (see Figure 4D, green). Confocal analyses of Z-stacks revealed that, at 4°C, the CMCOs were also solely intracellular, thereby traversing the cell membrane not by receptor-mediated uptake, but by diffusion or non-receptor-mediated endocytosis. Enlargements of two representative sections of a series through a cell that was incubated with the non-specific fvCMCO (see Table 1) are given in Figure 4E (red, CMCO; blue, nucleus).

Inhibition of HIV-1 Infection by Specific CMCOs

After verification by confocal microscopic analyses that the CMCOs are enriched inside cells, we next performed infection assays. These were intended to show that the CMCOs are not only self-transfecting, but are also able to inhibit infection at similar levels to the protease inhibitor IDV. We used two different approaches to determine the effectiveness of the CMCOs: (a) cells were preincubated with different concentrations of CMCOs (1.5625–25 μmol/L) before addition of HIV NL4-3 (single addition) (Supplementary Figures 2A, B, dark gray bars); and (b) the cells were preincubated with different concentrations of CMCOs (1.5625–25 μmol/L) before addition of NL4-3, and CMCOs were added every 48 h thereafter (repeated addition) (Supplementary Figure 2A, light gray bars). All experiments were repeated at least three times.

Single addition. CMCO1, CMCO2, nsCMCO (GFP) or fvCMCO were added to either M8166 cells (not shown) or PBMCs (Figure 4A and Supplementary Figure 2C) at concentrations of 1.5625, 2.5, 3.215, 6.25, 12.5 and 25 μmol/L before infection with NL4-3. Compared to cells pretreated with nsCMCO (6.25 μmol/L) or fvCMCO (2.5 μmol/L), a highly significant ($P < 0.0001$ [nsCMCO, Figure 3A] or $P = 0.0024$

