

#513 A CD38-derived peptide as a potential antiretroviral agent exploiting cellular mechanisms of antiviral defense

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ABSTRACT

Background: The leukocyte surface marker CD38 docks to CD4 on the cell surface and can prevent HIV-1 infection *in vitro* through an N-terminal peptide (CD38₅₁₋₇₄). We here model the structural interactions of CD38 with CD4 and evaluate the potential applicability of CD38₅₁₋₇₄ as a new antiretroviral agent.

Methods: CD38 binding to CD4 was evaluated using biotinylated probes in acellular and flow-cytometric assays. CD4/CD38 interactions were evaluated by molecular dynamics. Peptide toxicity was evaluated *in vitro* by standard methods. HIV-1 glycoprotein mediated fusion was evaluated by syncytium assays. HIV-1 replication was assessed as p24 production in acutely infected MT-2 cells.

Results: The HIV-1 inhibitory peptide CD38₅₁₋₇₄ bound recombinant human CD4 in acellular assays ($P < 0.05$; *t*-test for regression) and selectively bound to CD4⁺ transfectants of the CD4-murine cell line SR-D10 ($P < 0.01$, Kolmogorov Smirnov statistics). Accordingly, CD38₅₁₋₇₄ participates to the CD38/CD4 docking interface. The lowest-energy docking of CD38 to CD4 also involves an interaction of CD38₅₁₋₇₄ with the CD4 Phe43 residue fundamental for HIV-1 gp120/CD4 binding. In line with this model, CD38₅₁₋₇₄ dose-dependently antagonized gp120/CD4 binding and inhibited HIV-1 glycoprotein mediated fusion in the nanomolar range ($P < 0.05$, *t*-test for regression). The EC₅₀ of CD38₅₁₋₇₄ on both fusion and acute infection assays was decreased to average values < 100 nM when the peptide was subjected to lysine-PEGylation. Instead, CD38₅₁₋₇₄ and its PEGylated counterpart, at concentrations up to 1 mM did not inhibit viability of several cell types such as lymphoid cell lines, PBMC, HeLa cells and human spermatozoa ($P = 0.6-0.9$), thus showing extremely high selectivity indexes (103-104). Despite CD38 is a marker of lymphocyte activation, the CD38₅₁₋₇₄ peptide neither induced lymphocyte activation, nor altered production of IL-2, IL-4, IL-5, IL-6, IL-10 or the chemokines MIP-1alpha and MIP-1beta ($P = 0.5-0.7$).

Conclusions: Human CD38 might represent a natural HIV-1 attachment inhibitor that might be exploited for the development of new and safe antiretrovirals or topical microbicides.

INTRODUCTION

Human CD38 is a leukocyte surface antigen expressed on naïve and/or activated T-lymphocytes and other cell types. It is a type-II transmembrane protein whose extracellular domain presents two domains enclosing a NAD⁺ hydrolysing catalytic site and an HIV-1-inhibitory peptide at the membrane-proximal portion [1]. As a molecule whose expression increases during lymphocyte activation, it has long been used as a progression marker of HIV infection. It is well known that expression of CD38 on CD8⁺ T-lymphocytes acts as a negative prognostic marker when associated with HLA-DR expression [2-3].

It is intriguing that, although CD38 is a negative prognostic marker of HIV-1 progression, at the early stage of HIV-1 infection, CD38 can actually prevent the lymphocytes from HIV-1 infection *in vitro* [4]. The molecular relationship between CD38 and HIV-1 was first identified by independent studies reporting a lateral association between CD38 and CD4 [5] and a negative correlation between CD38 expression and susceptibility to HIV-1 infection in CD4⁺ T-cell lines [6]. The anti-HIV-1 effects of CD38 were recently attributed to its HIV-1 inhibitory peptide [7]. This peptide obstructs HIV-1 attachment to CD4⁺ T-lymphocytes and is likely to be involved in the lateral interactions with other cell surface molecules including CD4. When MT-4 human T cells were incubated with the soluble CD38 amino acid sequence 51-74 (CD38₅₁₋₇₄), it inhibited HIV-1 infection, syncytium formation and binding of gp120 to CD4 cells at micromolar concentrations [7]. CD38₅₁₋₇₄ also inhibited phylogenetically unrelated HIV-1 and HIV-2 primary isolates in lymphoid cells and in peripheral blood mononuclear cells [7]. The details of the molecular interplay behind these events have been unknown. Here, we i) model the structural interactions of CD38 with CD4, and ii) evaluate the potential of CD38₅₁₋₇₄ for becoming a new anti-HIV agent.

