Conjugation of Cholesterol to HIV-1 Fusion Inhibitor C34 Increases Peptide-Membrane Interactions Potentiating Its Action

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Abstract
Recently, the covalent binding of a cholesterol moiety to a classical HIV-1 fusion inhibitor peptide, C34, was shown to potentiate its antiviral activity. Our purpose was to evaluate the interaction of cholesterol-conjugated and native C34 with membrane model systems and human blood cells to understand the effects of this derivatization. Lipid vesicles and monolayers with defined compositions were used as model membranes. C34-cholesterol partitions more to fluid phase membranes that mimic biological membranes. Importantly, there is a preference of the conjugate for liquid ordered membranes, rich in cholesterol and/or sphingomyelin, as observed both from partition and surface pressure studies. In human erythrocytes and peripheral blood mononuclear cells (PBMC), C34-cholesterol significantly decreases the membrane dipole potential. In PBMC, the conjugate was 14- and 115-fold more membranotropic than T-1249 and enfuvirtide, respectively. C34 or cholesterol alone did not show significant membrane activity. The enhanced interaction of C34-cholesterol with biological membranes correlates with its higher antiviral potency. Higher partitions for lipid-raft like compositions direct the drug to the receptor-rich domains where membrane fusion is likely to occur. This intermediary membrane binding step may facilitate the drug delivery to gp41 in its pre-fusion state.

Introduction
The development of new drugs against the human immunodeficiency virus type 1 (HIV-1) has been the focus of intense research since its discovery [1]. The virus fusion with the cell membrane and the consequent entry into the host cell is a critical moment of its life cycle. Efficiently blocking this process prevents all the subsequent intracellular steps. Most importantly, the integration of the viral genome, which can stay silent for years, does not occur. Despite this promising approach, only two HIV-1 fusion inhibitor peptides have been approved for human use: enfuvirtide (T-20) [2] and maraviroc [3]. Enfuvirtide, a fusion inhibitor peptide targeting gp41 in its pre-fusion conformation [4], interacts with adjacent receptor domains of CD4 and CCR5, creating the so-called fusion pore used for the entry of the viral content into the target cell [7]. Most of these peptides were derived from the C-terminal heptad repeat (CHR) region of gp41, and hence bind to the opposite domain in order to prevent bundle formation. The rational design of higher efficacy peptides compared to the native sequence from gp41 CHR include rearrangements and substitutions in order to improve intrinsic characteristics such as solubility, helical stability, oligomerization, lipophilicity and resistance to proteolysis [4].

In our previous works, we emphasized the importance of the lipophilicity and membrane interaction properties of the fusion inhibitor peptides enfuvirtide, T-1249 and sifuvirtide [8–10]. The fact that these peptides can partition to the membranes and to the microdomains, where receptors are preferentially located, facilitates the interaction with gp41 in its exposed conformation. This intermediate is temporally and spatially constrained; thus, a pre-concentration effect in the membrane can be a strategy to overcome this poorly accessible target.

Previously, the peptide C34, a classical fusion inhibitor used also as a template for the structural characterization of gp41 [7], was conjugated with a cholesterol moiety in order to obtain higher efficacy and lifetime in vivo [11]. C34-cholesterol (with cholesterol covalently bound to the C-terminus of the peptide; also known as L’644) was shown to be 50-fold more potent than C34 alone in terms of IC50 (HXB2 strain). Moreover, in a mouse model, the

Citation: Hollmann A, Matos PM, Augusto MT, Castanho MARB, Santos NC (2013) Conjugation of Cholesterol to HIV-1 Fusion Inhibitor C34 Increases Peptide-Membrane Interactions Potentiating Its Action. PLoS ONE 8(4): e60302. doi:10.1371/journal.pone.0060302

Editor: Stefan Pöhlmann, German Primate Center, Germany

Received December 13, 2012; Accepted February 25, 2013; Published April 2, 2013

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Funding: This work was funded by Fundação para a Ciência e Tecnologia, Portugal (fellowships SFRH/BPD/72037/2010 and SFRH/BD/42205/2007 to A.H. and P.M.M., respectively, and projects PTDC/QUI-BIO/104787/2008, PTDC/QUI-BIO/112929/2009 and VH/SAU/0047/2011). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Conjugated peptide was still detected 24 h after injection and with higher concentration, when compared with the unconjugated peptide, which was undetectable after 6 h [11]. In another recent work, a comparative pre-clinical evaluation of four fusion inhibitors (C34, T1249, enfuvirtide and C34-cholesterol) as potential microbicides showed that C34-cholesterol exhibits the highest anti HIV-1 activity as well as an extended window of activity. Moreover, C34-cholesterol is able to inhibit both wild type and reverse transcriptase inhibitors-resistant isolates [12].