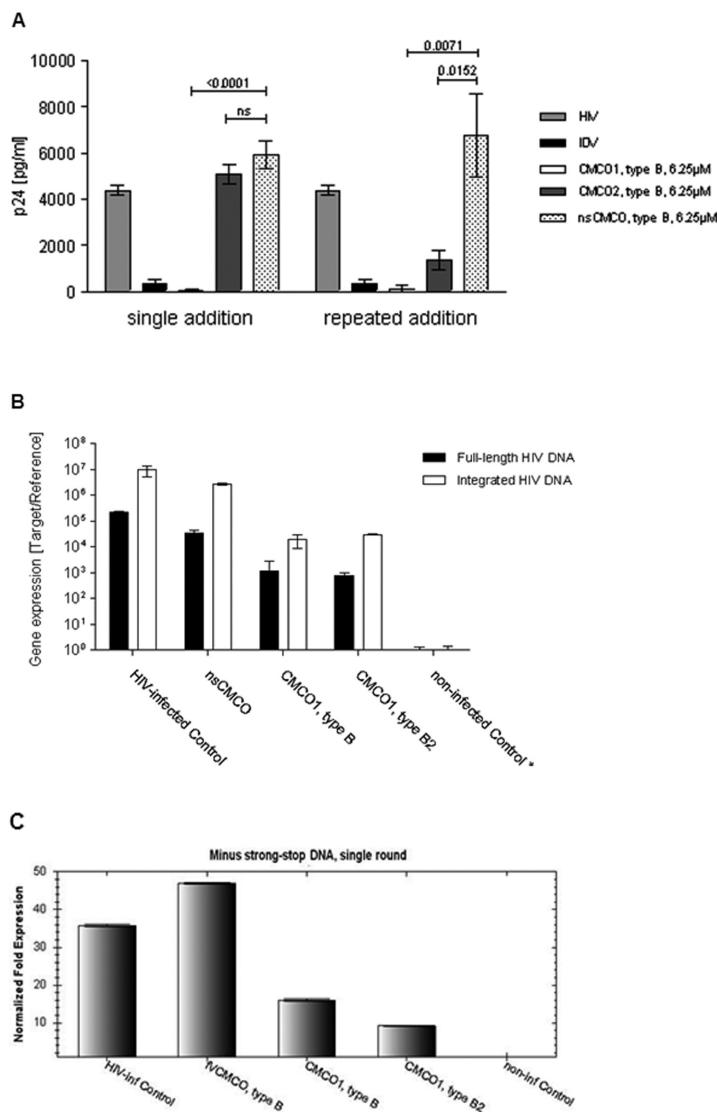


Figure 3. HIV-specific CMCOs, type B, significantly block HIV infection in stimulated PBMCs. (A) PBMCs (1×10^5 /condition) were preincubated once (left panel) using $6.25 \mu\text{mol/L}$ of HIV-specific (CMCO1 and CMCO2) and nsCMCOs for 24 h at 37°C before addition of HIV NL4-3 (500 pg p24/mL). The right panel depicts p24 production in cultures repeatedly treated with the compounds, as described in Results. Light gray bars show cells infected with HIV only; black bars IDV (2.5 nmol/L); white bars CMCO1, type B; dark gray bars CMCO2, type B; and dotted bars nsCMCO, type B, pretreated and infected cells. The infection experiments were repeated five times in triplicates and the graph represents mean values ($\pm\text{SD}$) from triplicates of a representative experiment. ns, nonsignificant (left panel). (B) DNA from single and repeatedly CMCO-pretreated and HIV-infected PBMCs (1×10^5) was extracted after infection and monitored for GAPDH, minus-strong-stop, full-length and integrated HIV DNA using TaqMan probes. The amount of target genes (full-length, integrated HIV DNA) was relatively quantified to the reference gene GAPDH ($2^{-\Delta\Delta\text{Ct}}$ method) at d 7. Inhibition between 2.5 and 3 logs in full-length (black bars) and integrated (white bars) HIV DNA transcripts by HIV-specific CMCOs 1, type B and B2, was detected. As negative controls for the specificity of the PCR, DNA from uninfected PBMCs was isolated. Figure 3B summarizes the results from three different donors. (C) Additionally, a single-cycle infection was performed, and minus-strong-stop HIV DNA in cells pretreated or not with different CMCOs before HIV infection was detected (gray bars). The analyses were performed three times in duplicates.

[fvCMCO, Supplementary Figure 2C]) reduction of HIV production comparable to 2.5 nmol/L IDV (see Figure 3A, dark gray bar) was observed in cells preincubated with $6.25 \mu\text{mol/L}$ CMCO1, type B (not shown) and type B2 (see Figure 3A, gray bar [CMCO1]) or with $2.5 \mu\text{mol/L}$ CMCO1, type B (Supplementary Figure 2C). CMCO2, type B, only exhibited a slight but nonsignificant reduction in p24 production compared with nsCMCO at this concentration (see Figure 3A, black [CMCO2] and white [nsCMCO] bars). At higher concentrations (12.5 and $25 \mu\text{mol/L}$), CMCO2 gained antiviral activity in both single and repeated addition experiments (Supplementary Figure 2A, dark and light gray bars). Additionally, the nonspecific CMCOs slightly reduced the productive infection of cells compared with nontreated, infected PBMCs (not shown). The inhibitory action of the effective, once-applied CMCOs was observable over 10–14 d during the duration of the infection experiment. Figure 3A depicts p24 mean values from supernatants taken on d 10 after infection, and Supplementary Figure 2C shows mean p24 values on d 3, 6 and 10 after infection from cells incubated with IDV (2.5 nmol/L); CMCO1, type B ($2.5 \mu\text{mol/L}$); or fvCMCO, type B ($2.5 \mu\text{mol/L}$). Whereas similar p24 concentrations were measured on d 3 under all conditions (HIV-infected control, IDV, CMCO1, fvCMCO), the HIV-infected control showed elevated p24 levels on d 6 after infection. On d 10 after infection, only the HIV-specific CMCO1, type B, and IDV showed the same p24 concentrations as measured on d 3, whereas a significantly higher productive infection was measured in HIV-infected cells (HIV-infected control, $P < 0.0001$) and in cells preincubated with the non-HIV-specific fvCMCO ($P = 0.0024$). The time course shown in Supplementary Figure 2C also demonstrates the lack of off-target effects of the CMCOs, since, besides IDV, only the HIV-specific CMCO strongly inhibited the productive infection of the cells during all time points.

