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Bovine Lactoferrampin, Human Lactoferricin, and Lactoferrin 1-11 Inhibit Nuclear Translocation of HIV Integrase

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Abstract This study aimed to investigate fragments derived from human and bovine lactoferrins for ability to inhibit nuclear translocation of HIV-1 integrase. It was shown that human lactoferricin, human lactoferrin 1-11, and bovine lactoferrampin reduced nuclear distribution of HIV-1 integrase. Bovine lactoferrampin could inhibit both the activity and nuclear translocation of HIV-1 integrase. Human lactoferrampin, bovine lactoferricin, and bovine lactoferrin 1-11 had no effect on HIV-1 integrase nuclear translocation. Human lactoferrampin which inhibited the activity of integrase did not prevent its nuclear translocation. Human lactoferricin and lactoferrin 1-11 did not inhibit HIV-1 integrase nuclear translocation despite their ability to attenuate the enzyme activity. The discrepancy between the findings on reduction of HIV-1 activity and inhibition of nuclear translocation of HIV-1 integrase was due to the different mechanisms involved. A similar reasoning can also be applied to the different inhibitory potencies of the milk peptides on different HIV enzymes, i.e., nuclear translocation.

Keywords HIV-1 integrase · Nuclear translocation · Milk peptides

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Introduction

Lactoferrin is a mammalian single-chain iron-binding glycoprotein in whey with a molecular weight in the vicinity of 80 kDa. Its diversity of activities includes bone regenerating, anti-obesity, anti-viral, anti-bacterial, and immunomodulatory activities [1–6]. Oral administration of lactoferrin exerted an immunomodulatory action in HIV-infected, anti-retroviral therapy-naïve children [7]. Many protein anti-HIV activities, including trypsin inhibitor, lectins, and ribonuclease, have been reported [8–10]. The lactoferrin-derived peptides lactoferricin and lactoferrampin display anti-bacterial, anti-viral, anti-fungal, anti-inflammatory, immunomodulatory, anti-angiogenic, and anti-metastatic activities [11].

Bovine lactoferrin exerted pronounced HIV-1 reverse transcriptase inhibitory activity but only meager suppressive activity on HIV-1 protease and integrase. In the presence of bovine and human lactoferrin fragments, the activities of reverse transcriptase, protease, and integrase of the HIV-1 were attenuated [12]. It was found that human lactoferricin attenuated the activity of HIV-1 reverse transcriptase with the highest potency ($IC_{50} = 2$ μM), followed by bovine lactoferricin ($IC_{50} = 10$ μM) which had a dissimilar sequence, and then bovine lactoferrampin ($IC_{50} = 150$ μM). Bovine lactoferrin [1–11], human lactoferrin [1–11], and human lactoferrampin failed to affect HIV-1 reverse transcriptase activity at 1 mM concentration. Bovine but not human lactoferriampin inhibited HIV-1 RT, although they were structurally similar. The peptides exhibited only a weak (from slightly below 2 to 6 % inhibition) HIV-1 protease inhibitory activity [13].

Integrase is not present in humans, and this explains why HIV-1 integrase is a target for combating HIV [14]. In recent years, the research on HIV-1 integrase inhibitors has been intensive [15]. A Triumeq® tablet which contains dolutegravir (an integrase strand transfer inhibitor) and abacavir and lamivudine nucleoside/nucleotide reverse transcriptase inhibitors is deployed against HIV-1 [16]. Dolutegravir appears to be the best HIV-1 integrase inhibitor [17]. Nevertheless, resistance may arise under certain circumstances [18–20].

Research efforts have been directed to inhibitors of nuclear translocation of HIV-1 integrase [21]. We have previously identified some lactoferrin-derived peptides as inhibitors of HIV-1 integrase activity. In view of the fact that inhibition of HIV-1 integrase activity and inhibition of nuclear translocation of HIV-1 are two different processes, we tested the lactoferrin-derived peptides for the ability to inhibit nuclear translocation of the retroviral enzyme in the present investigation.