The conjugation approach to improve fusion inhibitor peptides, such as by attaching acyl chains of various sizes [13-15] or human serum albumin [16,17], has been tried before; however, none has reached beyond pre-clinical tests. C34-cholesterol is a promising candidate [12].

In this article, we aimed at characterizing the interaction of C34 and C34-cholesterol with biomembranes to elucidate the peptide-membrane interaction and possible relations with the efficiency of these drugs. Our approach first covered a detailed study of the peptide partition towards biomembrane model systems, namely large unilamellar vesicles (LUV), by following the intrinsic fluorescence changes of the peptide tryptophan residues, located at the N-terminal region, when it adsorb or penetrate in the lipid environment. This methodology allows testing a diversity of lipid compositions, in order to dissect the role of the different lipid types. Insertion of the fusion inhibitors in lipid monolayers was also evaluated on a Langmuir-Blodgett trough. Finally, we studied the interaction with membranes of human blood cells, in an ex vivo setting. In this case, we used a lipophilic fluorescent probe (di-8-ANEPPS) that is sensitive to the membrane dipole potential and can report interactions of molecules that disturb the membrane order.

Experimental Section

Reagents

C34 (WMEDREINNYTSLHSLIESQNQEQKNEQELL) was obtained from the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). C34-cholesterol (L’64) was a kind gift from the International Partnership for Microbicides (licensed by Merck) and its sequence is C34-GSGG-Cholesterol. 5NS (5-doxyl-stearic acid) and 16NS (16-doxyl-stearic acid) were from Aldrich (Milwaukee, WI, USA). L-Tryptophan, acrylamide, HEPES and NaCl were from Merck (Darmstadt, Germany). POPC [1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine], DPPC [1,2-dipalmitoyl-sn-glycero-3-phosphocholine], POPG [1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac(1-glycerol)], DHSM (N-dodecanoyl-sphinganine-1-phosphocholine), SM (egg sphingomyelin), POPE [1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine) and POPS [1-palmitoyl-2-oleoyl-sn-glycerol-3-phospho-L-serine] were purchased from Avanti Polar Lipids (Alabaster, AL, USA), while cholesterol (Chol) were from Sigma (St. Louis, MO, USA).

Fluorescence Spectroscopy Measurements

C34 and C34-cholesterol peptides contain tryptophan residues, which make fluorescence techniques suitable tools to probe these molecules. The working buffer used throughout the studies was HEPES 10 mM pH 7.4 in NaCl 150 mM. C34 (150 μM) and Trp (500 μM) stock solutions were prepared in buffer. C34-cholesterol (500 μM) stock solutions were prepared in DMISO. Large unilamellar vesicles (LUV) were prepared by extrusion methods, as described elsewhere [18,19].

Membrane partition and fluorescence quenching studies using acrylamide were carried out in a Varian Cary Eclipse fluorescence spectrophotometer (Mulgrave, Australia) and time-resolved fluorescence spectroscopy studies in a LifeSpec II Fluorescence Lifetime spectrometer (Edinburgh Instruments).

The fluorescence spectral characterization of C34, C34-cholesterol and Trp was performed with an excitation wavelength of 280 nm, except for 5NS, 16NS and acrylamide quenching experiments, where the excitation was performed at 290 nm to minimize the relative quencher/fluorophore light-absorption ratios. For the quenching experiments, fluorescence emission was collected at a fixed wavelength of 350 nm and for the partition studies integrated spectra from 310 to 450 nm were used. Typical spectral bandwidths were 5 nm for excitation and 10 nm for emission. Excitation and emission spectra were corrected for wavelength-dependent instrumental factors [20]. During the quenching and partition experiments, emission was also corrected for successive dilutions, scatter [21] and simultaneous light absorptions of quencher and fluorophore. All the fluorescence measurements in this study were performed at room temperature (approximately 25°C).

Partition Coefficient Determination

Membrane partition studies were performed by successive additions to a 5 μM C34 or C34-cholesterol solution of small amounts of LUV suspensions with different lipid compositions, including: POPC, POPC:Chol (2:1 and 1:1), DPPC, POPC-Chol:SM (1:1:1 and 2:2:1), POPC:SM (2:1), POPC:POPG (5:1), POPC:DHSM (1:0.06), and HIV membrane-like mixture with and without DHSM (POPC 5.3%, DPPC 3.5%, cholesterol 45.3%, SM 18.2%, POPE 19.3% and POPS 8.4%) [22], with a 10 min incubation time between each addition. The partition coefficients \( K_p \) were calculated using the equation [23]:

\[
\frac{I}{I_L} = \frac{1 + K_p \gamma_L [L]}{1 + K_p [L]}
\]

where \( I_W \) and \( I_L \) are the fluorescence intensities in aqueous solution and in lipid, respectively, \( \gamma_L \) is the molar volume of the lipid [24,25], and [L] its concentration.

