REPORT

Identification of a new therapeutic target and a potential therapeutic candidate for the treatment of HIV infection

✍Celia Fernández-Ortega[1], Anna C Ramirez[1], Dionne Casillas[1], Taimi Paneque[1], Raimundo Ubieta[1], Marta Dubed[2], Leonor Navea[2], Lila Castellanos-Serra[1], Carlos Duarte[1], Viviana Falcón[1], Osvaldo Reyes[1], Hilda E Garay[1], Eladio Silva[2], Enrique Noa[2], Yassel Ramos[1], Vladimir Besada[1], Lázaro Betancourt[1]

[1] Dirección de Investigaciones Biomédicas, Centro de Ingeniería Genética y Biotecnología, CIGB
Ave. 31 entre 158 y 190, Cubanacán, Playa, CP 11600, La Habana, Cuba
[2] Laboratorio de Investigaciones sobre Sida, LISIDA
Carretera de Tapaste y Autopista Nacional, San José de las Lajas, CP 32700, Mayabeque, Cuba
✍celia.fernandez@cigb.edu.cu

ABSTRACT

A comparative proteomics analysis conducted in MT4 cells showed that the expression levels of vimentin were down-modulated after treatment with a fraction of the dialyzable leukocyte extract which shows anti-HIV-1 activity. The relationship of this intermediate filaments (IF) forming protein with the viral replication cycle was then elucidated by establishing a knockdown cell line expressing low levels of vimentin. The early stages of HIV-1 replication were safely evaluated in this cell line, by using a third-generation lentiviral vector which expressed the green fluorescent protein. Using this challenge system as well as the replicative HIV infection assay, it was evidenced for the first time that a reduction in vimentin expression levels drastically affects HIV-1 replication. It was demonstrated by transmission electron microscopy and fluorescence microscopy techniques that a synthetic peptide derived from the keratin-10 protein (denominated CIGB-210) modifies vimentin IFs, also showing a potent inhibitory activity and remarkably low cell toxicity. This makes of CIGB-210 a promising therapeutic candidate against HIV/AIDS. Altogether, our results showed that vimentin is involved in the early stages of viral infection and could be a potential target for the development of novel drugs against HIV/AIDS. This work was granted with the Annual Award of the National Academy of Sciences of Cuba for the year 2016.

Keywords: vimentin, intermediate filaments, cytoskeleton, HIV, anti-HIV activity, proteomics

Biotecnología Aplicada 2017;34:1501-1504

RESUMEN

Identificación de un nuevo blanco terapéutico y de un novedoso candidato a fármaco para el tratamiento de la infección por el virus de la inmunodeficiencia humana. Mediante un estudio de proteómica comparada se determinó que el tratamiento de la línea celular MT4 con una fracción leucocitaria con actividad anti-VIH-1, modula negativamente los niveles de vimentina. Para dilucidar la relación de esta proteína formadora de filamentos intermedios (FI) del citoesqueleto con la infección del VIH se generó una línea celular que expresa bajos niveles de vimentina. En esta línea celular se evaluaron las primeras etapas de la replicación del VIH de manera segura mediante un sistema basado en un vector lentiviral de tercera generación que expresa la proteína verde fluorescente. Utilizando este sistema de reto, así como el ensayo de infección con VIH replicativo, se evidenció por primera vez que la disminución en los niveles de vimentina reduce drásticamente la replicación viral. Se demostró mediante microscopía electrónica de trasmisión y microscopía de fluorescencia, que un péptido sintético derivado de la queratina 10 y denominado CIGB-210 modifica los FI de vimentina, exhibe una potente actividad inhibitoria y baja toxicidad celular, lo cual lo convierte en un promisorio candidato a fármaco contra el VIH/sida. En su conjunto, los resultados de esta investigación demostraron que la vimentina está involucrada en las etapas tempranas de la infección y que representa una diana potencial para el desarrollo de nuevos medicamentos contra el VIH/sida. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2016.