Materials and Methods

Peptide Synthesis

All peptides were synthesized following manual solid phase synthesis protocols and were synthesized by Shanghai Biotech Bioscience and Technology Company, China. Purity was confirmed by high-performance liquid chromatography (HPLC) analyses. Mass spectrum was also used for confirmation of product quality. Fmoc solid-phase peptide synthesis was employed. In the case of bovine lactoferricin (peptide with free C-terminal carboxyl group), the C-terminal amino acid residue (Fmoc-Ser(tBu)) was attached to the 2-chlorotrityl chloride resin (substitution of Cl 1.46 meq/g; Calbiochem-Novabiochem AG, Switzerland) in the presence of an equimolar amount of DIPEA in relation to the amino acid in anhydrous

DCM solution. Peptide chains were elongated in the consecutive cycles of deprotection and coupling. Deprotection was performed with 20 % piperidine in the mixture of DMF/NMP (1:1, *v*/*v*) with addition of 1 % Triton X-100, whereas the chain elongation was achieved with standard DIC/HOBt chemistry; three equivalents of protected amino acid derivatives were used. After completing the syntheses, the peptides were cleaved from the resin simultaneously with the side chain deprotection in a one-step procedure, using a mixture of TFA/phenol/triisopropylsilane/H2O (88:5:2:5, *v*/*v*/*v*/*v*). The crude peptides were purified by HPLC on a Beckman Gold System (Beckman, USA) using an RP Kromasil-100, C8, 5-μm column (8 × 250 mm; Knauer, Germany). The solvent system was 0.1 % TFA (A) and 80 % acetonitrile in A (B). Either isocratic conditions or a linear gradient were applied (flow rate 3.0 ml/min, monitored at 226 nm). The purity of the synthesized peptides was checked on another RP Kromasil 100, C8, 5-μm column (4.6 × 250 mm) (Knauer, Germany). The solvent system was 0.1 % TFA (A) and 80 % acetonitrile in A (B). Linear gradient from 10 to 90 % B in 40 min with a flow rate 1 ml/min was employed and monitored at 226 nm. The mass spectrometry analysis was carried out on a MALDI MS (a Biflex III MALDI-TOF spectrometer, Bruker Daltonics, Germany) using α-cyano-4-hydroxycinnamic acid as a matrix. The analysis confirmed the correctness of molecular weight values of all six synthesized peptides [22].

Assay of Hemolytic Activity

Rabbit erythrocytes were washed four times with 10 mM phosphate-buffered saline (PBS; pH 7.5) and adjusted to a final concentration of 2 % (*v*/*v*) in PBS. A sample solution (50 μl) was mixed with rabbit erythrocytes (50 μl) and incubated at room temperature for 1 h before centrifugation at 400×*g* for 5 min. The amount of hemoglobin released from disrupted erythrocytes was determined spectrophotometrically. One-hundred-percent hemolysis was defined as OD540 of hemoglobin released from erythrocytes treated with 0.1 % Triton X-100.

Assay of Inhibitory Activity on Translocation of HIV-1 Integrase into the Nucleus

The translocation of HIV-1 integrase assay was conducted by transfected pcDNA3.1/HIV IN into HeLa cells, and the HIV-IN nuclear distribution was probed by using both nuclear protein western blot and localization image analysis.

First of all, HeLa Tet-Off Advanced Cells (Clontech) were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 μg/ml G418, and 1 % of streptomycin–penicillin (Invitrogen) at 37 °C in a 5 % CO_2 incubator. Twenty-four hours before transfection, 5×10^6 HeLa Tet-Off Advanced Cells were seeded onto a 12-well plate (BD) in DMEM containing 10 % FBS. IN expression vector was transfected into HeLa Tet-Off Advanced Cells using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instruction. Then, the medium was removed 4 h after transfection. Fresh medium containing test peptide was added. Compound *Y*, a newly synthesized compound from our collaborator, was used as a positive control. At the end of another 24 h, for western blot analysis, the cells were rinsed with PBS, dislodged, pelleted by centrifugation, and resuspended in the cell lysis buffer [10 mM HEPES; pH 7.5, containing 0.5 mM PMSF, 0.5 % Nonidet-40, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 10 mM KCl, together with the protease inhibitor cocktail (Sigma)] and left to swell on ice for 15–20 min with intermittent mixing. Tubes were vortexed to disrupt cell membranes followed by centrifugation for 10 min at 12,000*g* and 4 °C. The supernatant was discarded. The nuclei in the

pellet were rinsed thrice with the cell lysis buffer and resuspended in the nuclear extraction buffer containing 20 mM HEPES (pH 7.5), 1 mM PMSF, 1 mM DTT, 1 mM EDTA, and 400 mM NaCl, with protease inhibitor cocktail, followed by incubation in ice for 30 min. After centrifugation at 12,000*g* for 15 min at 4 °C, the nuclear extract was collected. Determination of protein concentration of the nuclear extract was carried out using Bradford's reagent (Bio-Rad, USA). The extract was immediately used for western blot.