Surface Pressure

Changes on the surface pressure of lipid monolayers induced by C34, C34-cholesterol, enfuvirtide and T-1249 were measured in a Langmuir-Blodgett trough NIMA ST900 (Coventry, UK), at constant temperature (23±0.5°C). The surface of a HEPES buffer solution contained in a Teflon trough of fixed area was exhaustively cleaned by surface aspiration. Then, a solution of lipids in chloroform was spread on this surface, reaching a surface pressure of 20.5±1 mN/m. Peptide solutions were injected in the subphase and the changes on the surface pressure were followed during the necessary time to reach a constant value. The surface pressure of an air—water interface upon injecting the largest concentration of each peptide used throughout the studies was always below 15 mN/m (data not shown). For this reason, the lowest initial surface pressure of the lipid monolayers before the addition of the peptides to the subphase was above that value. In this condition, the changes in surface pressure observed upon the injection of the peptide can be attributed to an effect of the peptide on the monolayer interfacial tension.

The dissociation constant \( K_d \) was calculated from the adsorption Langmuir isotherm:
where $\Delta\Pi$ are the changes of surface pressure, $\Delta\Pi_{\text{max}}$ is the maximum change of pressure achieved and $[\text{peptide}]$ is the peptide concentration.

**Acrylamide Quenching**

The fluorescence quenching of 5 μM C34 or C34-cholesterol by acrylamide (0–60 mM) [8] was studied in buffer and in the presence of POPC or DPPC 3 mM LUV, by successive additions of small volumes of the quencher stock solution. For every addition, a minimal 10 min incubation time was allowed before measurement. Quenching data were analyzed by using the Stern–Volmer equation [23]:

$$\frac{I_0}{I} = 1 + K_{SV}[Q]$$

where $I$ and $I_0$ are the fluorescence intensity of the sample in the presence and absence of quencher, respectively, $K_{SV}$ is the Stern–Volmer constant and $[Q]$ the concentration of quencher.

**5NS and 16NS Quenching**

Fluorescence quenching assays with the lipophilic probes 5NS and 16NS were performed by time-resolved fluorescence spectroscopy. These assays were carried out at the same peptide and lipid concentrations used for the acrylamide quenching, by successive additions of small amounts of these quenchers (in ethanol) to samples of peptide in POPC and POPC:Chol (2:1), keeping the ethanol concentration below 2% (v/v) [26].

The effective lipophilic quencher concentration in the membrane was calculated from the partition coefficient of both quenchers to the lipid bilayers [27]. For every addition, a minimal 10 min incubation time was allowed before measurement. Quenching data were analyzed by using the Stern–Volmer equation (eq. 1), or the Lehrer equation [27,28] when a negative deviation to the Volmer equation (eq. 1),

$$\frac{R}{R_0} = 1 + \frac{[\text{peptide}]}{K_d}$$

where $R$ is the fluorescence intensity at a given peptide concentration, $R_0$ is the fluorescence intensity in the absence of peptide, and $K_d$ is the dissociation constant.

**Results**

**Membrane Partition**

UV-visible absorption and fluorescence spectra in buffer of C34 are comparable to those of Trp in aqueous solution (Fig. 1); however, C34-cholesterol presents a blue shift, indicating that there is a change in the Trp surrounding environment in this conjugate.

As shown in Fig. 2, there is an increase in the fluorescence intensity of C34-cholesterol in the presence of LUV. The partition coefficient between the lipid and aqueous phases, $K_p$, was determined by fitting eq. 1 to the fluorescence intensity data, in order to quantify the extent of interaction of the peptides to the lipid bilayers (Table 1). For POPC, a lipid with packing density and fluidity properties similar to biological membranes, a $K_p$ value of 1.57×10^4 was obtained, showing a significant interaction. When gel phase DPPC membranes were tested, a decreased $K_p$ was found, probably due to the lower bilayer rigidity. In cholesterol-rich membranes (POPC:Chol, POPC:Chol:SM 2:2:1 and HIV-like mixture) a significant increase in the partition was observed, when compared with pure POPC, also with a decrease on $L_\alpha$. Mixtures with cholesterol and SM mimic membrane microdomains usually named lipid rafts [33]. The same variation on $L_\alpha$ occurred for the addition of SM or DHSM (an unusual sphingolipid which does not contain a 4,5-trans double bond in its sphinganine backbone, significantly enriched in HIV-1 membranes [22]). The HIV-like mixture had the highest partition,
hinting to an importance of the viral membrane in capturing the drug besides the cell membrane.

In the case of C34, except for DPPC, no significant changes in the fluorescence intensity were observed in the presence of membranes (Fig. 2A), indicating an absence of significant peptide-membrane interactions.

