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Albumin-conjugated C34 Peptide HIV-1 Fusion Inhibitor

*EQUIPOTENT TO C34 AND T-20 IN VITRO WITH SUSTAINED ACTIVITY IN SCID-HU THY/LIV MICE**

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Entry inhibitors of human immunodeficiency virus, type 1 (HIV-1) have been the focus of much recent research. C34, a potent fusion inhibitor derived from the HR2 region of gp41, was engineered into a 1:1 human serum albumin conjugate through stable covalent attachment of a maleimido-C34 analog onto cysteine 34 of albumin. This bioconjugate, PC-1505, was designed to require less frequent dosing and less peptide than T-20 and was assessed for its antifusogenic activity both *in vitro* and *in vivo* in the SCID-hu Thy/Liv mouse model. PC-1505 was essentially equipotent to the original C34 peptide and to T-20 *in vitro*. In HIV-1-infected SCID-hu Thy/Liv mice, T-20 lost activity with infrequent dosing, whereas the antiviral potency of PC-1505 was sustained, and PC-1505 was active against T-20-resistant ("DIV") virus with a G36D substitution in gp41. The *in vivo* results are the direct result of a significantly improved pharmacokinetic profile for the C34 peptide following albumin conjugation. Contrary to previous reports that the gp41 NHR trimer is poorly accessible to C34 fused to protein cargoes of increasing size (Hamburger, A. E., Kim, S., Welch, B. D., and Kay, M. S. (2005) *J. Biol. Chem.* 280, 12567–12572), these results are the first demonstration of the capacity for a large, endogenous serum protein to gain unobstructed access to the transient gp41 intermediates that exist during the HIV fusion process, and it supports further development of albumin conjugation as a promising approach to inhibit HIV-1 entry.

Entry of human immunodeficiency virus type 1 (HIV-1)[3] into uninfected cells encompasses three main steps: the binding of gp120 to the CD4 receptor, the subsequent binding to coreceptor CXCR4 or CCR5, followed by the conformational changes of the ectodomain of HIV-1 gp41 critical to membrane fusion that ultimately permits the infection process. Several small molecule drug candidates, including those that inhibit binding to CD4 or to the CCR5 coreceptor, are either in human clinical trials or are close to market approval (1, 2). However, T-20 (DP-178, enfuvirtide, Fuzeon, Trimeris; Roche Applied Sciences), a synthetic peptide based on the C-terminal helical region (CHR) sequence of HIV-1 gp41, remains the only compound marketed to date that targets the conformational rearrangements of gp41. It had been widely believed that T-20 inhibition was due to its ability to bind to the hydrophobic grooves of the N-terminal helical region (NHR) of gp41, resulting in the inhibition of six-helix bundle formation (3). Despite the identification of less common escape mutants against T-20 with mutations in the NHR of gp41 (4, 5), recent studies suggest that T-20 is capable of targeting multiple sites in gp41 and gp120 (6–8). In contrast, another C-peptide, C34, composed of a peptide sequence that overlaps with T-20 but contains the gp41 coiled-coil cavity binding residues, ^{628}WMEW^{631}, is known to compete with the CHR of gp41 for the hydrophobic grooves of the NHR yet is incapable of functioning at a post-lipid mixing stage (6). Despite the successes of T-20, its commercial utility has been somewhat restricted to salvage therapy (9, 10). The challenge in developing therapeutic peptides is complicated primarily by their rapid renal clearance, poor distribution, and susceptibility to peptidase degradation. Despite recent predictions that cross-linking C-peptide inhibitors to larger proteins will likely reduce their antiviral activity (11), we used albumin conjugation as a vehicle to achieve superior pharmacokinetic profiles of C34 peptide as has been performed with other classes of maleimido peptides (12–17). Such conjugation reactions may be performed *in vivo* by administering the compound directly into the human patient followed by conjugation to endogenous serum albumin. Similarly, conjugation reactions

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[3] The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; gp, glycoprotein; CHR, C-terminal helical region; NHR, N-terminal helical region; PBMC, peripheral blood mononuclear cells; HSA, human serum albumin; rHA, recombinant human albumin; AEEA, aminoethoxy ethoxy acetic acid; maleimido-1505, C34 peptide modified with maleimide proprionic acid-AEEA at N-terminal Trp1; PC-1505, preformed HSA-conjugate of maleimido-1505; DMF, *N*,*N*-dimethylformamide; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HPLC, high pressure liquid chromatography.

may also be carried out *in vitro* by reacting the maleimido peptide with albumin prior to administering the bioconjugate to a subject. In this study, the C34 peptide derived from the CHR of gp41 (18, 19) was engineered into preformed albumin conjugates whereby specific covalent linkage to albumin was carried out through either the N terminus or the C terminus of the fusion inhibitor. Similarly, preformed albumin conjugates composed of maleimido-T-20 analogs were also generated. Each drug construct represented a 1:1 complex through specific and stable covalent attachment of the peptide to cysteine 34 of albumin, and each construct was assessed for its antiviral activity *in vitro* following infection in a peripheral blood mononuclear cell (PBMC)-based assay with the HIV-1 strain III_B (20–22). Furthermore, using the SCID-hu Thy/Liv mouse model of HIV-1 infection (23, 24), we evaluated the antiviral activity of one C34 human serum albumin (HSA) conjugate, PC-1505 and found that although T-20 lost activity with infrequent dosing, the antiviral potency of PC-1505 was sustained.