Palabras clave: vimentina, filamentos intermedios, citoesqueleto, VIH, actividad anti-VIH, proteómica

*I*ntroduction

Despite notable advances in antiretroviral therapy for human immunodeficiency virus (HIV) during the last decade, the acquired immunodeficiency syndrome (AIDS) continues to be one of the top ten causes of death worldwide, and the second in low-income countries [1]. Antiretroviral Therapy (ART) has been effective to control viral replication to minimum levels, reduce AIDS related comorbidities and increase the life expectancy of patients [2-4]. However, the long-term efficacy of ART is limited primarily by the emergence of antiretroviral-resistant viral variants, among other problems [5]. One of the most promising current research lines is the generation of inhibitors targeting cellular components involved in viral replication. This kind of inhibitors against genetically stable targets must, in theory, delay the emergence and selection of such resistant viral variants [5, 6]. Several candidates of this sort are currently at advanced stages of clinical development [7, 8].

The cytoskeleton is a dynamic cellular structure, integrated by many network-forming proteins [9]. Some of these proteins have been connected with different events of the HIV replication cycle [10]. Previous reports have associated vimentin, an intermediate filaments (IF) forming protein expressed in cells of mesenchymal origin, with some HIV proteins,

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although the specific functions of these interactions and their consequences for HIV replication remain to be established [11, 12].

Our group had previously described the presence of HIV replication inhibitory activity in the dialyzable leukocyte extract (DLE) *in vitro* [13], and particularly in one fraction of DLE denominated B1, when MT4 cells are treated for seven days before addition of the virus. In this work, we summarize a sequence of experiments that began with the finding that DLE is able to inhibit HIV replication in MT4 cells after 24 h incubation prior to HIV infection; continues with the identification of vimentin as a novel therapeutic target mediating such effect; and ends with the proposal of a vimentin IF network modulating peptide as a novel HIV drug candidate.

Results

DLE inhibits HIV replication in MT4 cells after 24 h incubation of cells prior to HIV infection

MT4 cells were treated for 24 h before HIV virus infection. DLE variants produced in both, virally induced leukocytes (DLEind) or non-induced leukocytes (DLEn/i), were tested. Cell cultures were treated with 0.3 U DLE per mL, for either 24 h or 7 days, and further infected with HIV-1 BRU strain at 0.05 or 0.1 multiplicity of infection (m.o.i.). Over 80 % of HIV-1 inhibition was observed when cells were pre-incubated for 24 h with DLE. These results allowed us to shorten the time of DLE treatment required to induce a non-permissive state for HIV replication in these cells to 24 h [14, 15].

The B1 fraction of DLE was able to inhibit HIV replication after 24 h incubation of MT4 cells

To further delimit the molecules responsible for the anti-HIV activity of DLE, the extract was fractionated by molecular exclusion chromatography and it was shown that one of the fractions, denominated B1, retained the inhibitory capacity after 24 h of MT4 cells pretreatment, but not after just 3 h [16].

Treatment of MT4 cells with B1 negatively downmodulates vimentin expression

A comparative proteomics study was conducted in MT4 cells, by comparing cells treated with B1 for 24 and 3 h, respectively. Untreated cells were used as controls. The underlying hypothesis was that some of the proteins modulated after 24 h but not after 3 h of B1 treatment could be involved in the observed antiviral effect. A marked reduction in vimentin expression was documented after 24 h treatment, whereas this protein only experienced a modest reduction after 3 h of incubation with B1. Hence, we hypothesized that a causal relationship could exist between fraction B1, the reduced vimentin expression and the inhibition of HIV replication [16].

Generation of a cell line with a permanent reduction of vimentin levels and a control cell line

To further investigate the relationship between vimentin and HIV, a knockdown MT4 cell line was constructed through the introduction of a shRNA (short hairpin RNA) specific for the vimentin gene into its genome (MT4sh/Vim). This cell line was generated by transduction of MT4 cells with a lentiviral vector (pLenti-shRNAvim) expressing the shRNA specific for vimentin. In parallel, a control shRNAvim-deficient cell line was generated by transduction with a modified variant of the lentiviral vector lacking the expression cassette with the shRNA and the U6 promoter (MT4mock). Both cell lines showed similar morphology and doubling times as compared to the parental MT4 cell line they originated from [15]. This work allowed us to have a vimentin knockdown cell line and its control to further analyze the underlying role of vimentin in HIV replication [16].