CONCLUSIONS

On the whole, the results of the present study show that the human CD38 antigen-derived peptide, namely CD38₅₁₋₇₄, inhibits HIV-1 replication by hindering the very first step of HIV-infection (gp120/CD4 binding) and that it displays no toxicity in cell culture models. PEG-conjugated CD38₅₁₋₇₄ (CD38₅₁₋₇₄ PEG) exerts potent anti-HIV-1 effects in the low nanomolar range. These results encourage further studies aimed at evaluating CD38₅₁₋₇₄ PEG as a topical microbicide or a new antiretroviral. Studies of sCD38₅₁₋₇₄ PEG using an animal model and clinical field trials will be necessary before drawing conclusions on the usefulness of the drug in clinical settings.

METHODOLOGY

Peptide biotinylation and binding to SR-D10 cells. Peptides were synthesized at a purity rate $\geq 85\%$ by Primus srl (Milan, Italy, <http://www.primus.it>) using the N- α -fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis method. The CD38₅₁₋₇₄ and CD38₅₁₋₇₄K57G peptides were synthesized using NRS-L-Clonina (Pharco, Rockford, IL, U.S.A.) (Zhang et al. N- α -dimethylformamide) CD38₅₁₋₇₄ and CD38₅₁₋₇₄K57G (1 mg/ml) were incubated for 1 h in an mixture of borate solution in DMF and peptide solution (1:20 v/v). The reactions were stopped by adding glycine (50 mM, 2 v/v) in the final volume [8]. Mouse MCF-7 SR-D10 cells and SR-D10 cells transfected with human CD4 (hCD4⁺ SR-D10) were incubated with CD38₅₁₋₇₄, biotin and CD38₅₁₋₇₄K57G biotin (100 nM) for 30 min. *in situ*. The binding between MCF-7 and peptides was detected using streptavidin fluorescent isothiocyanate (FITC) (100 nM) (Dako, Denmark). Cells were then washed once in PBS A-4, and fluorescence was acquired by flow cytometry. Fluorescence data were expressed as MFI.

Virological assays
The effects of CD38₅₁₋₇₄ and CD38₅₁₋₇₄K57G peptides on viral envelope-mediated fusion were evaluated by syncytium assays based on co-cultivation of MT-2 cells with chronically HIV-1-infected TH9 cells in a 10:1 ratio. Soluble peptide concentration range 10⁻¹⁰ (10⁻¹⁰ M) were added in the culture medium in which the cells were resuspended. The number of syncytium field was evaluated after 20h of incubation at 37°C [9].
For acute infection assays, MT-2 cells were incubated at 37°C for 1 h in the presence or absence of the CD38 peptides, and then for 2 h at 37°C with infectious virus at a multiplicity of infection (MOI) of ~ 1 . After three washes, cells were incubated in fresh culture medium for 7 days at 37°C, and cell-free supernatant at different intervals post-infection were harvested for ELISA measurement of HIV-1 p24 (NEN Life Science, Boston MA, USA).

PEGylation of CD38₅₁₋₇₄ peptide
Methoxy-PEG-conjugated lysine (mPEG-SPA, M 5000) was purchased from NIKTAR Transforming Therapeutics (Huntsville, AL, U.S.A.). The CD38₅₁₋₇₄ and CD38₅₁₋₇₄K57G peptides were diluted at 2 mg/ml in deionized water and stored at -20°C . Then, 50 μg of peptides were first treated with 1.85 μg of mPEG in 10 μL of sodium acetate in a buffer containing 0.9 mM tryethylamine (TEA) at 25°C for 30 minutes. The reaction was stopped by adding glycine 2M (one volume, 10:1).

The presence of monoPEGylated species were verified by SDS-PAGE on 10% acrylamide gel and Coomassie or silver staining.

Toxicity assays
Cell proliferation was assessed by sequential counts of the number of live cells per milliliter of cell cultures by the Trypan blue exclusion method. Cell viability and apoptosis were analyzed by the methyl tetrazolium (MTT) method (Diagen-Diagnostica) and by propidium iodide/annexin V-FITC staining (Molecular Diagnostic GmbH, Vienna, Austria) following the manufacturer's instructions.
The levels of cytokines IL-2, IL-4, IL-5, IL-6, IL-10 in cell culture media was evaluated with a CBA kit (MxPlexHuman-Cytokine Assay) (BD, Franklin Lakes, NJ, USA) which allow the simultaneous evaluation of different cytokines and while the MIP-1alpha and MIP-1beta secretion was assessed by ELISA kit (R&D Systems, Inc, Minneapolis, MN, USA).