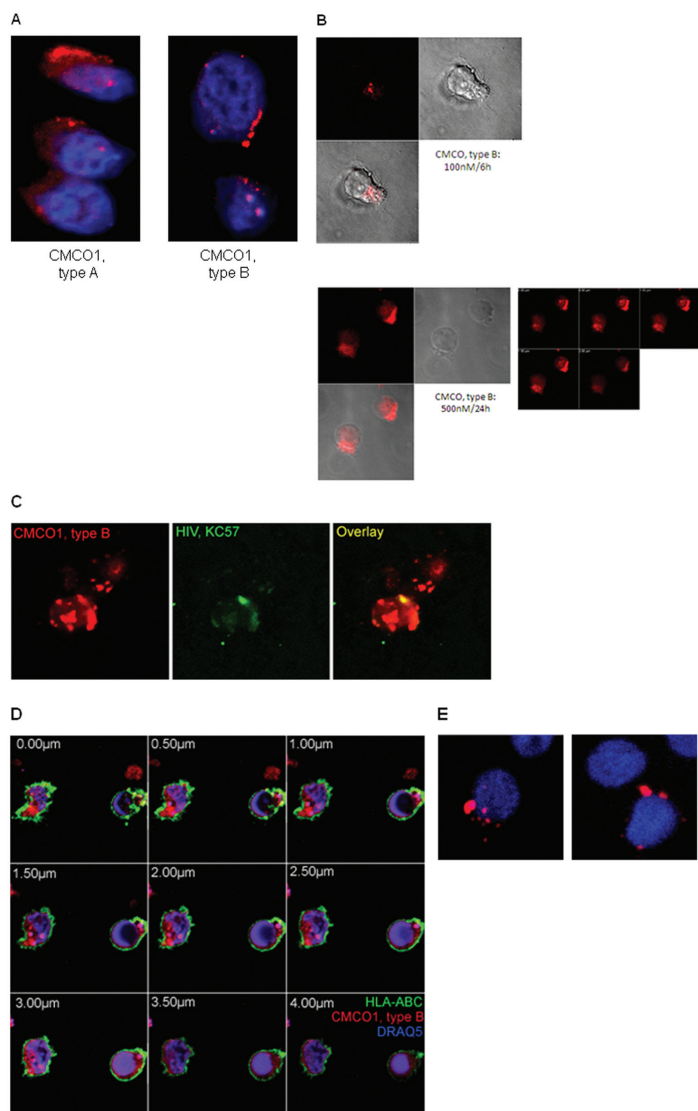


Figure 4. Differently modified CMCOs internalize into cells without any further coupling to CPPs or any other adjuvant. (A) To confirm that the CMCOs are internalized and not only attached to the cell surface, confocal fluorescence microscopic analyses of FITC-labeled type A- and type B-modified CMCOs were performed in PBMCs. For this, 5×10^4 cells/condition were incubated overnight with $25 \mu\text{mol/L}$ FITC-labeled CMCOs, type A and type B (red). After adhering on poly-L-lysine-treated glass coverslips, the nucleus was counterstained with DRAQ5 (blue). (B) Low concentrations of type B-modified, HIV-specific CMCOs (100 nmol/L (upper panel) and 500 nmol/L (lower panel)) after a 6-h (upper panel) and 24-h (lower panel) incubation period were tested. (C) PBMCs (5×10^4) were preincubated with Lissamine-labeled CMCO1, type B (red), overnight at $37^\circ\text{C}/5\% \text{ CO}_2$, and the next day, HIV (NL4-3, 100 ng p24/mL) was added for 24 h. Adhered cells were fixed and permeabilized before staining HIV using the FITC-labeled anti-HIV p24^{99g} Ab KC57. Quantifications of colocalization were performed on an average of 25 cells for each condition as previously described (26). (D) PBMCs (5×10^4 /slide) were incubated overnight at 4°C with $25 \mu\text{mol/L}$ of a Lissamine-labeled, HIV-specific CMCO1, type B (red). The cell surface was stained with a FITC-labeled mAb against human leukocyte antigen (HLA)-ABC (green). The nucleus was labeled using the dye DRAQ5 (blue). The slides were again analyzed by confocal microscopy. The gallery on Figure 2C represents a Z-stack through labeled cells. (E) An enlargement within the cells that were incubated at 4°C overnight depicts the intracellular distribution of the CMCO near the nucleus.