EXPERIMENTAL PROCEDURES

Peptide Synthesis, Purification, and Characterization—Chemicals were purchased from commercial suppliers and were used as received. The synthesis of all derivatives was performed using an automated solid phase procedure on a Symphony peptide synthesizer with manual intervention during the generation of the drug affinity complex moiety, which is the combination of a maleimide proprionic acid, a linker, and the peptide. The synthesis was performed on Fmoc-protected Ramage amide linker resin using Fmoc-protected amino acids methodology. Coupling of the amino acids was achieved by using *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate as activator in *N*,*N*-dimethylformamide (DMF) solution and diisopropylethylamine as base. The Fmoc protective group was removed using 20% piperidine/DMF. When needed, a Boc-protected amino acid was used at the N terminus to generate the free N_α terminus after the peptide is cleaved from resin. All amino acids used during the synthesis possess the L-stereochemistry. When needed, the selective deprotection of the Lys (Aloc) group was performed manually and accomplished by treating the resin with a solution of 3 eq of $Pd(PPh_3)_4$ dissolved in 5 ml of C_6H_6 $CHCl_3$ (1:1), 2.5% *N*-methyl morpholine (v/v), 5% acetic acid (v/v) for 2 h. The resin was then washed with $CHCl_3$ (6 × 5 ml), 20% acetic acid in dichloromethane (6 × 5 ml), dichloromethane (6 × 5 ml), and DMF (6 × 5 ml). When needed, the synthesis was reautomated for the addition of the Fmoc-AEEA-acetic acid followed by the 3-maleimidopropionic acid. Between every coupling, the resin was washed three times with DMF and three times with isopropanol. The different peptides were cleaved from the resin using 85% trifluoroacetic acid, 5% triisopropyl-silane, 5% thioanisole, and 5% phenol, followed by precipitation by dry ice-cold diethyl ether prior to purification.

The peptide crude products were purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system: gradient elution of 32–42% solvent B in solvent A (0.045% trifluoroacetic acid in H_2O; solvent A and 0.045% trifluoroacetic acid in CH_3CN; solvent B) over 180 min at 9.5 ml/min using a Phenomenex Luna 10-μ phenyl-hexyl, 21-mm × 250-mm column and UV detector (Varian Dynamax UVD II) at 214 and 254 nm resulting in peptides (native and drug affinity complex) of >95% purity, as determined by reverse phase HPLC. Purified peptides were analyzed by reverse phase HPLC with gradient elution of 20–60% solvent B in solvent A over 20 min at 1.0 ml/min using a Luna 10-μ phenyl-hexyl, 4.6-mm × 250-mm column and UV detector at 214 and 254 nm. In addition, the mass of the purified peptides was measured by an Agilent 1100 series (liquid chromatography/electron impact-mass spectrometer with a gradient elution of 20–60% solvent B in solvent A over 30 min at 1.0 ml/min using a Luna 10-μ phenyl-hexyl, 4.6-mm × 250-mm column and UV detector at 214 nm followed by mass detection on the 1100 series electron impact-mass spectrometer.

Preparation of Albumin Conjugates—The conjugation of maleimido-C34 and maleimido-T-20 derivatives to cysteine 34 of HSA and subsequent purification were performed as reported previously (13–17). Mass spectrometry of each purified sample confirmed the most abundant protein product corresponded to a 1:1 covalent complex of HSA with each maleimido derivative, and reverse phase HPLC analysis of each purified sample confirmed the removal of essentially all unbound (free) maleimido derivative.

Viruses—The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health: $HTLV\text{-}III_B$/H9 from Dr. Robert Gallo (20–22) and pNL4-3 from Dr. Malcolm Martin (25). pNL4-3 from the AIDS Reagent Program contains an unexpected variant DIV (G36D) mutation in gp41, which confers 8-fold resistance to T-20 *in vitro* (26). A T-20-sensitive NL4-3 D36G (NL4-3G) was altered by site-directed mutagenesis to match the consensus sequence at amino acid position 36 (aspartic acid replaced by glycine) of gp41. Stocks of NL4-3G and NL4-3D (original clone) were prepared by transfection of 293T cells and collection of supernatants on day 3. The virus stocks were titrated by 50% end point assay in phytohemagglutinin-activated PBMC with p24 detection by enzyme-linked immunosorbent assay.

Anti-HIV Efficacy Evaluation in Fresh Human PBMC—Antiviral activity and cytotoxicity were assayed in fresh human PBMC infected with HIV-1 IIIB as previously described (27) for determination of IC_{50} (50% inhibition of virus replication), IC_{90} (90% inhibition of virus replication), TC_{50} (50% reduction in cell viability), and selectivity index (IC_{50}/TC_{50}). The data are expressed as the means of three independent assays.

Pharmacokinetic Profiles of C34 Peptide, PC-1505, and Recombinant Human Albumin (rHA) in Rats—Male Sprague-Dawley rats ($n = 4$) were given a single injection of C34 peptide, PC-1505, or rHA either at 100 nmol/kg intravenously or at 500 nmol/kg subcutaneously. Blood samples were collected before injection and up to 96 h after dosing, and plasma samples were analyzed by three different sandwich enzyme-linked immunosorbent assays. C34 peptide was measured using a pair of rabbit polyclonal antibodies directed against each end of the peptide. PC-1505 was captured via the albumin portion using a goat anti-HSA antibody and detected with a rabbit polyclonal antibody specific to the C terminus of the C34 peptide. Human albumin concentrations were determined using a commercial

TABLE 1

Structure and antiviral activity of HIV-1 entry inhibitors

Peptides were synthesized using an automated solid phase procedure and purified by HPLC as explained under "Experimental Procedures." The HIV peptides are numbered as appropriate for HIV-1 HXB2. The letters stand for the one-letter amino acid code unless otherwise designated.