RESULTS

We first modeled the CD4/CD38 interaction *in silico*. Docking modeling using the program Hex suggests that the extracellular portions of CD38 and CD4 may form stable heterodimers (Fig. 1A). In the lowest-energy configuration, the simulated CD4/CD38 docking involves an interaction between CD4's Phe43 and CD38's Lys57. This interaction is apolar and does not involve the NH₂ group of Lys (Fig. 1B). This molecular interaction is intriguing because Phe43 is the principal residue responsible for gp120 binding to CD4, and Lys57 resides within the HIV-1-inhibitory peptide of CD38. These results raise the hypothesis that CD38₅₁₋₇₄ might bind to CD4 thus inhibiting HIV-1 infection.

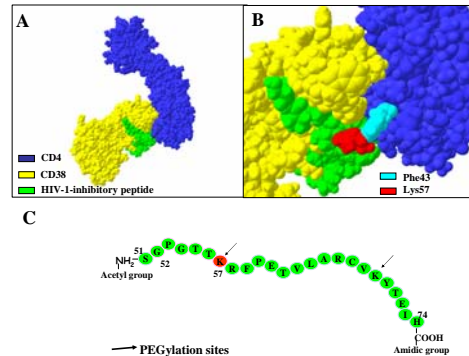


Fig. 1. Modeling of CD4/CD38 interaction: CD4 and CD38 may form stable heterodimers involving CD4's Phe43 and CD38's Lys57. A: full view; -B: zoom-in of A; C: primary structure of the HIV-1 inhibitory peptide CD38₅₁₋₇₄ showing possible PEGylation sites.

As a CD38₅₁₋₇₄ peptide was previously shown to inhibit the early stages of HIV-1 replication *in vitro*, we tested whether this peptide might bind to CD4. For this purpose, we analysed the binding of biotin-conjugated peptides to mouse SR-D10 cells transfected with human CD4 (hCD4). Following incubation with streptavidin-PE, biotin CD38₅₁₋₇₄ significantly increased the fluorescence of hCD4⁺ SR-D10 cells, but not that of hCD4⁻ SR-D10 cells (Fig. 2). Fluorescence was instead not intensified using a CD38₅₁₋₇₄ peptide with Lys57 substituted with a Gly residue (CD38₅₁₋₇₄K57G) (Fig. 2). In line with previous observations that CD38₅₁₋₇₄ inhibited gp120/CD4 binding in acellular models, binding of gp120 to human CD4⁺ cells was down-modulated in the presence of CD38₅₁₋₇₄ (data not shown). We conclude that the CD38 HIV-1 inhibitory peptide displays a specific interaction with CD4 involving CD38's Lys57, and that it obstructs gp120 binding.

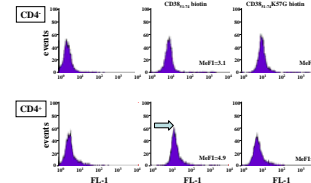


Fig. 2. Flow-cytometric detection of the binding of CD38₅₁₋₇₄ and CD38₅₁₋₇₄K57G peptides to hCD4 SR-D10 cells and hCD4⁻ SR-D10 cells. x-axis = fluorescence intensity; y-axis = number of events. One experiment out of three with similar results.

We tested whether the interaction of CD38's Lys57 with the gp120-binding site of CD4 might be associated with functional impairment of HIV-1 fusion. We found that CD38₅₁₋₇₄, but not CD38₅₁₋₇₄K57G, inhibited syncytia formation in MT-2/H9 cells co-cultures (Fig. 3). Of note, the inhibitory concentrations were decreased by approx. 150 fold by conjugation with PEG (Fig. 3). We conclude that: i) CD38₅₁₋₇₄ inhibits HIV-1 fusion, ii) Lys57 is fundamental for these effects and iii) the effects are amplified by PEG-conjugation.

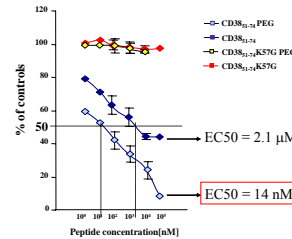


Fig. 3. Syncytia formation MT-2/H9 cell co-cultures in presence of CD38₅₁₋₇₄ and CD38₅₁₋₇₄K57G peptides (results from three independent experiments; means \pm SEM).

We then tested whether the effects of CD38₅₁₋₇₄ PEG on p24 production by cells acutely infected with HIV-1. For this purpose, MT-2 cells were infected with HIV-1_{IIIIB} in the presence or absence of the peptides, and viral replication was evaluated as p24 production at five days post-infection. As shown in Fig. 4, the EC₅₀ of CD38₅₁₋₇₄ on HIV-1 p24 production was decreased by > 200 fold by PEG conjugation. We conclude that CD38₅₁₋₇₄ PEG displays HIV-1 inhibitory concentrations worthy of pharmacological attention.