Repeated addition. IDV, CMCO1, CMCO2 or nsCMCO were added every 48 h after the infection of pretreated cells with HIV. Under these conditions, both HIV-specific CMCOs (1 and 2) showed an antiviral capacity similar to IDV (see Figure 3A) and a highly significant inhibition of HIV infection at concentrations of $25 \mu\text{mol/L}$, $12.5 \mu\text{mol/L}$ (Supplementary Figure 2A, light gray bars) and $6.25 \mu\text{mol/L}$ (see Figure 3A, gray and black bar). Again, the nonspecific CMCO (see Figure 3A, white bar) slightly decreased the productive infection compared with HIV-infected control cells (not shown). Figure 3A depicts p24 mean values from supernatants taken on d 10 after infection.

The CMCOs inhibited infection of M8166 cells also at lower concentrations (3.125 and $1.5625 \mu\text{mol/L}$), although these cells are susceptible to HIV infection due to high levels of the CD4 receptor and the CXCR4 coreceptor on the cell surface (Supplementary Figure 2B). The HIV inhibitory effect was similar in cells pretreated with 2.5 nmol/L IDV, $6.25 \mu\text{mol/L}$ and $3.125 \mu\text{mol/L}$ CMCO1, type B, and was still apparent upon pretreatment of the cells with $1.5625 \mu\text{mol/L}$ of the self-transfecting oligonucleotide compared with nontreated HIV-infected cells (Supplementary Figure 2B).

Impairment of HIV Provirus Formation in PBMCs by CMCO Pretreatment

To assess the mechanism underlying the observed inhibitory effect of the HIV-specific CMCOs, we explored relative HIV DNA levels using a real-time PCR assay for HIV-1-dynamics (21). This assay was designed to simultaneously measure DNA synthesis and integration efficiency of HIV-1 in untreated, CMCO-pretreated and HIV-infected PBMCs. Again, single and repeated addition of the CMCOs were tested. Uninfected PBMCs served as the control for the specificity of the PCR, and all products were quantified relative to a reference gene (GAPDH) as described (26). We analyzed CMCO- or untreated PBMCs at 7 d (Figure 3B) and 14 d (not

shown) after infection with HIV by real-time PCR for minus-strong-stop DNA (not shown), full-length HIV DNA and proviral integrated HIV DNA by a nested Alu-HIV PCR and minus-strong-stop DNA. The relative amount of minus-strong-stop HIV DNA reduction was measured by a single-cycle infection of the cells (Figure 3C).

Single addition. Compared to untreated, infected PBMCs, which served as positive controls for virus integration, the HIV-specific CMCOs 1, type B and B2, and CMCO 2, type B, reduced virus integration into the host genome up to three orders of magnitude (not shown). As expected, no signal was detectable in uninfected cells (not shown). Expression of minus-strong-stop DNA transcripts was similar in all samples independent of CMCO addition (not shown).

Repeated addition. Repeated addition of the CMCOs every 48 h after infection of CMCO-pretreated cells with HIV improved the impairment of HIV integration in the case of CMCO1, type B and B2, to a >3 log reduction (see Figure 3B, white bars). A slight reduction in HIV integration was observed in cells pretreated with nsCMCO compared with HIV-infected cells (see Figure 3B, white bar). Full-length HIV DNA was decreased about 2 logs in CMCO1, type B and B2, pretreated cells compared with HIV-infected control cells (see Figure 3B, black bars). A slight decrease in full-length transcripts was detected in cells preincubated with nsCMCO similar to the integrated transcripts (see Figure 3B, black bar). Minus-strong-stop DNA transcripts were lower by about one order of magnitude in cells repeatedly treated with the HIV-specific CMCO1, type B and B2, compared with HIV- or nsCMCO-incubated control cells (not shown).