Compound	Structure	Activity against HIV-1 III_B in human PBMCs		
		IC_{50} (nM)	IC_{90} (nM)	Selectivity index
HSA[a]	Human serum albumin	NA[g]	NA	NA
C34	(628)WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL(661)-$CONH_2$[b]	0.6	2.8	>255
A (maleimido-1505)	MPA[c]-AEEA[d]-(628)WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL(661)-$CONH_2$[b]	NP[h]	NP	NP
B (PC-1505)	[HSA[a]-Cys34[e]]-MPA[c]-AEEA[d]-(628)WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL(661)-$CONH_2$[b]	1.8	13.5	>81.5
C	[HSA[a]-Cys34[e]]-MPA[c]-(628)WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL(661)-$CONH_2$[b]	2.1	17	>70
D	(628)WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL(661)K(εN)-AEEA[d]-MPA[c]-[Cys34[e]-HSA[a]]	11.2	30.2	>22.4
T-20	Ac[f]-(638)YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF(673)-$CONH_2$[b]	2.2	9.5	>109
E	[HSA[a]-Cys34[e]]-MPA[c]-AEEA[d]-(638)YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF(673)-$CONH_2$[b]	10.7	31.7	>23.4
F	Ac[f]-(638)YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF(673)K(εN)-AEEA[d]-MPA[c]-[Cys34[e]-HSA[a]]	87.0	>2,000	>23.0

[a] HSA, human serum albumin.
[b] $CONH_2$, carboxamide.
[c] MPA, maleimide proprionic acid.
[d] AEEA, amino ethyl ethoxy acetic acid.
[e] Cys34, cysteine 34 of albumin.
[f] Ac, acetyl.
[g] NA, no activity.
[h] NP, not performed.

kit using polyclonal goat anti-HSA antibodies (Bethyl Labs E80–129).

SCID-hu Thy/Liv Mice—Coimplantation of thymus and liver fragments under the kidney capsule to create SCID-hu Thy/Liv mice and inoculation of the Thy/Liv implants with HIV-1 was carried out as described (23, 24), and cohorts of 50–60 SCID-hu Thy/Liv mice each were implanted with tissues from a single donor. PC-1505 was prepared in 8 mM sodium octanoate in 1.5% polysorbate 80, and maleimido-1505 was prepared in 20 mM sodium phosphate buffer (pH 7.5). T-20 (Fuzeon; obtained from the San Francisco General Hospital pharmacy) was dissolved in sterile water for injection and adjusted to pH 7 with HCl. The drugs were administered to mice ($n = 5–7$) by subcutaneous injection at the indicated dosage levels (peptide alone excluding albumin for PC-1505) beginning 24 h before direct injection of 1,000 $TCID_{50}$ HIV-1 NL4-3G, NL4-3D, or RPMI 1640 (mock infection) into each Thy/Liv implant. Implants were collected 21 days after inoculation and dispersed into single-cell suspensions for quantification of p24 by enzyme-linked immunosorbent assay and viral RNA by branched DNA assay and were stained with antibodies to CD3, CD4, and CD8 for analysis of T-cell subsets by flow cytometry as described previously (23, 24, 28). The limit of detection of HIV-1 RNA by the bDNA assay was 1.5 $\log_{10}$ copies/10^6 implant cells. The animal protocols were approved by the University of California, San Francisco Institutional Animal Care and Use Committee.

RESULTS

Antiviral Activities In Vitro Using PBMC-based Assays—The antiviral activity of each albumin conjugate was compared with the original peptide inhibitors *in vitro* using a PBMC-based assay against HIV-1 III_B. Interestingly, the antiviral activity of PC-1505 (compound B), compound C, and compound D were all found to be essentially equipotent to C34 peptide and T-20 *in vitro* (Table 1). Following albumin conjugation to T-20, there was excellent retention of antiviral activity when the reactive peptide is designed so that conjugation occurs at the N-terminal end of the peptide (compound E), whereas a significant decrease in the antiviral activity was observed when conjugated at the C-terminal end of the peptide (compound F).

Pharmacokinetic Profiles of C34 Peptide, PC-1505, and rHA in Rats—To ensure that the antiviral activities observed in this study resulted from the action of the albumin conjugates rather than to the free peptide or to the reversibility of the covalent bond between maleimide and cysteine 34, all of the albumin conjugates were purified to remove any unbound peptide before testing, and the pharmacokinetic profile of PC-1505 was compared with C34 peptide and rHA in rats (Fig. 1). Clearly, exposure of C34 peptide is improved dramatically following albumin conjugation, and the virtually identical pharmacokinetic profiles of PC-1505 and rHA confirm that the conjugated C34 peptide has a half-life closer to that of albumin. Superimposition of pharmacokinetic curves measuring C34 peptide and HSA have also been observed in BALB/c mice for at least 30 h following either intravenous or subcutaneous administration of PC-1505 (data not shown; $T_{½}$ of albumin is shorter in mice than in rats). Conversely, a slow and continuous release of C34 peptide from the conjugate would cause the two pharmacokinetic profiles to no longer superimpose because the total exposure of PC-1505 would be inferior to that of rHA. Furthermore, C34 peptide released from the conjugate would be subject to a very short half-life *in vivo* with limited antiviral effectiveness as compared with the long lasting PC-1505. Hence, the bond linking maleimide to cysteine 34 is highly stable *in vivo*, and C34 peptide is rendered more stable against rapid renal clearance and