For localization image analysis, after 24-h transfection, cells were fixed in 4 % paraformaldehyde in PBS for 15 min at room temperature. The cells were rinsed in PBS, postfixed, and made permeable in 0.5 % Triton X-100 at room temperature for 15 min. After rinsing with PBS, the cells were incubated in 3 % BSA for 1 h, washed in PBS, and incubated in anti-integrase-1 antibody (Santa Cruz Biotechnology) diluted with PBS containing 1 % BSA for 1 h at 37 °C in a humid chamber. Then, cells were washed for 30 min in three changes of PBS at room temperature and then incubated in FITC-conjugated antibody (Santa Cruz Biotechnology) diluted with PBS containing 1 % bovine serum albumin for 1 h at 37 °C in a humid chamber. Then, cells were washed as before. Cells were stained with PI (1 μg/ml) for 20 min, and pictures were taken under a confocal fluorescence microscope.

Results

Peptide Synthesis

The amino acid sequences of the peptides used in this study are presented in Table 1, and their molecular masses are presented in Fig. 1.

Assay of Nuclear Translocation of HIV-1 Integrase

Western blot results for HIV-1 integrase nuclear translocation assay indicated that human lactoferricin (peptide 1), human lactoferrin 1-11 (peptide 2), bovine lactoferrampin (peptide 6), and compound *X* (positive control) could significantly reduce HIV-1 integrase nuclear distribution. Reduced protein concentration of HIV-1 integrase/lamin B1 was observed as indicated by the attenuated HIV-1 integrase after treatment with band intensity human lactoferricin

Table 1 Amino acid sequences of human and bovine lactoferricins, lactoferrins 1-11, and lactoferrampins

Abbreviation	Amino acid sequence	Molecular mass	Name of peptide
Peptide no. 1	NH_2-GRRRRSVQWCAVSQPEATKCFQWQRNMRK VRGPPVSCIKRDSPIQEIQA-COOH	5743	Human lactoferricin
Peptide no. 2	NH_2-GRRRRSVQWCA-COOH	1374	Human lactoferrin 1-11
Peptide no. 3	NH_2-WNLLRQAQEKFGKDKSPK-COOH	2174	Human lactoferrampin
Peptide no. 4	NH_2-MFKCRRWQWRMKKLGAPSITCVRRAF-COOH	3257	Bovine lactoferricin
Peptide no. 5	NH_2-MFKCRRWQWR-COOH	1628	Bovine lactoferrin 1-11
Peptide no. 6	NH_2-DLIWKLLSKAQEKFGKNKSR-COOH	2389	Bovine lactoferrampin

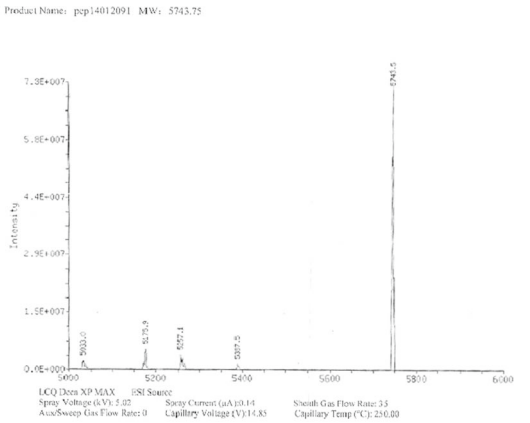
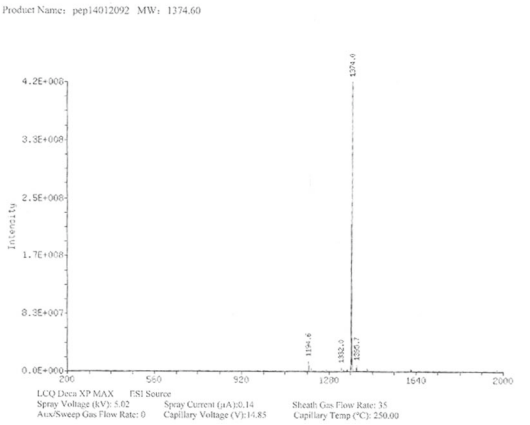
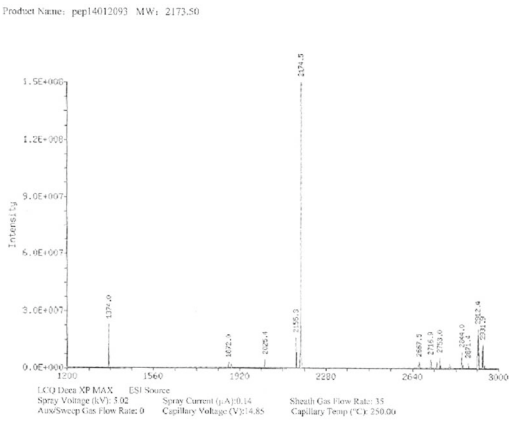
Peptides	Mass spectrometric analysis of peptides
Peptide 1	
Peptide 2	