The permanent reduction of vimentin levels in MT4sh/Vim cells inhibits the early stages of HIV replication

To determine if the reduction of vimentin levels would affect any of the early events taking place during HIV infection in vitro, the MT4sh/Vim and MT4mock cell lines were challenged with a HIV-1-based lentiviral vector (pLGW) expressing the enhanced green fluorescence protein (eGFP). The MT4sh/Vim and MT-4mock cell lines were incubated with the pLGW at different m.o.i. (1, 5 and 10) and eGFP expression was monitored after 72 h of culture by flow cytometry and fluorescence microscopy. In this experiment, a marked reduction of the eGFP expression in the MT4sh/Vim cell line was evidenced, as compared to the intense fluorescence observed in the MT4mock cell line for all m.o.i. tested (Figure 1A). Microscopy results were

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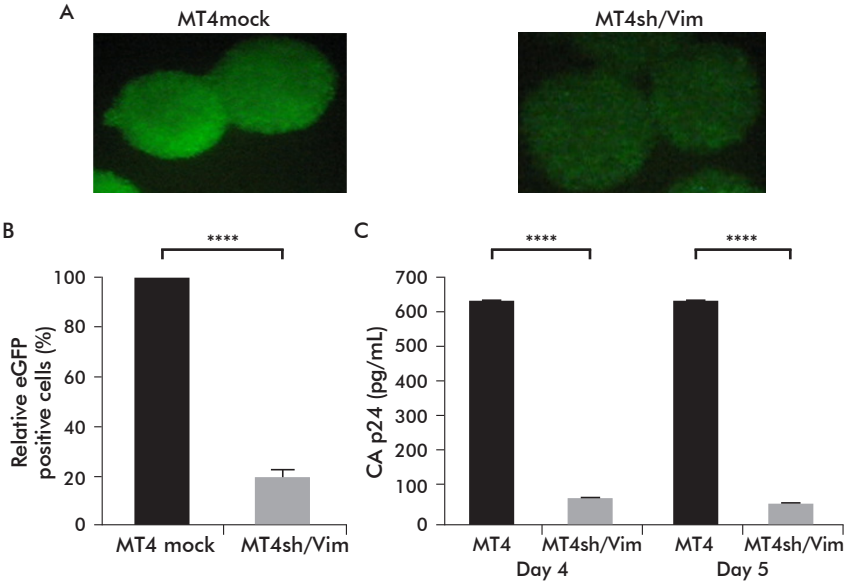

Figure 1. Vimentin silencing reduces the expression of an HIV-1 based lentiviral vector and a competent HIV-1. A) Fluorescence microscopy of MT4sh/Vim and MT4mock cell lines cultures, transduced with an eGFP expressing the lentiviral vector pLGW. 10× magnification. B) Relative percentage of eGFP positive cells measured by flow cytometry in MT4sh/Vim and MT4mock cells transduced with pLGW. Data are representative of three experiments. C) MT4sh/Vim and MT4 were infected with the HIV-1 BRU viral strain at a m.o.i. of 0.001. Virus was removed 1 h after viral challenge and CAp24 antigen was measured by ELISA at days 4 and 5 after infection. Samples were run in triplicate and the experiment was repeated three times. Data represent the mean ± standard deviation of one representative experiment. Bonferroni multiple comparisons test was applied, (****$p < 0.0001$). Adapted from: Fernández-Ortega C, *et al*. Viruses. 2016;(8):98. doi:10.3390/v8060098.

confirmed by quantification of the number of fluorescent cells by flow cytometry, where a decrease of about 80% in the number of fluorescent cells in MT4sh/Vim was found (Figure 1B). These evidences suggested that vimentin is involved in at least one of the early steps of the HIV replication cycle, specifically in one or several of the events mediating from the release of the viral nucleocapsid into the cytoplasm until the integration of the proviral DNA into the cell genome and its subsequent transcription [16, 17].