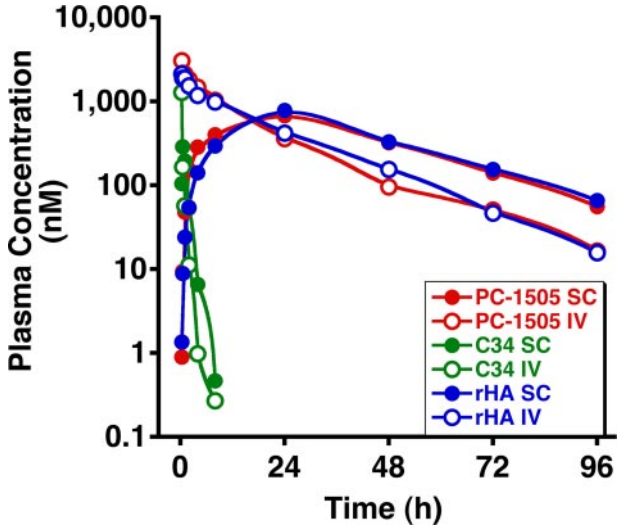

FIGURE 1. **Pharmacokinetic curves of PC-1505, C34 peptide, and rHA following either subcutaneous (*SC*) or intravenous (*IV*) administration to Sprague-Dawley rats.** The superimposition of the PC-1505 and rHA curves provides definitive supporting evidence for the stability of the chemical bond linking maleimido-1505 to cysteine 34 of human serum albumin as well as the stability of PC-1505 against renal clearance and peptidase degradation.

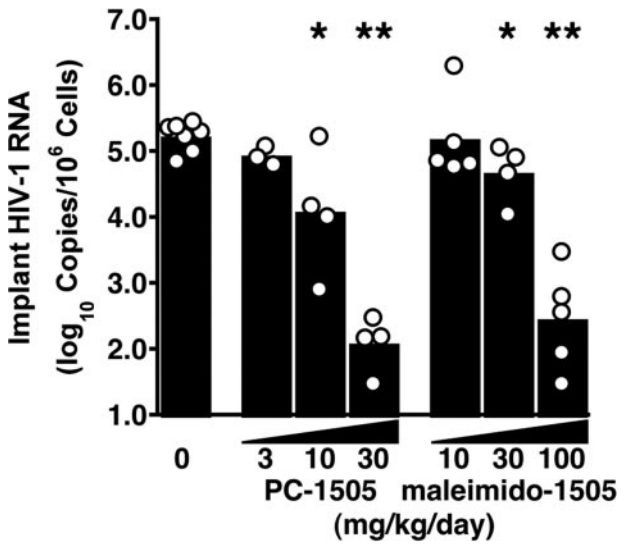

FIGURE 2. **PC-1505 is three times more potent than maleimido-1505 in reducing viral RNA in SCID-hu Thy/Liv mice.** Mice were treated twice daily by subcutaneous injection beginning 1 day before inoculation of Thy/Liv implants with 1,000 $TCID_{50}$ HIV-1 NL4-3G. The *columns* represent means, and the *open circles* represent individual animals from the same cohort 21 days after inoculation. *, $p < 0.05$; **, $p < 0.01$ compared with untreated mice (0 mg/kg/day) by the Mann-Whitney *U* test.

against peptidase degradation. Taken together, it may be concluded the antiviral activities for all of the albumin conjugates *in vitro* and *in vivo* are solely the result of the action of chemically stable conjugates rather than of reversibility of the maleimide-cysteine 34 bond.

Antiviral Activity in SCID-hu Thy/Liv Mice—To determine whether albumin conjugation could improve the antiviral potency of C34 peptide *in vivo*, we employed the SCID-hu Thy/Liv mouse model of HIV-1 infection. The human thymus implant in these mice supports long term differentiation of human T cells, and the model has been standardized (23) and validated with four classes of licensed antiretrovirals (24) for the preclinical evaluation of antiviral compounds against HIV-1. Importantly, new classes of agents such as the CCR5 antagonist SCH-C (the predecessor of vicriviroc) (29) and the HIV-1 maturation inhibitor bevirimat (30) have shown potent dose-dependent antiviral activity in these mice after oral administration.

The activity of the albumin conjugate, PC-1505, was compared with the unconjugated peptide, maleimido-1505, which forms a conjugate with mouse albumin *in vivo* after subcutaneous administration. When PC-1505 was compared head-to-head to maleimido-1505 in SCID-hu Thy/Liv mice treated twice daily beginning 24 h before inoculation with NL4-3G, PC-1505 was approximately three times more potent than maleimido-1505 in reducing HIV-1 RNA in the implants (Fig. 2). Specifically, viral RNA was reduced by 3.1 log_{10} with 30 mg/kg/day PC-1505 and by 2.7 log_{10} with 100 mg/kg/day maleimido-1505.

When PC-1505 and T-20 were compared in mice treated twice daily beginning 24 h before inoculation with NL4-3G, the two drugs had comparable dose-dependent activity at 1, 3, and 10 mg/kg/day. Treated mice had statistically significant reductions in viral RNA, p24, Gag-$p24^+$ thymocytes, and interferon-α-induced MHC-I expression on DP thymocytes compared with untreated mice (Fig. 3*A*). Reductions in viral load were accompanied by virtually complete protection of the Thy/Liv implants from thymocyte depletion, in terms of total cellularity, thymocyte viability, percentage of immature cortical $CD4^+CD8^+$ (double-positive, DP) thymocytes, and CD4/CD8 ratio (Fig. 3*B*). In a third SCID-hu study to compare the activity of PC-1505 and T-20 against T-20-resistant NL4-3D, 10 mg/kg/day of PC-1505 reduced viral RNA by 2 log_{10} more than did T-20 at 100 mg/kg/day (Fig. 4*A*). When the data for viral RNA reductions in seven independent SCID-hu mouse experiments are analyzed together, it is clear that antiviral potency against T-20-resistant NL4-3D was maintained for PC-1505 (Fig. 4*B*).