**Surface Pressure Perturbation of Monolayers**

Despite the negligible partition and quenching results, adsorption of C34 to the membrane surface cannot be discarded, as it may leave the Trp residues exposed to the bulk aqueous environment, with unchanged quantum yield (i.e., not contributing for \( K_s \) calculation) [10]. In order to discard that situation, surface pressure measurements on POPC monolayer were carried out, using a low initial surface pressure (20.5 mN/m). Usually, the adsorption and penetration of molecules is favored at low surface density (loosely packed lipid monolayers), whereas high surface pressure (compact lipid packing) hinders the adsorption and penetration of molecules [34]. As shown in Fig. 3A, C34 is not able to induce any significant change in the surface pressure, in contrast to C34-cholesterol.

We also measured the changes in surface pressures induced by enfuvirtide and T-1249, yielding similar results to C34-cholesterol (Fig. 3A). In contrast, with POPC:Chol (2:1) or POPC:Chol:SM (1:1:1) monolayers, C34-cholesterol was able to induce larger changes than the other inhibitors (Figure 3B,C). \( K_s \) and \( \Delta \Pi_{\text{max}} \) values were determined by fitting eq. 2, in order to quantify the interaction of each peptide with the Langmuir monolayers (Table 2).

Direct comparison between the three peptides is difficult since they have different membrane binding domains. Therefore, they are not expected to modify the same way the surface tension of the membrane. However, comparing the same peptide for different lipids, we observe that C34-cholesterol presents an increased affinity to cholesterol rich membranes: an approximate ten-fold increase for POPC:Chol (2:1) and around six-fold for POPC::Chol:SM (1:1:1), when compared to pure POPC. This is in good agreement with data obtained from partition studies. In the case of enfuvirtide, no significant changes in the affinity were observed among each membrane composition tested, with just a slight decrease in the affinity for POPC:Chol, in a good agreement with previously published data [35]. Finally, T-1249 presented lower affinity for POPC:Chol, when compared with the other two compositions.

When the interaction kinetics were tested, a faster variation was observed for C34-cholesterol (Figure 3D). Control experiments were carried out using DMSO without C34-cholesterol and no significant changes were observed.

### Localization in the Lipid Bilayer

In order to test the accessibility of the fluorophores to the aqueous environment, the fluorescence quenching of the Trp residues of the peptide by acrylamide was studied. Linear Stern-Volmer plots were obtained in the absence of lipid (Fig. 4) and the quenching for C34-cholesterol is less efficient than C34, indicating that the fluorophore in the conjugate is less exposed to the surrounding aqueous medium. The cholesterol moiety may create hydrophobic pockets due to the formation of micro-aggregates, leading to this difference in the quenching in aqueous solution. Stern-Volmer plots and \( K_s \) constants in the presence of 3 mM of lipid and 5 \( \mu \)M of C34 or C34-cholesterol were obtained in order to complement the partition assays (Fig. 4). For C34-cholesterol, both assays with lipids yielded \( K_s \) values lower than the obtained in their absence (7.23 ± 0.15 mM\(^{-1}\) in solution, 5.58 ± 0.14 mM\(^{-1}\) with DPPC and 5.16 ± 0.11 mM\(^{-1}\) with POPC). In contrast, for C34 only, DPPC was able to reduce the \( K_w \) value from

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**Table 1. Partition coefficients.**

| Lipid Mixture            | \( K_p \)  | \( K_w \)  |
|--------------------------|------------|------------|
| POPC                     | 1574 ± 261 | 1.88 ± 0.05|
| DPPC                     | 331 ± 44   | 2.98 ± 0.16|
| POPC:Chol 2:1            | 3454 ± 718 | 1.25 ± 0.01|
| POPC:Chol:SM 2:2:1       | 4050 ± 885 | 1.24 ± 0.01|
| POPC:Chol 1:1            | 4612 ± 436 | 1.32 ± 0.01|
| POPC:DHS1 6%             | 755 ± 232  | 1.72 ± 0.09|
| HIV-like mixture         | 8084 ± 1190| 1.14 ± 0.004|
| HIV-like mixture with DHS1| 1758 ± 688 | 1.15 ± 0.002|

Parameters obtained from the fitting of the fluorescence data of the partition assays of C34-cholesterol 5 \( \mu \)M using eq. 1. All measures were made at least in triplicate. Values are presented as mean ± standard error of mean (SEM). doi:10.1371/journal.pone.0060302.t001
13.6 ± 0.01 mM⁻¹ in solution to 11.5 ± 0.39 mM⁻¹ in DPPC vesicles. These reductions on the Stern–Volmer constant indicate that the interaction with the membrane occurs with the fluorophores partially exposed to the aqueous environment.