The permanent reduction of vimentin levels in MT4sh/Vim cells inhibits the replication of an HIV infective strain

We next investigated whether the reduction of vimentin was also able to inhibit the replication of an HIV infective strain in a multiround infection assay. For this, the MT4sh/Vim and MT4 cell lines were infected with the BRU strain. Viral p24 antigen production was reduced in more than 90 % in the MT4sh/Vim cell line, as compared with the MT4 cell line (Figure 1C). These results provided strong evidence supporting the idea that reduced vimentin levels certainly affect HIV replication, further indicating that this protein plays an important role in the HIV replication cycle [16, 17].

A synthetic peptide of keratin-10 is capable of inhibiting HIV-1 replication

To validate the potentiality of vimentin as a target for ART against HIV, we designed, synthesized and tested the effect of various peptides from the central domain 1A region of vimentin and keratin-10, previously associated with the disassembly of vimentin IFs [20]. In particular, an 18 mer peptide derived from the 1A region of keratin-10 (coined CIGB-210), was evaluated in an inhibition assay with the HIV-1 BRU strain. MT4 cells were pre-treated for 24 h with the peptide prior to a viral challenge at m.o.i. 0.001. A strong inhibition of HIV replication was evidenced, with an IC_{50} estimated at 7.92 ± 1.76 nM (Figure 2A). On the other hand, CIGB-210 show very low cell toxicity on the MT4 cell line, with a CC_{50} of 1702 ± 255 μM. These values were very encouraging for a drug candidate in the early stages of development, because the effective therapeutic concentration is very distant from the cytotoxic concentration. These evidences made CIGB-210 an attractive drug candidate against HIV-1 [16].

Effect of CIGB-210 on the intermediate filaments of vimentin

To corroborate the effect of CIGB-210 on vimentin IFs, MT4 cells treated with CIGB-210 for 24 h were analyzed by transmission electron microscopy [17] and by fluorescence microscopy. The images obtained by both techniques evidenced changes in the IF structure in the cells treated with CIGB-210. Fluorescence microscopy showed a rearrangement of vimentin IF network around the nucleus (Figure 2B). These results revealed that CIGB-210 specifically causes a rearrangement of vimentin filaments in the MT4 cell line, where it exerts anti-HIV-1 activity [16].

CIGB-210 is able to penetrate the MT4 cells

A very relevant question to elucidate the mechanism of action of CIGB-210 is whether this peptide is able to cross the plasma membrane and interact with vimentin, or other proteins, directly inside the cell. To answer this question, MT4 cells were treated with 10, 20 or 40 μM of fluorescein-labeled CIGB-210 for 15 min, 60 min or 24 h. The percentages of fluorescent cells, determined by flow cytometry, increased proportionally to the peptide concentration and the incubation time [16] (Figure 2C). An external fluorescence quenching step with Trypan Blue was performed to exclude the possibility that the signal recorded by the flow cytometer were caused by peptides anchored on the cell surface [18]. The maximum percentage of internalization of CIGB-210 in MT4 was 83 ± 3 % fluorescent cells, after 24 h of incubation at 40 μM. Moreover, a biotinylated variant of CIGB-210 was visualized in the cells cytoplasm by fluorescence microscopy [16]. These results demonstrate that CIGB-210 is able to penetrate the plasma membrane without the aid of a penetrating peptide.