Finally, to determine whether the superior pharmacokinetics of PC-1505 would require less frequent dosing compared with T-20, we compared the activities of both drugs following dosing every fourth (Q4D) or seventh (Q7D) day and beginning 1 day before or 3 days after inoculation (Fig. 5). Contrary to T-20, there was excellent conservation of antiviral activity for PC-1505 with infrequent dosing and with initiation of dosing delayed until 3 days after exposure. With the Q4D and Q7D dosing regimens, T-20 caused no significant reduction in HIV-1 RNA or p24, nor did it protect from thymocyte depletion. Finally, following a single elevated pre-exposure dose of each drug 1 day before NL4-3G inoculation (Fig. 6), only PC-1505 was highly effective at reducing viral RNA by nearly 3 log_{10} and p24 to almost undetectable levels as well as complete protection of immature human T cells from virus-mediated depletion.

DISCUSSION

The NHR trimer of gp41 remains an attractive target for drug discovery (31–34). Synthetic peptides based upon NHR and CHR sequences of HIV gp41 have been shown to inhibit HIV

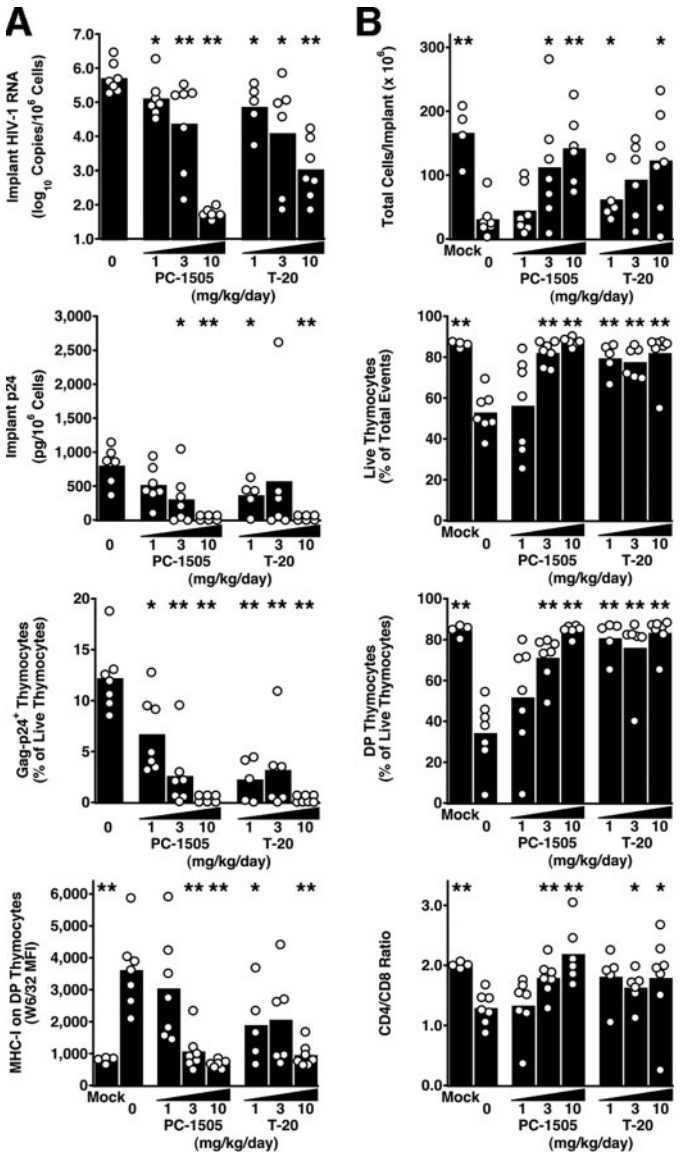

FIGURE 3. **PC-1505 and T-20 are equipotent against NL4-3G when administered twice daily.** SCID-hu Thy/Liv mice were treated by subcutaneous injection beginning 1 day before inoculation. *A*, antiviral efficacy was assessed by determining HIV-1 RNA, p24, percentage of Gag-p24$^+$ thymocytes, and MHC-I expression on DP thymocytes. *B*, thymocyte protection was assessed by total implant cellularity, thymocyte viability, percentage of DP thymocytes, and CD4/CD8 ratio for treated *versus* untreated mice. The *columns* represent the means, and the *open circles* represent individual animals from the same cohort 21 days after virus (or mock) inoculation. *, $p < 0.05$; **, $p < 0.01$ by the Mann-Whitney *U* test.