Fluorescence quenching measurements were also used to evaluate the depth of insertion of Trp residues of the peptides inserted in POPC or POPC:Chol (2:1) vesicles. Stearic acid molecules derivatized with doxyl (quencher) groups either at carbon 5 (5NS) or 16 (16NS) were used. 5NS is a better quencher for molecules inserted in the membrane in a shallow position, close to the lipid-water interface, while 16NS is better for molecules buried deeply in the membrane [36]. Fig. 5A and B shows the Stern–Volmer plot obtained for C34-cholesterol on POPC or POPC:Chol (2:1), respectively, using the effective concentration of 5NS and 16NS in the bilayer matrix. For POPC, the $K_{sv}$ with 5NS ($4.43±0.41 \text{mM}^{-1}$, calculated with the Lehrer equation) was higher than for 16NS ($1.38±0.052 \text{mM}^{-1}$, calculated with the linear Stern–Volmer equation). In the case of POPC:Chol (2:1), $K_{sv}$ for 5NS was also higher ($9.23±0.67 \text{mM}^{-1}$, with the Lehrer equation) than for 16NS ($6.94±0.44 \text{mM}^{-1}$, with the Lehrer equation). Fluorescence lifetime quenching data enabled the application of the SIMEXDA method [36] to obtain the depth of insertion of the Trp residues of C34-cholesterol (Fig. 5C). A similar mean shallow location, close to the membrane interface, was observed for both lipid membranes. However, in the presence of cholesterol, the depth distribution is narrower, and the location more superficial (more exposed to the aqueous media), in a good agreement with acrylamide quenching data. The location distribution of C34-cholesterol does not differ much from the determined previously for enfuvirtide and T-1249 [10,35]. As expected, no changes in the fluorescence intensity were detected in the presence of 5NS or 16NS for C34 (data not shown).

Interaction with Blood Cell Membranes

After the characterization using membrane-model systems, we studied this peptide-membrane interaction in biological settings. We used isolated human erythrocytes and PBMC, labeled with the fluorescent probe di-8-ANEPPS, which is sensitive to the membrane dipole potential [30]. If the peptides interact by inserting or adsorbing on the membrane, it is expected that they change the membrane order to some extent, inducing variations in its dipole potential. These changes can be reported by di-8-ANEPPS excitation spectrum shifts [30,37], becoming easier to assess in differential spectra (Fig. 6A–C). The fluorescence red shift indicates a decrease in the membrane dipole potential in a peptide concentration-dependent manner. In order to quantify this interaction, we measured the ratio $R$ of the intensities at the excitation wavelengths 455 and 525 nm (with emission at 670 nm) for a range of peptide concentrations. $R$ is a quantitative descriptor of spectral shifts and, hence, of the relative variation of dipole potential. The membrane dipole potential significantly decreased in the presence of C34-cholesterol (Fig. 6 D–F), in sharp contrast to the unconjugated C34 peptide that had almost no effect. This shows that, in agreement with the model membrane studies, C34 does not have tendency to interact with cell membranes, while C34-cholesterol does. Affinity constants derived from the dipole potential variation curves indicate a much higher affinity when compared with the previously studied HIV fusion inhibitor peptides enfuvirtide and T-1249 (Table 3).

For the sake of comparison with the data obtained for blood cell membranes, and in order to validate the comparison with previous data, we also labeled lipid vesicles with di-8-ANEPPS (Fig. 6A, D). Lipid vesicles were composed of POPC, POPC:Chol 2:1 and POPC:Chol 1:1. In a good agreement with the partition and pressure data, we found that the increase of the cholesterol percentage on the membrane also increased the interaction of C34-cholesterol. Additions of DMSO, cholesterol or C34 were also tested as control and no changes on the dipole potential were observed (data not shown).

Discussion

In previous works, we showed that the interaction of HIV fusion inhibitor peptides with membranes (using lipid membrane models
and blood cells) is a key aspect of their mechanism of action. The membranotropic properties of a peptide can increase its local concentration at the membrane and boost the efficiency of the drug [8–10,29,35,38]. In this context, we studied the membrane interactions of the C34 peptide, in comparison with its cholesterol conjugated version, C34-cholesterol, in order to understand the

**Figure 3. Interaction of the fusion inhibitor peptides with lipid monolayers.** (A–C) Changes in surface pressure as a function of HIV-1 fusion inhibitor addition to pure POPC (A), POPC:Chol 2:1 (B) or POPC:Chol:SM 1:1:1 (C) monolayers. Dashed lines are fittings of eq. 2 to the experimental data. (D) Changes in surface pressure as a function of time after addition of HIV fusion inhibitors to achieve a final concentration of 31 nM on a POPC:Chol 2:1 monolayer. All assays were carried out at 25°C, using an initial pressure of 21.5 ± 0.5 mN/m. Each point is the average of at least triplicates of independent samples. Error bars represent the standard error of mean (SEM).