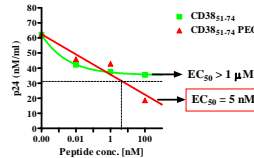


Fig. 4. Typical results showing p24 production in MT-2 cells infected with HIV-1_{IIIIB} in the presence of CD38₅₁₋₇₄ and CD38₅₁₋₇₄PEG (results at five days post-infection).

CD38₅₁₋₇₄ and its PEGylated counterpart, at concentrations up to 1 mM did not inhibit viability of several cell types such as lymphoid cell lines, PBMC and HeLa cells (ANOVA; $P = 0.9$; data not shown), thus showing extremely high selectivity indexes (>1000). Moreover, CD38₅₁₋₇₄ did not affect the viability of human spermatozoa from six healthy and six astenospermic donors ($P > 0.06$; Wilcoxon ranked test). Despite CD38 is a marker of lymphocyte activation, the CD38₅₁₋₇₄ peptide neither induced lymphocyte activation (evaluated as expression of CD69, CD38 CD25 and HLA-DR), nor altered production of IL-2, IL-4, IL-5, IL-6, IL-10 or the chemokines MIP-1 α and MIP-1 β ($P = 0.5-0.7$; data not shown).

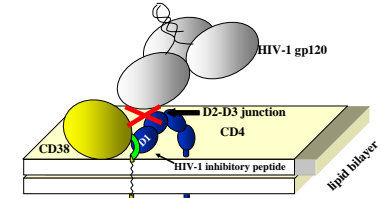


Fig. 5. A model for the interplay between CD38, CD4 and HIV-1 gp120.

DISCUSSION

Our results are consistent with a model wherein CD38 competes, through its HIV-1 inhibitory peptide CD38₅₁₋₇₄, with gp120 binding to CD4. Steric constraints due to the location of CD38₅₁₋₇₄ at the cell membrane-proximal portion of CD38 and the gp120-binding site at the membrane-distal portion of CD4 (in the D1 domain) can be overcome by the flexibility of the CD4 molecule at the D2-D3 junction [11]. This flexibility allows the D1 domain to be transported close to the cell membrane (Fig. 5). An alternative hypothesis is that the CD4/CD38 interaction might occur *in trans*. This hypothesis is however unlikely because previous studies using fluorescence resonance energy transfer indicate that the CD4/CD38 interaction occurs *in cis* on the cell surface [7].

Whether there are any connections between CD38 as a marker of disease progression and its anti-HIV-1 effects remains a mystery at present. CD38 overexpression has been characterized better on CD8⁺ T-lymphocytes. However, a number of studies indicate that also the proportion CD4⁺ CD38⁺ T-cells increases during disease progression [3]. It is possible that CD38⁺ cells are preferentially depleted during HIV-1 infection. This hypothesis is in line with evidence that HIV-1 preferentially infects and depletes memory CD4⁺ T-cells (CD38⁺) as compared to naïve lymphocytes (CD38⁻). It will thus be intriguing to design studies aimed at connecting overexpression of CD38 in HIV-1/AIDS with its HIV-1 inhibitory properties.

The effects of CD38₅₁₋₇₄ on the very first step of HIV-1 infection and the lack of toxicity to HeLa cells support the idea of considering the CD38₅₁₋₇₄ sequence in topical microbicide strategies to prevent transmission of HIV to women. Indeed, the HeLa cell line is representative of the epithelium of the uterine cervix. Part of this epithelium faces into the vaginal cavity and is highly susceptible to damage by potentially toxic substances introduced into the vagina. The lack of spermicidal activity should preserve the reproductive capacity of a couple, thus favoring acceptance of such a strategy by those cultures refusing contraceptive methods. Although CD38₅₁₋₇₄ is derived from a molecule linked to lymphocyte activation, it did not induce secretion of pro-inflammatory cytokines. Being derived from an endogenous molecule, CD38₅₁₋₇₄ is unlikely to be recognized as a non-self antigen by the immune system and thus to induce inflammatory reactions as many therapeutic peptides do. Finally, PEGylation of CD38₅₁₋₇₄ increased its HIV-1-inhibitory properties by approximately 1000 fold. This observation is in line with previous studies showing that PEGylation of proteins/peptides increases their therapeutic window [10]. The inhibitory concentrations displayed by CD38₅₁₋₇₄ PEG are comparable to those of clinically used and candidate HIV-1 fusion inhibitors. The effects of CD38₅₁₋₇₄ PEG on a step of HIV-1 attachment/fusion upstream to that affected by fusion inhibitors might represent a valuable addition to HAART.

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