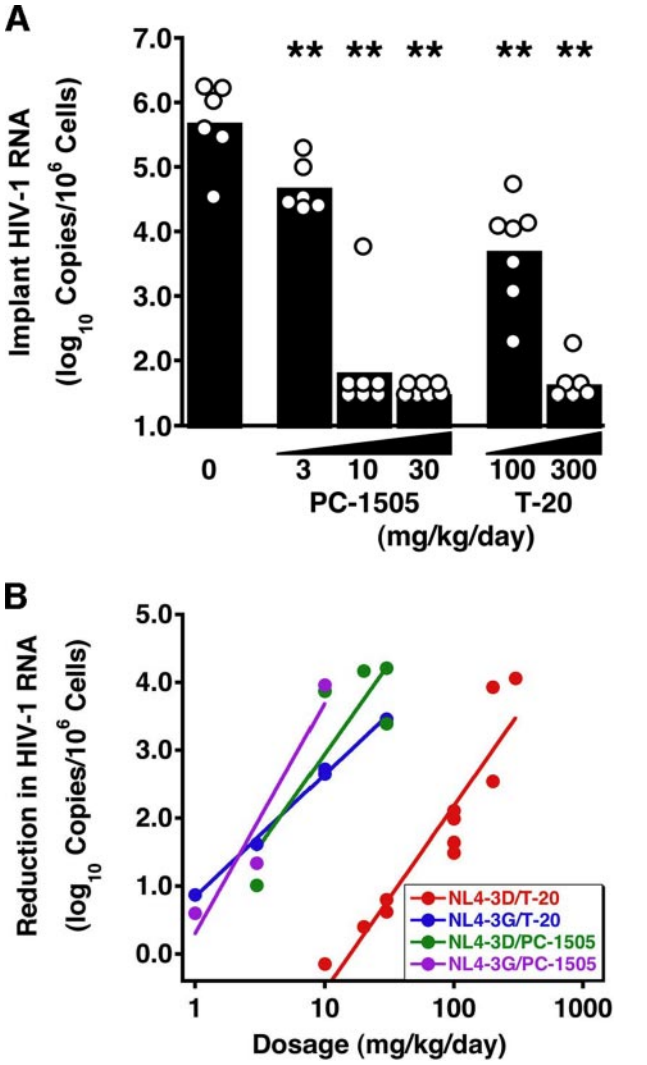

FIGURE 4. **PC-1505 is >10 times more potent than T-20 against T-20-resistant NL4-3D.** SCID-hu Thy/Liv mice were treated twice daily by subcutaneous injection beginning 24 h before inoculation. *A*, viral RNA means (*columns*) and individual animals (*open circles*) from one cohort 21 days after inoculation. **, $p < 0.01$ by the Mann-Whitney *U* test. *B*, reduction in viral RNA by treatment with PC-1505 or T-20 in NL4-3G- and NL4-3D-infected mice. Each point represents the $\log_{10}$ difference in means between treated and untreated groups (5–7 mice/group) in seven separate experiments with a total of 202 mice.

entry by competing for exposed gp41-binding sites during the multistep fusion process (18, 19). In the clinic, the most successful of these peptides is T-20 derived from the CHR of gp41. As compared with small molecules, the commercial utility of peptides is often limited by their high cost as well as their short half-lives and poor distribution *in vivo*. In this study, we sought to address these shortcomings by engineering CHR peptides (C34 and T-20) to bond covalently to cysteine 34 of human albumin. Given the steric block associated with the NHR-trimer region of gp41 (11) and given the current dearth of neutralizing antibodies against this target, it was impossible to predict whether such conjugates could access the hydrophobic grooves of the NHR of gp41 that are exposed only transiently once the viral and target membranes are in close contact (35), *i.e.* within the contact zone referred to as the "entry claw" (36).

The results reported here are surprising for several reasons. First, our results suggest that NHR of gp41 is more accessible than originally postulated. Therefore, with a molecular mass of ~71 kDa for PC-1505, our results suggest the molecular mass cut-off for accessing the NHR-trimer of gp41 is much greater than previously reported, *i.e.* <25 kDa (11). Second, the N-terminal segment of the C34 peptide, ^{628}WMEW^{631}, represents the gp41 coiled-coil cavity binding residues postulated to be essential for the ability of C34 peptide to inhibit HIV-1 entry (18, 19). Therefore, in the case of either PC-1505 (composed of aminoethoxy ethoxy acetic acid (AEEA linker)) or compound C (absence of AEEA linker), one possible explanation for the retention of antiviral activity for these two compounds is the