Fig. 1 Mass spectrometric analysis of peptides

(peptide 1), human lactoferrin 1-11 (peptide 2), and bovine lactoferrampin (peptide 6). However, human lactoferrampin (peptide 3), bovine lactoferricin (peptide 4), and bovine lactoferrin 1-11 (peptide 5) and raltegravir did not inhibit HIV-1 integrase nuclear distribution (Fig. 2). A comparison with previously published data on HIV-1 integrase inhibitory activity revealed that only bovine lactoferrampin was capable of inhibiting nuclear translocation as well as activity of HIV-1 integrase.

Figure 3 shows green HIV integrase staining with FITC, red nucleus staining with PI, and merged picture obtained after treatment with the various peptides. Accumulation of HIV integrase in the cytoplasm signifying inhibition of nuclear translocation of HIV integrase

Peptide 3



Peptide 4

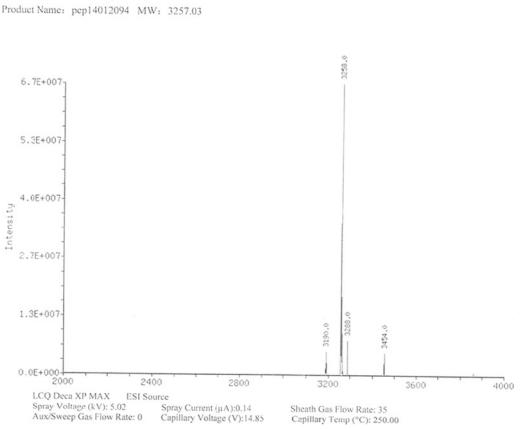
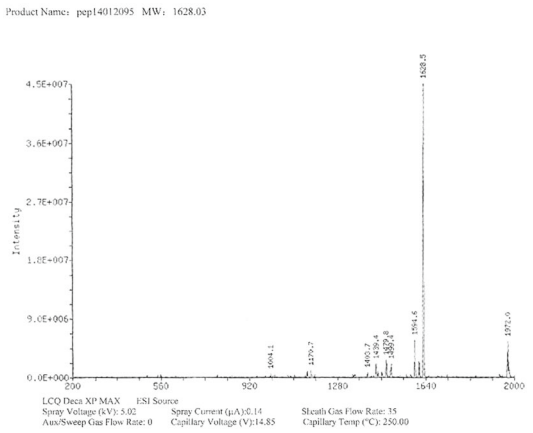


Fig. 1 (continued)

was observed following treatment with human lactoferricin (peptide 1), human lactoferrin 1-11 (peptide 2), bovine lactoferrampin (peptide 6), and compound *X* (positive control) but not after treatment with human lactoferrampin (peptide 3), bovine lactoferricin (peptide 4), and bovine lactoferrin 1-11 (peptide 5). The merged picture shows merging of the green and red colors after treatment with human lactoferrampin (peptide 3), bovine lactoferricin (peptide 4), and bovine lactoferrin 1-11 (peptide 5), indicating absence of an inhibitory action on nuclear translocation of HIV integrase. The green fluorescence due to HIV integrase did not merge with the red nucleus staining with PI after treatment with human lactoferricin (peptide 1), human lactoferrin 1-11 (peptide 2), bovine lactoferrampin (peptide 6), and compound *X* (positive control) indicating an inhibitory action on nuclear translocation of HIV integrase.

Peptide 5



Peptide 6

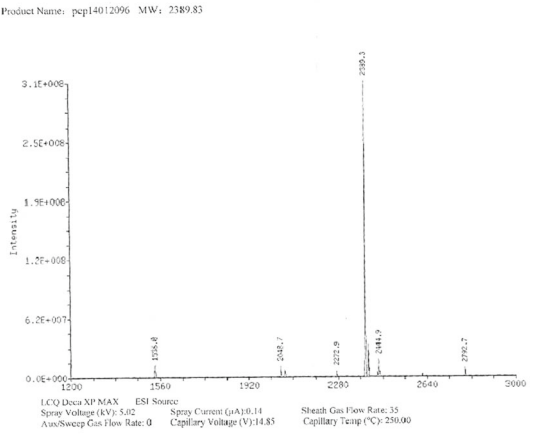


Fig. 1 (continued)