doi:10.1371/journal.pone.0060302.g003

**Table 2.** Dissociation constants, $K_d$, and $\Delta \Pi_{\text{max}}$ determined from surface pressure changes.

|          | C34-cholesterol | Enfuvirtide | T1249 |
|----------|-----------------|-------------|-------|
| POPC     | $K_d$ (nM)      | $\Delta \Pi_{\text{max}}$ (mN/m) | $K_d$ (nM) | $\Delta \Pi_{\text{max}}$ (mN/m) | $K_d$ (nM) | $\Delta \Pi_{\text{max}}$ (mN/m) |
| POPC     | 59±15           | 3.5±0.3     | 31±3.7 | 3.1±0.1 | 12±0.6 | 3.3±0.02 |
| POPC:Chol (2:1) | 5.3±1.4         | 2.5±0.05    | 41±6.8 | 1.4±0.06 | 65±3.5 | 1.9±0.03 |
| POPC:Chol:SM (1:1:1) | 9.2±1.3         | 2.6±0.02    | 27±4.6 | 1.6±0.03 | 13±1.1 | 1.8±0.02 |

Parameters obtained from the fitting of the surface pressure data from Fig. 3 using eq. 2. All measures were made at least in triplicate. Values are presented as mean ± standard error of mean (SEM).

doi:10.1371/journal.pone.0060302.t002
molecular basis of the dramatically increased antiviral efficiency of the conjugate [11].

This study demonstrates the membrane-binding ability of C34-cholesterol. Overall the results obtained from partition, surface pressure, quenching and dipole potential show that the addition of the cholesterol moiety to the C-terminus of C34 renders the peptide membranotropic. The partition coefficients for POPC obtained for this conjugated peptide are of the same order as the previously determined for enfuvirtide and T-1249 (Table 1). In contrast, C34 shows no partition for membranes of this lipid. Enfuvirtide and C34 share a common CHR sequence as a structural domain; however, C34 lacks a putative lipid-binding domain (LBD) that is Trp rich, which may explain its low membrane partition (Fig. 1A) [39]. This is also the case of the LBD-lacking fusion inhibitor peptide sifuvirtide, which also presents residual binding to POPC vesicles and the same preference for DPPC membranes (on the gel phase) [8,29]. When the interactions with cholesterol-rich membranes were studied, in contrast with the result previously obtained for enfuvirtide [35], membrane partition of C34-cholesterol still takes place and with a relatively high \( K_p \) (Table 1). A similar behavior was found for lipid raft-mimicking membranes (POPC:Chol:SM).

In order to interpret results on POPC:Chol binary mixtures, their phase diagram at 25°C needs to be considered [33]. In the case of pure POPC, the membrane is in the liquid disordered phase (Ld). For POPC:Chol 2:1 there is a phase coexistence between liquid ordered (Lo) and liquid disordered (Ld) phases, while for POPC:Chol 1:1 only Lo phase occurs (but close to the...
Table 3. Antiviral activity and cell membrane interaction parameters compared of HIV-1 fusion inhibitors.

|                | C34-cholesterol | T-1249 | Enfuvirtide (T-20) |
|----------------|-----------------|--------|--------------------|
| IC50 (nM)*     | 0.36 | 8.3   | 20                 |
| JRFL           | 0.09 | 2.6   | 6.1                |
| NL4-3          | 0.58 ± 0.049   | 4.16 ± 0.13 | 35.4 ± 1.33        |
| £ (μM) Erythrocytes | 0.54 ± 0.052 | 7.71 ± 0.61 | 62.2 ± 10.2       |

*11[1]; 19[9].

Surface pressure measurements (Fig. 3) showed a higher membrane affinity of C34-cholesterol towards cholesterol and SM-rich membranes, when compared to pure POPC. Taking into account that C34 was unable to induce any change in any of the tested monolayers (data not shown) these experiments also showed that the cholesterol moiety boosts the adsorption of C34 specifically on cholesterol-rich membranes. In comparison, enfuvirtide showed much less distinction between the three mixtures tested. Furthermore, regarding the kinetics of the interactions of the three HIV fusion inhibitors in POPC:Chol monolayers (Fig. 3D), we found that C34-cholesterol also exhibit a faster binding kinetic, which could only be assigned to the “membrane anchoring” cholesterol moiety.

This preference of the drug for cholesterol and “lipid raft-like” mixtures is very relevant regarding inhibiting HIV entry. The most important receptors for HIV entry, CD4 and CCR5, were shown to be associated with lipid raft microdomains and DRM (detergent resistant membranes) and this association is important for the binding of the virus to cells [40]. Several studies also demonstrated that depleting cholesterol from the host cells greatly boundaries between Lipid/Ld coexistence). Our partition data indicate that C34-cholesterol preferentially partitions to the Lo phase, displaying a $K_p$ three-fold higher than for the Ld phase (pure POPC), and an intermediate behavior when both Ld and Lo phases coexist. Additionally, using di-8-ANEPPS labeled LUV (Fig. 6A, D), we showed that the interaction of the conjugated peptide increases with the amount of cholesterol present in the POPC:Chol binary mixture, suggesting again a preference for cholesterol-rich membranes.