Single-cycle infection. To detect whether the early steps of replication are affected by the compounds and to make out the differences in minus-strong-stop DNA in more detail, a single-cycle infection was performed. These analyses revealed that the HIV-specific compounds

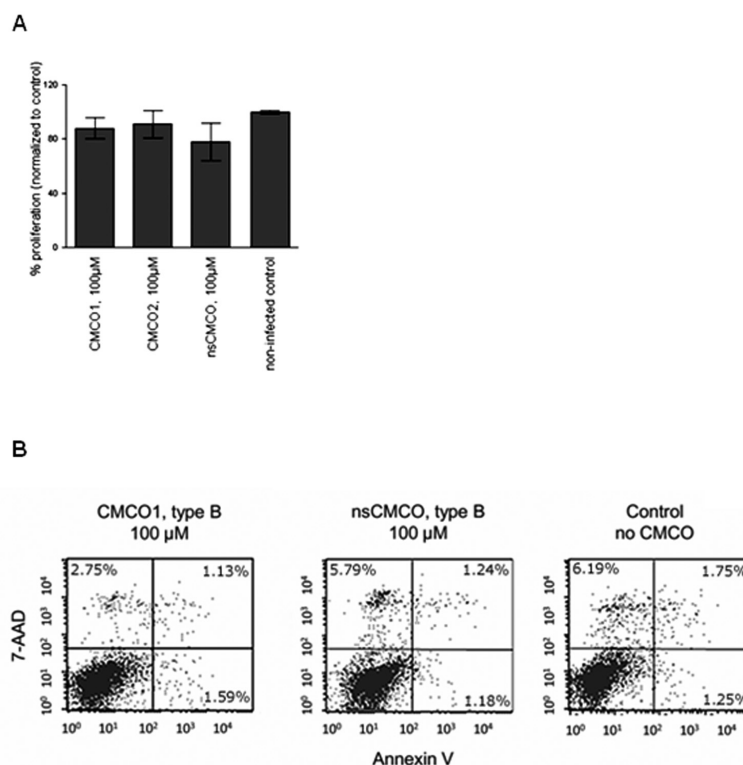


Figure 5. HIV-specific CMCOs, type B, are not impeding cell proliferation and are not cytotoxic. (A) The cell proliferation was assessed using a CellTiter96® nonradioactive cell proliferation assay (Promega) according to the manufacturer's instructions. Briefly, 50 µL M8166 cells or PBMCs (1×10^6 cells/mL) were incubated in the presence of high CMCO concentrations (100 µmol/L) and compared with untreated control cells. After 3 d, the dye solution was added and the reaction was terminated after 4 h and measured using an ELISA reader (570 nm/650 nm). The proliferation experiment was repeated three times in duplicates. (B) Untreated cells or cells treated with the non- or HIV-specific CMCOs were analyzed for dying/apoptotic (annexin V) or dead (7-AAD) cells by FACS.

CMCO1, type B and B2, significantly reduced the relative amount of the minus-strong-stop DNA in contrast to the non-HIV-specific fvCMCO, type B, and HIV-infected control cells (Figure 3C). As expected, no signal was measured using DNA from noninfected cells (see Figure 3C).

HIV-Specific CMCOs Do Not Interfere with Cell Proliferation

Subsequent to demonstrating the efficiency of the HIV-specific CMCOs, we next showed that the observed effects were not due to interference of the CMCOs with cell proliferation. We found that the HIV-specific CMCO1 and CMCO2 and the nsCMCO interfered

slightly with PBMC proliferation at a concentration of 100 µmol/L (Figure 5A), but not at lower concentrations (25 and 6.25 µmol/L; not shown).