Discussion

A great deal of effort has been dedicated to ascertain compounds with ability to inhibit nuclear translocation of HIV-1 integrase which is a focus of anti-HIV research. 3-(1,3-benzothiazol-2-yl)-8-{[bis(2-hydroxyethyl)amino]methyl}-7-hydroxy-2H-chrom en-2-one (D719) demonstrated HIV-1 integrase nuclear translocation inhibitory activity in cell imaging [23]. 1,4-bis(5-(naphthalen-1-yl)thiophen-2-yl) naphthalene [24] and wikstroelide obstructed nuclear translocation of HIV-1 integrase by attenuating the interaction between LEDGF/p75 and the enzyme. Importin α3 (Impα3) interacts with HIV-1 integrase via the (211) KELQKQITK and (262) RRKAK regions of the C-terminal domain of retroviral enzyme and plays a role in viral cDNA nuclear import [25]. Ivermectin hindered importin α/β-mediated transport of HIV-1

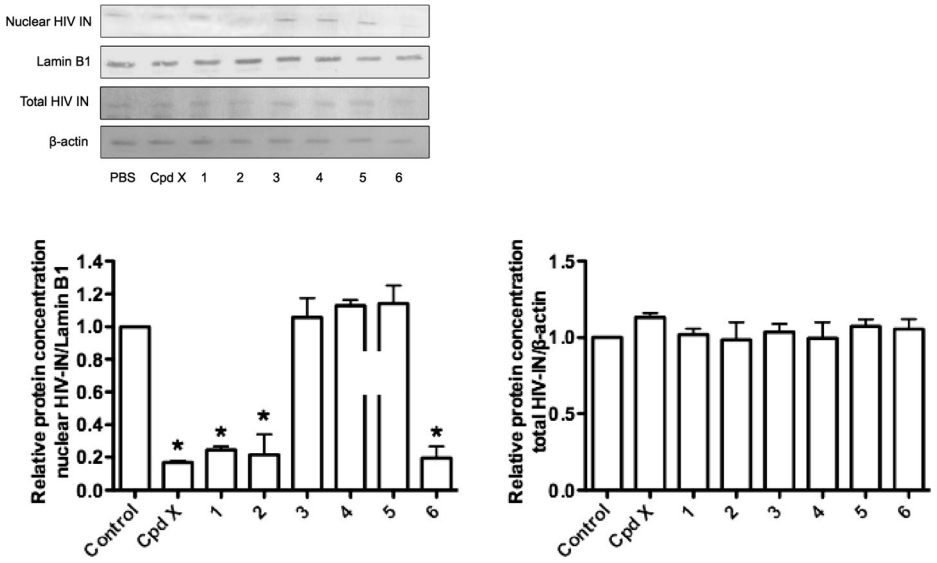


Fig. 2 Western blot results for HIV-IN translocation assay (*upper panel*) and graphical presentation of western blot results in Fig. 1 after scanning of band intensities (*lower panel*). At *lower panel*, *left-hand-side graph* is showing the concentration of HIV IN in nucleus; *right-hand-side graph* is showing the concentration of HIV IN in whole cells. *Numbers* 1 to 6 present results for peptides 1 to 6, respectively. Data represent mean ± SD ($n = 3$). *Asterisk* indicates statistically significant difference ($p < 0.01$) compared with the other treatments (control, peptide 3, peptide 4, and peptide 5) when analyzed by ANOVA