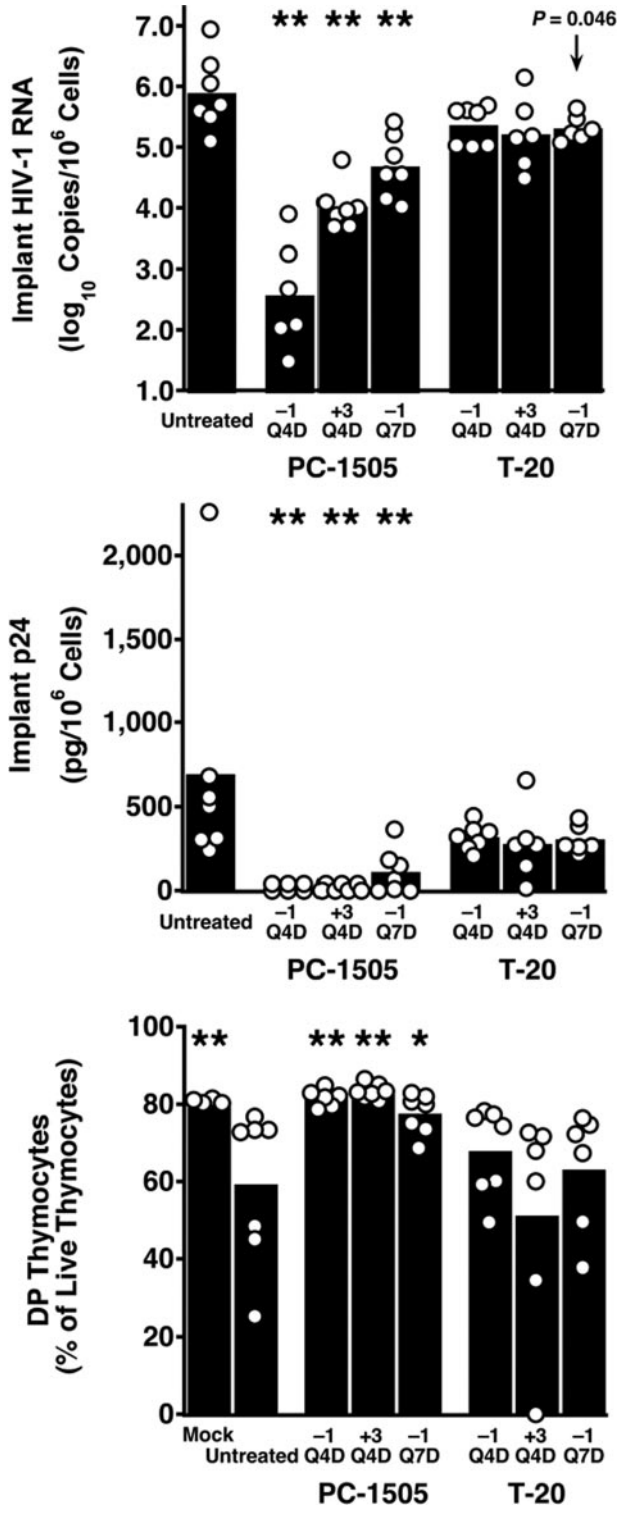

FIGURE 5. **PC-1505 has more sustained activity than T-20 against NL4-3G when administered every fourth or seventh day.** SCID-hu Thy/Liv mice were treated by subcutaneous injection of 30 mg/kg beginning 1 day before (−1) or 3 days after (+3) inoculation and continued every fourth day (*Q4D*) or every seventh day (*Q7D*) until implant collection. Antiviral efficacy was assessed by determining HIV-1 RNA and p24, and thymocyte protection was assessed by percentage of DP thymocytes. The *columns* represent the means, and the *open circles* represent individual animals from the same cohort 21 days after virus (or mock) inoculation. *, $p < 0.05$; **, $p < 0.01$ by the Mann-Whitney *U* test.

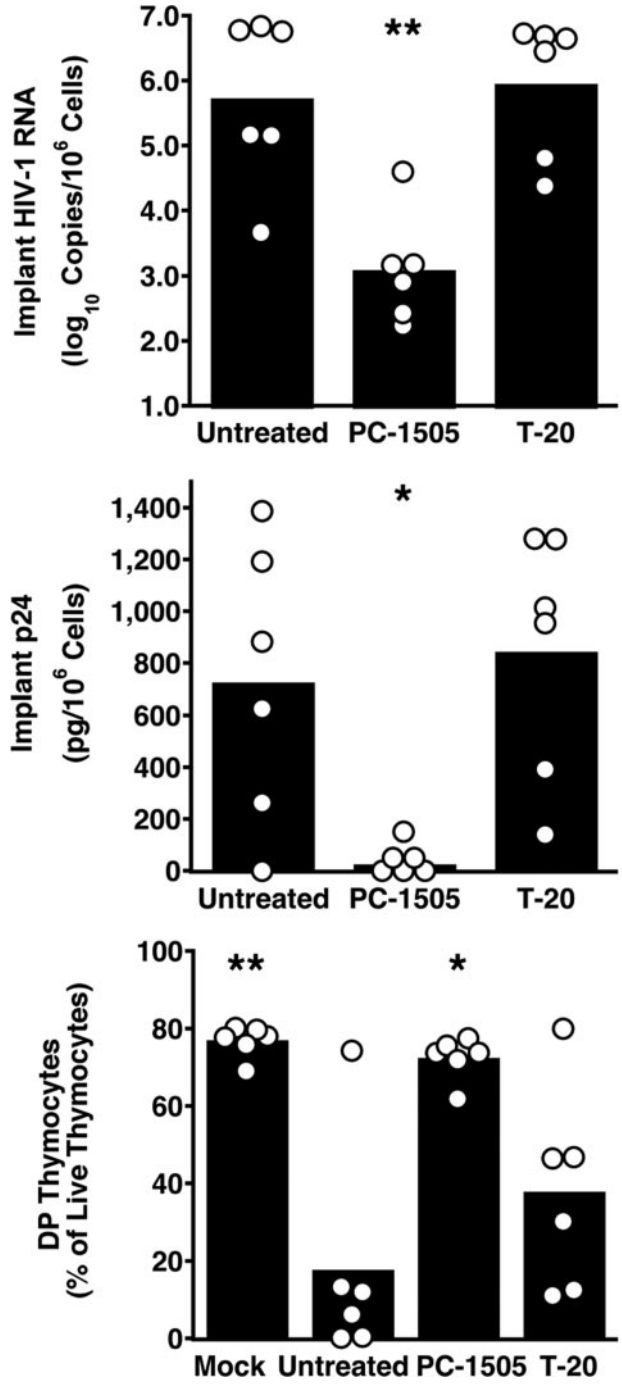

FIGURE 6. **PC-1505 has more sustained activity than T-20 against NL4-3G with a single pre-exposure dose.** SCID-hu Thy/Liv mice were treated with one subcutaneous injection of 200 mg/kg 1 day before inoculation. Antiviral efficacy was assessed by determining HIV-1 RNA and p24, and thymocyte protection was assessed by percentage of DP thymocytes. The *columns* represent the means, and the *open circles* represent individual animals from the same cohort 21 days after virus (or mock) inoculation. *, $p < 0.05$; **, $p < 0.01$ by the Mann-Whitney *U* test.