Figure 6. Peptide interactions with di-8-ANEPPS labeled LUV and cell membranes. (A–C) Differential spectra of di-8-ANEPPS bound to LUV (A) erythrocytes (B) and PBMC (C) membranes in the presence of C34-cholesterol and in its absence. Spectra were obtained by subtracting the excitation spectrum (normalized to the integrated areas) of labeled cells in the presence of peptide from the spectrum in the absence of the respective peptide. The shift to the red (decrease in dipole potential) is peptide concentration-dependent. For LUV, spectra traces represent C34-cholesterol 4 μM, in the presence of different lipid compositions: POPC, POPC:Chol 2:1 and POPC:Chol 1:1. For cells, spectra traces represent different C34-cholesterol concentrations: 0 μM, 0.25 μM, 1 μM and 5 μM. (D) Binding profiles of C34-cholesterol to LUV of POPC, POPC:Chol 2:1 and POPC:Chol 1:1, by plotting the di-8-ANEPPS excitation ratio, $R(455/525)$, normalized to the initial value, as a function of the peptide concentration. DMSO, cholesterol and C34 (unconjugated) were also tested, as controls, and no significant changes on the dipole potential were observed (data not shown). (E–F) Binding profiles of C34-cholesterol, C34 and cholesterol to erythrocytes (E) and PBMC (F) cell membranes. Controls for DMSO (empty circles) and DMSO:ethanol (empty square) were also performed. Dashed curves represent the fitting to the single binding site equation (eq. 5). Affinity parameters for the cells are indicated in Table 3. Error bars represent SEM, with n = 5 for C34-cholesterol curves in cells and n = 3 for the control curves and LUV.

doi:10.1371/journal.pone.0060302.g006
cholesterol moiety and also, with a putative weaker binding, via lipid rafts, which usually contain the receptors involved in HIV entry. C34-cholesterol anchors to the membrane via its cholesterol moiety and also, with a putative weaker binding, via its Trp (W)-rich N-terminal domain. In the context of HIV-1 gp41 engagement with the target cell, a confined space exists between the two membranes. Enrichment in DHSM in the viral membrane decreases the peptide partition, possibly shifting membrane partition equilibrium to the host cell membrane. The drug concentrated in the lipid raft environment may reach its target (gp41) more efficiently than through simple diffusion in aqueous solution. Moreover, the anchoring promoted by the cholesterol at the C-terminus brings the peptide into correct contact with the target cell membrane.

Recently, the lipid composition of HIV-1 membrane, constituting its lipidome, has been precisely determined [22]. That allowed us to study lipid vesicles with compositions mimicking the virus envelope. The high C34-cholesterol partition observed for the HIV membrane-like mixture of lipids indicates that the viral membrane itself can also extensively capture and carry the conjugate drug on its surface, enhancing its availability at the required site of action. However, this interaction seems to be sensitive to the presence of DHSM, a lipid found to be unusually enriched at the viral membrane, when compared to the host cell membrane [22]. When present, DHSM decreases C34-cholesterol partition (Table 1). It is tempting to speculate that, in a context of virus and host cell engagement, this may be a factor that shifts the equilibrium of partition towards the cell membranes, where it anchors in the right direction to interact with the transiently exposed gp41 NHR domain.

Regarding the location of C34-cholesterol at the membrane level, our data show that the Trp residues of the peptide insert in the external leaflet of the bilayer, at a shallow position. Previously, the Trp-rich regions of enfuvirtide and T-1249 were also shown to insert superficially in lipid bilayers [10,35]. As the peptide is conjugated with cholesterol in a defined position, the peptide should adopt a consistent orientation, with the C-terminus anchored to the membrane by the cholesterol moiety (Fig. 1A).

This is the proper orientation to align in an anti-parallel fashion with the NHR domain of gp41 when it extends towards the target membrane. The N-terminus, where the Trp residues are located, may interact freely with the nearby lipids, but cannot insert deep in the membrane.

In order to have a better understanding of what may happen in the bloodstream, we also studied the interaction of the peptides with human erythrocytes and mononuclear leukocytes. These peptide drugs must be injected, as oral administration is not feasible. HIV-1 is known to associate to the surface of erythrocytes in circulation [45–47], while PBMC are among the virus main targets. C34-cholesterol decreases the membrane dipole potential of the two cell types, indicating that an interaction is occurring (Fig. 6). In agreement with the partition data, C34 alone also does not interact with cell membranes. The same concentration of cholesterol alone is not sufficient to induce a detectable membrane potential change [48], indicating that the potential change is induced essentially by the peptide component of the conjugate. However, this change only occurs in the conjugate, emphasizing the role of cholesterol in inducing the membrane partition of the peptide to cell membranes.