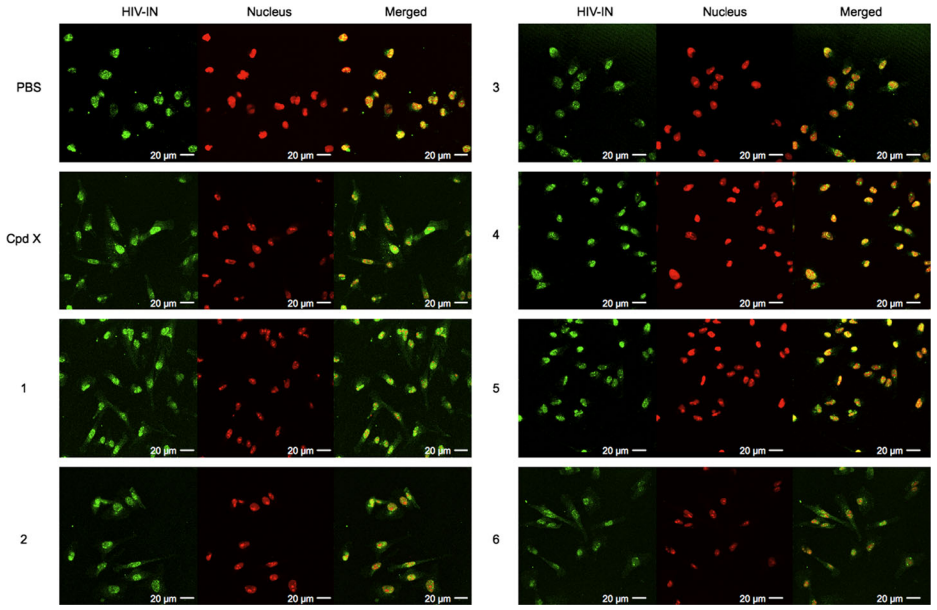


Fig. 3 Observation under a confocal fluorescence microscope for HIV-IN nuclear translocation assay. Human lactoferricin (peptide 1), human lactoferrin 1-11 (peptide 2), and bovine lactoferrampin (peptide 6) could reduce HIV-1 IN transport into cell nucleus. The *white arrows* pointed at the accumulation of HIV IN in the cytoplasm. Compound *Y* employed as a positive control in the present study was the next generation of compound *X* which was used as a positive control for the HIV-IN translocation assay

integrase and potently inhibited HIV-1 [26]. Mifepristone impeded nuclear translocation of HIV-1 integrase [27]. LEDGINs are HIV inhibitors targeting interaction between HIV integrase and LEDGF/p75 which is a coactivator of cellular transcription employed by HIV to tie the preintegration complex to the chromosome. Transportin-SR2 is utilized by HIV for nuclear import [28].

HIV-1 preintegration complex-associated integrase binds to the C-terminal domain of nucleoporin NUP153 for nuclear import of the preintegration complex [29]. The HIV-1 Rev protein facilitates nuclear export and prevents nuclear import of HIV-1 integrase [30]. Peptides that disrupt interaction of HIV-1 integrase with either importin α or TNPO3 impede nuclear import of the integrase-DNA complex and HIV-1 infection [31]. Amino acid residues in the C-terminal domain of HIV integrase play a role in HIV-1-cellular protein interaction. Importin alpha3, an HIV integrase-interacting cofactor, expedites nuclear import and replication of HIV-1 in cells undergoing cell division and also in nondividing cells [32].

Hence, the mechanism of inhibitory action of human lactoferricin, human lactoferrin 1-11, and bovine lactoferrampin on nuclear translocation of HIV-1 integrase awaits elucidation. We believe that the peptides inhibit IN nuclear translocation through blockade of p75/HIV-IN interaction. It is likely that these lactoferrin-derived peptides interact with some of the aforementioned entities. The efficacy of bovine lactoferrampin in attenuating HIV-1 integrase activity and preventing HIV-1 integrase nuclear translocation makes it a promising candidate for development into an anti-HIV-1 drug. Attempts have been made to search for natural products and synthetic compounds with an inhibitory action on activity of HIV enzymes including HIV-1 integrase and on HIV-1 integrase nuclear translocation [33–36]. The finding of bovine lactoferrampin with suppressive activity on both HIV-1 integrase activity and on HIV-1 integrase nuclear translocation may facilitate anti-HIV therapy.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

References

1. Włodarski, K. H., Galus, R., Brodzikowska, A., Włodarski, P. K., & Wojtowicz, A. (2014). The importance of lactoferrin in bone regeneration. *Polski merkuriusz lekarski : organ Polskiego Towarzystwa Lekarskiego, 37*(217), 65–67.
2. Brimelow RE, West NP, Williams LT, Cripps AW, Cox AJ. A role for whey-derived lactoferrin and immunoglobulins in the attenuation of obesity-related inflammation and disease? Crit Rev Food Sci Nutr. 2015 [Epub ahead of print].
3. Redwan, E. M., Uversky, V. N., El-Fakharany, E. M., & Al-Mehdar, H. (2014). Potential lactoferrin activity against pathogenic viruses. *Comptes Rendus Biologies, 337*(10), 581–595.
4. Tang, X. S., Shao, H., Li, T. J., Tang, Z. R., Huang, R. L., Wang, S. P., Kong, X. F., Wu, X., & Yin, Y. L. (2012). Dietary supplementation with bovine lactoferrampin-lactoferricin produced by *Pichia pastoris* fed-batch fermentation affects intestinal microflora in weaned piglets. *Applied Biochemistry and Biotechnology, 168*(4), 887–898.