fact that serum albumin is a highly flexible protein capable of adopting several conformational states (37). For example, because C34 peptide is permanently attached to cysteine 34 of albumin, it is possible that local conformational rearrangements within the unconstrained N-terminal domain of albumin cause partial unwinding so as to facilitate correct insertion of the fusion inhibitor onto the NHR of gp41. Therefore, it is not

known whether positioning of C34 peptide elsewhere within the albumin molecule other than on cysteine 34 will lead to similar conservation of antiviral activity for this fusion inhibitor (*e.g.* lysine residues, N-terminal or C-terminal ends) or whether similar conservation of antiviral activity would be observed following permanent conjugation of C34 peptide to other abundant serum proteins of higher molecular mass. Hence, it is also possible the albumin molecule plays an active participatory role rather than merely serving as a protein cargo. For example, maleylated, aconitylated, and succinylated albumin function as potent HIV-1 entry inhibitors *in vitro* (38–41).

Recent evidence suggests the mechanism of HIV-1 fusion inhibition caused by T-20 is distinct from that of C34 peptide (6–8). For example, the insertion of T-20 into the plasma membrane at a post-lipid mixing step was shown to be responsible for the ability of T-20 to inhibit both the recruitment and oligomerization of gp41 with the C-terminal segment of T-20, ^{666}WASLWNWF^{673}, critical for effecting such hydrophobic interactions (6–8). The loss in antiviral activity observed for compound F, where the ^{666}WASLWNWF^{673} sequence is positioned directly adjacent to the albumin molecule, may be attributed to the inability of the T-20 moiety of compound F to insert into the plasma membrane at a post-lipid mixing step as efficiently as the unconjugated (free) T-20 peptide. Conversely, the ^{666}WASLWNWF^{673} sequence is less conformationally constrained in the design of compound E, thus leading to significantly improved retention of antiviral activity for the T-20 moiety.

The antiviral activity of PC-1505 was also assessed *in vivo* using the SCID-hu Thy/Liv mouse model and compared with that for maleimido-1505 (Fig. 2) and T-20 (Figs. 3–6). By using an *in vivo* model, the advantages of dosing a fusion peptide inhibitor prefixed onto a carrier protein such as albumin become obvious. For example, we attribute the 3-fold increase in potency for PC-1505 over maleimido-1505 to incomplete conjugation efficiency *in vivo* for the latter following its subcutaneous administration to SCID-hu Thy/Liv mice. Therefore, the reduced potency for maleimido-1505 may be accounted for by the fact that the unreacted peptide is less stable against proteolytic enzymes and is subject to normal rapid clearance pathways.

The equipotent activity of PC-1505 and T-20 *in vitro* (Table 1) was corroborated using the T-20-sensitive NL4-3G clone in the *in vivo* model. However, the advantages of PC-1505 over T-20 become obvious when either the frequency of dosing is reduced from twice daily to every fourth day (Fig. 5) or when a single elevated pre-exposure dose is administered (Fig. 6). Clearly, the ability of PC-1505 to outperform T-20 *in vivo* is due primarily to the significantly improved exposure and stability of the C34 peptide following albumin conjugation (Fig. 1) without abrogating the antiviral activity of the original peptide (Table 1). Finally, PC-1505 has also been shown to be highly active *in vivo* against the T-20-resistant NL4-3D (Fig. 4). Given that the amino acid sequences of the C34 peptide and T-20 overlap and that Gly^{547} positioned within the NHR of gp41 is expected to bind near the C-terminal end of C34 peptide, the conserved antiviral activity of PC-1505 against NL4-3D provides definitive supporting evidence for the importance of the gp41 coiled-coil cavity binding residues, ^{628}WMEW^{631}, which are absent in the structure of T-20 (18, 19). Taken together, these data confirm the highly potent *in vivo* anti-HIV activity of albumin-conjugated C34 peptide fusion inhibitor.

The results presented herein establish a proof-of-principle for this new class of albumin-peptide conjugates for inhibition of HIV or other viruses that have adopted similar mechanisms of membrane fusion and viral entry. As compared with unconjugated (free) peptide inhibitors, albumin conjugation may lead to a significantly improved exposure to the lymphatic system representing the anatomical home of ~98% of total HIV-infected cells (42), primarily because of significant steady-state lymph to plasma concentration ratios observed for serum albumin and for subcutaneously injected proteins >16–20 kDa (43, 44). Prefixing antifusogenic peptides to albumin may also overcome the low solubility limits commonly associated to this family of inhibitors (45), thus rendering these peptides more amenable to subcutaneous delivery with less frequent and less severe injection site reactions (46, 47). In summary, conjugation of antifusogenic peptides through cysteine 34 of albumin overcomes the steric block commonly associated with the NHR trimer of gp41 and thus offers hope for the discovery of novel, larger molecular mass molecules exhibiting potent and broadly neutralizing activity. One example of an albumin-conjugated C34 peptide HIV-1 fusion inhibitor, PC-1505, may require less frequent dosing than T-20 and is likely to be an effective agent against T-20-resistant HIV-1 in humans. The potent activity we observed for a single elevated pre-exposure dose of PC-1505 supports further preclinical and clinical development of this promising antiviral approach and confirms the utility and flexibility of the SCID-hu Thy/Liv mouse model for the preclinical evaluation of *in vivo* antiretroviral efficacy and drug resistance.

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