HIV-Specific CMCOs Are Not Cytotoxic

To further exclude a possible cytotoxic effect of the CMCOs, 7-AAD and annexin V staining was performed. 7-AAD intercalates into double-stranded nucleic acids and is excluded by viable cells (staining of dead cells), and annexin V detects cells expressing phosphatidylserine on the cell surface, a feature found in apoptosis. These studies revealed that the CMCOs (non- and HIV-specific) did not exert any cytotoxic effect also at high

concentrations used (100 $\mu\text{mol/L}$) (Figure 5B). CMCO1, type B, showed 2.75% 7-AAD⁻, 1.59% annexin V⁻ and 1.13% double-positive cells (see Figure 5B). A total of 5.79% 7-AAD⁻, 1.18% annexin V⁻ and 1.24% 7-AAD/annexin V⁻-positive cells were stained in nsCMCO-treated cells; and a total of 6.19% 7-AAD⁻, 1.25% annexin V⁻ and 1.75% 7-AAD/annexin V⁻-labeled cells were found in control cells (see Figure 5B).

DISCUSSION

To date, an efficient method for delivering siRNA-like molecules *in vivo* still poses a challenge because of several problems, such as half-life of siRNA in serum, cell permeability and tissue targeting. In this study, we demonstrate for the first time the effective introduction of specifically modified inhibitory oligomers into cell lines or primary cells without any further adjuvant, such as electroporation, transfection, microinjection or coupling to CPPs, independent of the growth property of cells (7). Our CMCOs were directed against highly conserved regions in the HIV-1 genome and differently modified to allow autonomous passage via the cell membrane. In initial experiments, the CMCOs were confirmed as stable in serum and to cross cell membranes solely by diffusion or nonreceptor-mediated uptake in the absence of other external factors. Next, the HIV-directed CMCOs were found to accumulate in HIV-infected cells compared with uninfected cells and to specifically inhibit HIV infection of and integration into cells in a concentration-dependent manner and in accordance with the modification. In addition, the CMCOs did not interfere with cell proliferation and were shown to be noncytotoxic, even at the highest concentrations tested.

There is an urgent need for new anti-HIV agents that are more efficient, cheaper (thus affordable worldwide) and have fewer side effects than those currently available. Therefore, intense efforts are underway to develop a method for introducing siRNA-like molecules into primary cells *in vivo*. Furthermore, to

apply the antisense technology *in vivo*, a high serum stability and an even distribution of the agents in the body must be achieved, as well as targeted delivery (14,27). A significant improvement with respect to the stability of antisense agents was achieved by changing the oligonucleotides backbone to phosphorothioate, as shown nearly 20 years ago (28). GEM-91, a phosphorothioate, exerted high efficiency in long-term cultures up to 3 months without any viral escape mutants, but eventually failed because of nonspecific side effects and a short half-life in serum (29,30). As recently shown by Stein *et al.* (7), “gymnosis” uses the normal growth properties of the cell to take up oligonucleotides without the need for any transfection reagent or any additives to serum. In agreement with these results, our self-transfecting anti-HIV agents also exerted high stability in serum and were not degraded within lysosomal cell compartments during 48 h of incubation. Additionally, the CMCOs exerted a prolonged antiviral effect, since after 14 d in culture, no HIV replication was detectable using CMCO1. They do not depend on normal cellular growth properties for uptake, since we also observed that HIV-specific and nonspecific CMCOs are internalized into cells at 4°C. Because of comparable structural and stability properties of the CMCOs with PNAs, it can be assumed that the CMCOs also behave similarly with regard to distribution and traversing the blood-brain barrier (31). However, this result must be confirmed by *in vivo* analyses. The *in vivo* distribution of the two most promising CMCOs that were used in these experiments is the subject of ongoing studies. CMCOs directed against targets distinct to HIV have been previously applied *in vivo*, namely, targeting the gastrointestinal tract in Medaka fish and cholesterol and the genes apolipoprotein (Apo)-B100 and ApoB48 in mice. The compounds were detected after 5 d in the gastrointestinal tract of fish after a single administration, and a significant and concentration-dependent decrease of cholesterol,

ApoB100 and ApoB48 was measured in the blood of mice 4 d after application of the CMCOs (18).