5. Tang, X. S., Tang, Z. R., Wang, S. P., Feng, Z. M., Zhou, D., Li, T. J., & Yin, Y. L. (2012). Expression, purification, and antibacterial activity of bovine lactoferrampin-lactoferricin in *Pichia pastoris*. *Applied Biochemistry and Biotechnology, 166*(3), 640–651.
6. Siqueiros-Cendón, T., Arévalo-Gallegos, S., Iglesias-Figueroa, B. F., García-Montoya, I. A., Salazar-Martínez, J., & Rascón-Cruz, Q. (2014). Immunomodulatory effects of lactoferrin. *Acta Pharmacologica Sinica, 35*(5), 557–566.
7. Zuccotti, G. V., Vigano, A., Borelli, M., Saresella, M., Giacomet, V., & Clerici, M. (2007). Modulation of innate and adaptive immunity by lactoferrin in human immunodeficiency virus (HIV)-infected, antiretroviral therapy-naïve children. *International Journal of Antimicrobial Agents, 29*(3), 353–355.
8. Fang, E. F., & Ng, T. B. (2015). A trypsin inhibitor from rambutan seeds with antitumor, anti-HIV-1 reverse transcriptase, and nitric oxide-inducing properties. *Applied Biochemistry and Biotechnology, 175*(8), 3828–3839.
9. Yuan, S., Yan, J., Ye, X., Wu, Z., & Ng, T. (2015). Isolation of a ribonuclease with antiproliferative and HIV-1 reverse transcriptase inhibitory activities from Japanese large brown buckwheat seeds. *Applied Biochemistry and Biotechnology, 175*(5), 2456–2467.
10. Ng, T. B., Chan, Y. S., Ng, C. C., & Wong, J. H. (2015). Purification and characterization of a lectin from green split peas (*Pisum sativum*). *Applied Biochemistry and Biotechnology, 177*(6), 1374–1385.
11. Yin, C., Wong, J. H., & Ng, T. B. (2014). Recent studies on the antimicrobial peptides lactoferricin and lactoferrampin. *Current Molecular Medicine, 14*(9), 1139–1154.
12. Ng, T. B., Lam, T. L., Au, T. K., Ye, X. Y., & Wan, C. C. (2001). Inhibition of human immunodeficiency virus type 1 reverse transcriptase, protease and integrase by bovine milk proteins. *Life Sciences, 69*(19), 2217–2223.
13. Wong, J. H., Liu, Z., Law, K. W., Liu, F., Xia, L., Wan, D. C., & Ng, T. B. (2014). A study of effects of peptide fragments of bovine and human lactoferrins on activities of three key HIV-1 enzymes. *Peptides, 62*, 183–188.
14. Li, Y., Xuan, S., Feng, Y., & Yan, A. (2015). Targeting HIV-1 integrase with strand transfer inhibitors. *Drug Discovery Today, 20*(4), 435–449.
15. Blanco, J. L., Whitlock, G., Milinkovic, A., & Moyle, G. (2015). HIV integrase inhibitors: a new era in the treatment of HIV. *Expert Opinion on Pharmacotherapy, 16*(9), 1313–1324.
16. Greig, S. L., & Deeks, E. D. (2015). Abacavir/dolutegravir/lamivudine single-tablet regimen: a review of its use in HIV-1 infection. *Drugs, 75*(5), 503–514.
17. Wainberg, M. A., & Han, Y. S. (2015). Will drug resistance against dolutegravir in initial therapy ever occur? *Frontiers in Pharmacology, 6*, 90.
18. Depatureaux A, Mesplède T, Quashie P, Oliveira M, Moisi D, Plantier JC, Brenner B, Wainberg MA. HIV-1 group O resistance against integrase inhibitors. Journal of Acquired Immune Deficiency Syndromes. 2015 [Epub ahead of print]
19. Seki, T., Suyama-Kagitani, A., Kawauchi-Miki, S., Miki, S., Wakasa-Morimoto, C., Akihisa, E., Nakahara, K., Kobayashi, M., Underwood, M. R., Sato, A., Fujiwara, T., & Yoshinaga, T. (2015). Effects of raltegravir or elvitegravir resistance signature mutations on the barrier to dolutegravir resistance in vitro. *Antimicrobial Agents and Chemotherapy, 59*(5), 2596–2606.
20. Smith, R. A., Raugi, D. N., Pan, C., Sow, P. S., Seydi, M., Mullins, J. I., Gottlieb, G. S., & University of Washington-Dakar HIV-2 Study Group. (2015). In vitro activity of dolutegravir against wild-type and integrase inhibitor-resistant HIV-2. *Retrovirology, 12*, 10.
21. Zhang, X., Huang, S. Z., Gu, W. G., Yang, L. M., Chen, H., Zheng, C. B., Zhao, Y. X., Wan, D. C., & Zheng, Y. T. (2014). Wikstroelide M potently inhibits HIV replication by targeting reverse transcriptase and integrase nuclear translocation. *Chin J Nat Med, 12*(3), 186–193.
22. Wong, J. H., Legowska, A., Rolka, K., Ng, T. B., Hui, M., Cho, C. H., et al. (2011). Effects of cathelicidin and its fragments on three key enzymes of HIV-1. *Peptides, 32*, 1117–1122.
23. Gu, W. G., Zhang, X., Ip, D. T., Yang, L. M., Zheng, Y. T., & Wan, D. C. (2014). Discovery of a novel HIV-1 integrase inhibitor from natural compounds through structure based virtual screening and cell imaging. *FEBS Letters, 588*(18), 3461–3468.
24. Gu, W. G., Ip, D. T., Liu, S. J., Chan, J. H., Wang, Y., Zhang, X., Zheng, Y. T., & Wan, D. C. (2014). 1,4-Bis(5-(naphthalen-1-yl)thiophen-2-yl)naphthalene, a small molecule, functions as a novel anti-HIV-1 inhibitor targeting the interaction between integrase and cellular Lens epithelium-derived growth factor. *Chemico-Biological Interactions, 213*, 21–27.
25. Jayappa, K. D., Ao, Z., Yang, M., Wang, J., & Yao, X. (2011). Identification of critical motifs within HIV-1 integrase required for importin α3 interaction and viral cDNA nuclear import. *Journal of Molecular Biology, 410*(5), 847–862.