Quantitatively, C34-cholesterol was found to have approximately 115 or 14 times more affinity towards PBMC membranes than enfuvirtide or T-1249, respectively. Furthermore, the cell membrane affinities of the different fusion inhibitor peptides (C34-cholesterol>T-1249> enfuvirtide) correlate with the antiviral potency against HIV-1 (Table 3). Regarding the comparison with the $K_p$ values determined with lipid vesicles, this is not straightforward, as the results obtained for the different compositions tested indicate that the process is lipid composition-dependent. Moreover, the partition is based on tryptophan fluorescence, which regards only the peptide part of the conjugate.

inhibits HIV infection [40–42]. Moreover, the substitution of the virion-associated cholesterol by the raft-inhibiting sterol analogues 4-cholestenone and coprostanol was shown to reduce infectivity, in contrast to other raft promoting sterols [43]. Overall, lipid rafts are considered to be essential for the virus entry process [44]. The preferential partition of C34-cholesterol to those domains would make the drug more available at the site where membrane fusion occurs.

Figure 7. Putative mode of action of HIV-1 fusion inhibitor C34-cholesterol. In aqueous solution, the conjugate may form micro-aggregates when membranes are not present, due to its cholesterol moiety. The drug was demonstrated to partition to cell membranes and virus-like membranes. A preference towards cholesterol and SM-rich compositions was identified. These lipids are characteristic of the membrane when membranes are not present, due to its cholesterol moiety. The drug was demonstrated to partition to cell membranes and virus-like virions. Make the drug more available at the site where membrane fusion occurs.

Figure 6. C34-cholesterol does not interact with cell membranes. The same concentration of cholesterol alone is not sufficient to induce a detectable membrane potential change [48], indicating that the potential change is induced essentially by the peptide component of the conjugate. However, this change only occurs in the conjugate, emphasizing the role of cholesterol in inducing the membrane partition of the peptide to cell membranes.

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However, if we focus on cholesterol-containing mixtures, C34-cholesterol has the highest partition, contrasting with enfuvirtide, with negligible interaction [35]. T-1249 should fall in between, cholesterol has the highest partition, contrasting with enfuvirtide, and membrane domain selectivity among HIV fusion inhibitor peptides. We demonstrated that C34-cholesterol has membranotropic behavior towards lipid vesicles and strongly interacts with circulating blood cells membranes. HIV fusion inhibitors have a restricted temporal and spatial window of opportunity to bind to their molecular target: the NHR region of gp41 in its extended conformation, when the virus and target cell are closely engaged. This makes aequous diffusion of the peptide to its target less efficient. It has also been demonstrated that the forces governing protein–protein interactions in a membrane environment are different from those in solution [49]. The ability that these peptides have to bind to cell membranes facilitates the delivery of the peptides to this confined environment, as some peptide is already locally present (Figure 7). In this context, C34-cholesterol should be able to concentrate at the cell surface and also on the viral membrane. In agreement with this, previous results with cells after 48 h of treatment with C34-cholesterol showed the persistence of antiviral activity of residual peptide not cleared by washing steps [11], supporting the idea that cell membranes could work as C34-cholesterol reservoir. Importantly, the concentration effect would be higher on the lipid rafts, where the HIV receptors are present and fusion is more likely to occur [40,44]. This persistent antiviral activity may be ideal for this drug to be applied as a topical microbicide, for example as vaginal gel, to prevent infection [12]. C34-cholesterol was generally the most active inhibitor when tested in the context of human mucosal tissue explants (colorectal, penile and cervical), when compared to C34, enfuvirtide and T-1249 [12]. The first microbicide formulations against HIV were based on unspecific polyamions to prevent viral attachment to the cell surface; however, they largely failed in clinical trials [50]. Only recently a trial using a tenofovir gel (a reverse transcriptase inhibitor) showed 39% reduction on HIV-1 infection [51]. Using an entry inhibitor such as C34-cholesterol could prevent the viral entry in the first encountered immunological cells of the mucosa, blocking the virus at the earliest stage of infection.

Ultimately, the membrane may act as a “catalyst” to the binding reaction between gp41 and the peptide inhibitors, as it has been postulated in other receptor-ligand scenarios in membrane environments [38]. Thus, in this case, the marked increase in antiviral efficiency of C34-cholesterol in relation to C34 (and other fusion inhibitors) correlates with its higher affinity towards model and blood cells membranes.

Acknowledgments

We thank Teresa Freitas (IMM, FUMUL) for the technical support on PBMC isolation.

Author Contributions

Conceived and designed the experiments: AH PMM MARBC NCS. Performed the experiments: AH PMM MTA. Analyzed the data: AH PMM NCS. Wrote the paper: AH PMM MARBC NCS.

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