Because the initial site of HIV entry into the host differs from the location of chronic infection, nontoxic and active HIV inhibitory substances that are able to reach the relevant target cells must be developed. Antisense reagents fulfill the basic requirements due to these and other properties (stability, distribution), although an improved and better-targeted delivery of the inhibitory molecules into infected cells is desirable. We have found that all of our specifically modified oligomers crossed the plasma membrane independently of their specific modification (type A or B) or their effective concentrations. CMCOs, type A, which carry phosphonic ester side chains at every nucleoside, revealed a uniform distribution within cells, as detected by confocal fluorescence microscopy. In contrast, type B- and type B2-modified CMCOs are a mix of modified and unmodified nucleosides, and these compounds were found to accumulate in vesicular intracellular compartments and near the nucleus. Intracellular localization near the nucleus also did not depend on infection of the cells, since the non-HIV-specific fvCMCO accumulated near the nucleus. Whether type B2-modified CMCOs are superior in their anti-HIV activity because of additional modulation of the methylation and acetylation site at the C terminus than type B-modified CMCOs has to be investigated *in vivo*. Uptake of fluorescently labeled CMCOs rapidly occurred in the nanomolar range, as detected by confocal microscopic analyses.

Upon performance of uptake and infection experiments, we found that CMCOs carrying the type B modification were more enriched inside HIV-infected cells and possessed superior HIV inhibitory activity in cell lines as well as primary cells compared with type A-modified CMCOs. CMCO1, type B, also significantly inhibited virus production (>30%) at low concentrations (1.5625 $\mu\text{mol/L}$) compared with nsCMCO-treated cells or HIV-infected M8166 cells. An extensive pro-

ductive infection with HIV is feasible in these cells because of the high expression of CD4 and CXCR4 on the surface. Thus, a more efficient inhibition in primary cells or *in vivo* systems can be expected using a similar or lower CMCO concentration. We also show a reduction of full-length HIV DNA transcripts and HIV integration into M8166 cells and stimulated PBMCs by preincubation of the cells with the specifically modified CMCOs. These results indicate that the anti-HIV CMCOs inhibit viral replication at more than one stage of the life cycle, since the incoming viral RNA constitutes a point of attack as well as the viral mRNA.

By our method, the tribulations of delivery of inhibitory oligonucleotides can be circumvented, since our CMCOs demonstrate that they can be transferred into cells without coupling to a cell-penetrating peptide; they therefore lay the foundation for further improvements. Additionally, targeted CMCO delivery to augment the local concentration in the desired cells or tissues by, for example, nanoparticles or antibodies (14,32) could improve the outcome of our newly designed molecules. This in turn would result in lower CMCO concentrations and enhanced delivery to HIV-infected cells. Beside optimizing the delivery problem *in vivo* using nanocarriers or antibodies, viral escape variants might also appear upon treatment with the HIV-specific CMCOs. Therefore, to bypass the emergence of viral escape from inhibitory oligomers in long-term studies, highly conserved target sites in the virus should be defined that allow the inhibition of a variety of HIV isolates. Thus, the best strategy for antisense-mediated HIV inhibition would be the use of inhibitory oligomers against multiple conserved viral sequences in combination with oligomers against host cell proteins that are responsible for viral entry, such as the HIV-1 coreceptors CCR5 or CXCR4 (33–35). Additionally, we showed that the anti-HIV-CMCOs did not exert effects on cell proliferation or cell viability. In contrast, the cytotoxicities of siRNA complexes, such as certain

polyethylenimine-based or dendrimer-siRNA complexes, present major limitations on their *in vitro* or *in vivo* use (36).

CONCLUSION

In conclusion, this study is a first step in demonstrating that the PNA backbone can be specifically modified to allow autonomous passage into the cell without an additional adjuvant. Efficient delivery of inhibitory oligomers by virtue of our modifications, in combination with novel targeting technologies, constitute an encouraging new method for inhibiting HIV replication. Our method has the following advantages: (a) easy preparation due to the absence of covalent linkage, (b) it can be adapted to any cellular target and (c) it was shown to work efficiently *in vitro*. Our CMCOs are now readily applicable to further studies, with the intention of verifying their suitability as antisense therapeutics *in vivo*.

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WO2008009470). T Lindhorst and H Bock are owners in ugichem GmbH.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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