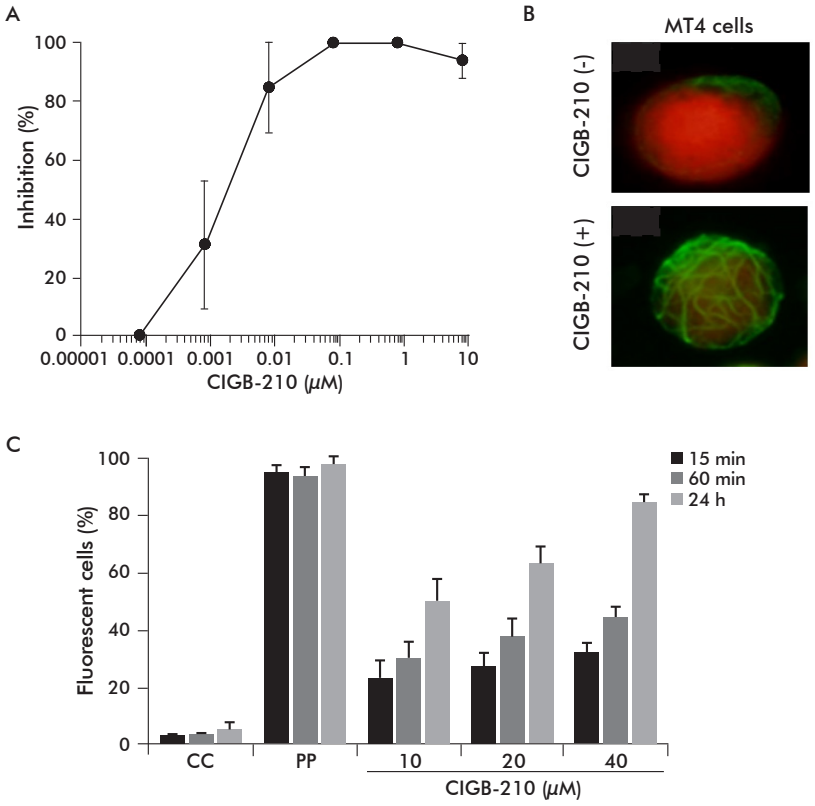

Figure 2. Anti-HIV activity, effect on FIs and uptake of the CIGB-210 peptide in the MT4 cell line. A) MT4 cells were treated with different concentrations of CIGB-210 for 24 h, prior to HIV-1BRU infection (m.o.i. = 0.001). CIGB-210 was added again after infection. CAp24 was measure five days post infection. The data shown are average values ± standard deviation for three experiments. IC50 of 7.92 ± 1.76 nM was calculated using CalcuSyn software. B) Vimentin IFs from cultured MT4 cells for 24 hours without CIGB-210, and treated for 24h with 40 μM CIGB-210. Arrows IF: Intermediate filaments, N: Nucleus. Bar = 0.2 μm, Red: Cell nucleus, Green: Vimentin. C) CIGB-210 uptake assessed by flow cytometry. CC: Control untreated MT4 cells; PP: fluorescein labeled peptide containing the Tat cell penetrating peptide used as positive control. Data represent the mean ± standard deviation of three experiments performed in triplicate. Adapted from: Fernández-Ortega C, *et al*. Viruses. 2016;(8):98. doi:10.3390/v8060098.

*R*elevance of the study

The theoretical novelty of this work can be summarized in the following aspects: i) Vimentin was

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identified as one of the proteins modulated by the action of DLE on MT4 cells. ii) It was shown for the first time, by using a vimentin knockdown cell line that a decrease in the levels of this protein in MT4 cells inhibits HIV replication. iv) This was the first report of the inhibition of HIV by a keratin-10 peptide, with an IC50 in the low nanomolar range and very low cell toxicity, making it a first-in-class drug candidate against HIV.

This study also provided the first documented evidence on the modification of the IF vimentin network as a feasible way to reduce HIV replication, at least *in vitro*. Overall, the results mentioned here strongly suggest that vimentin may be a novel and attractive host cell target molecule for HIV/AIDS therapy.

The practical importance of this research was that it not only defined vimentin as a potential target for AIDS, but also identified a peptide displaying a potent antiviral activity and a very attractive safety index. Particularly relevant is the property of this peptide to target vimentin, a highly stable cellular protein. These results set the stage for the development of this peptide as a drug candidate, with potential application against HIV/AIDS in humans. The process of preclinical development of this peptide, named CIGB-210, is currently ongoing.

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