26. Wagstaff, K. M., Sivakumaran, H., Heaton, S. M., Harrich, D., & Jans, D. A. (2012). Ivermectin is a specific inhibitor of importin α/β-mediated nuclear import able to inhibit replication of HIV-1 and dengue virus. *The Biochemical Journal, 443*(3), 851–856.
27. Wagstaff, K. M., Rawlinson, S. M., Hearps, A. C., & Jans, D. A. (2011). An AlphaScreen®-based assay for high-throughput screening for specific inhibitors of nuclear import. *Journal of Biomolecular Screening, 16*(2), 192–200.
28. Debyser, Z., & Christ, F. (2010). On the cell biology of HIV integration from basic research to development of novel antiviral drugs. *Verhandelingen-Koninklijke Academie voor Geneeskunde van België, 72*, 219–237.
29. Woodward, C. L., & Chow, S. A. (2010). The nuclear pore complex: a new dynamic in HIV-1 replication. *Nucleus, 1*(1), 18–22.
30. Levin, A., Hayouka, Z., Friedler, A., & Loyter, A. (2010). Nucleocytoplasmic shuttling of HIV-1 integrase is controlled by the viral Rev protein. *Nucleus, 1*(2), 190–201.
31. Levin, A., Hayouka, Z., Friedler, A., & Loyter, A. (2010). Transportin 3 and importin α are required for effective nuclear import of HIV-1 integrase in virus-infected cells. *Nucleus, 1*(5), 422–431.
32. Ao, Z., Danappa Jayappa, K., Wang, B., Zheng, Y., Kung, S., Rassart, E., Depping, R., Kohler, M., Cohen, E. A., & Yao, X. (2010). Importin alpha3 interacts with HIV-1 integrase and contributes to HIV-1 nuclear import and replication. *Journal of Virology, 84*(17), 8650–8663.
33. Deprez, E., Barbe, S., Kolaski, M., Leh, H., Zouhiri, F., Auclair, C., Brochon, J. C., Le Bret, M., & Mouscadet, J. F. (2004). Mechanism of HIV-1 integrase inhibition by styrylquinoline derivatives in vitro. *Molecular Pharmacology, 65*(1), 85–98.
34. He, Q. Q., Zhang, X., Yang, L. M., Zheng, Y. T., & Chen, F. (2013). Synthesis and biological evaluation of 5-fluoroquinolone-3-carboxylic acids as potential HIV-1 integrase inhibitors. *Journal of Enzyme Inhibition and Medicinal Chemistry, 28*(4), 671–676.
35. Suedee, A., Tewtrakul, S., & Panichayupakaranant, P. (2014). Anti-HIV-1 integrase activity of *Mimusops elengi* leaf extracts. *Pharmaceutical Biology, 52*(1), 58–61.
36. Li, B. W., Zhang, F. H., Serrao, E., Chen, H., Sanchez, T. W., Yang, L. M., Neamati, N., Zheng, Y. T., Wang, H., & Long, Y. Q. (2014). Design and discovery of flavonoid-based HIV-1 integrase inhibitors targeting both the active site and the interaction with LEDGF/p75. *Bioorganic & Medicinal Chemistry, 22*(12), 3146–3158.