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Short communication

Chemically modified bovine beta-lactoglobulin inhibits human papillomavirus infection

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Abstract

Previous studies have shown that 3-hydroxyphthalic anhydride-modified bovine beta-lactoglobulin is a promising anti-HIV microbicide candidate. Here we found that this chemically modified protein, designated JB01, exhibited highly potent antiviral activity against infection by human papillomaviruses (HPV), including HPV6, HPV16 and HPV18. Its anti-HPV activity was correlated with the percentage of modified lysine and arginine residues in JB01. This modified milk protein had no cytotoxicity at the concentration of 1 mg/ml, and it is highly stable at room temperature and 37 °C for at least 12 weeks. These results suggest that JB01 has good potential to be developed as an effective, safe and inexpensive antiviral agent for treatment and prevention of HPV infection.

Keywords: 3-Hydroxyphthalic anhydride; Chemical modification; Bovine beta-lactoglobulin; HPV infection; Cervical cancer; Anti-HPV agent

1. Introduction

Cervical cancer is the third most common cancer for women worldwide, and an estimated 274,000 women die from it each year [1]. This is more serious in the developing countries where cervical cancer is the second leading cause of cancer mortality in women 15–44 years old [2]. In China, about 135,000 women develop cervical cancer, and 50,000 die from this disease each year, or about 18% of total worldwide mortality [3]. Especially in this decade, the incidence of cervical cancer has rapidly increased, making this disease a great threat to Chinese women's health. Therefore, development of an effective and safe biologic to prevent cervical cancer is urgently needed.

Human papillomavirus (HPV) is now well recognized as the causal agent of cervical cancer [4]. HPV is a small non-enveloped virus, about 55 nm in size, containing double-stranded DNA. The whole genome consists of three regions. E and L regions control viral replication at the early and late stages, respectively, while the LCR region regulates gene functions [5]. So far, over one hundred different subtypes of HPV have been isolated, which could be further divided into high-risk and low-risk groups [6]. Since HPV types 6 and 11 mainly cause benign genital warts and mild cervical epithelial necrosis, they are considered as low-risk types. On the other hand, HPV16 and HPV18 are high-risk types since they are responsible for more than 70% of malignant tumors [7,8].

HPV usually infects the basal cells of stratified epithelium on human skin and mucosal tissues, particularly around the mouth, hand, feet and genitals. Direct contact between virus and human cells is a necessary requirement for viral invasion. When the skin or mucosa is damaged, free mature HPV particles have the opportunity to penetrate the microtrauma site and interact with tissue cells. Generally speaking, this is the beginning of the HPV lifecycle, which is also the best stage for prevention of the infection. Vaccines against HPV have now been licensed in over 100 countries [9]. However, their

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application in low-income, developing countries is limited by high cost and the lack of effectiveness against all HPV types. Furthermore, no effective antiviral agent is available for inhibiting HPV infection topically, necessitating the development of an anti-HPV agent for topical application.

Our previous studies have shown that 3-hydroxyphthalic anhydride-modified bovine beta-lactoglobulin, herein designated JB01, showed potent inhibition activity against HIV, HSV-1, HSV-2, as well as some Chlamydia [10–13]. In this study, we tested the potential inhibitory activity of this chemically modified milk protein against HPV infection. We found that JB01 exhibited potent antiviral activity against infection by HPV6, HPV16 and HPV18. Our previous studies have shown that this chemically modified bovine protein, which is very inexpensive, is highly stable in aqueous solution and can be easily formulated into a topical gel [10,14]. Therefore, we believe that JB01 has good potential to be further developed as an effective, safe and affordable topical biologic for prevention and treatment of HPV infection.

2. Materials and methods

2.1. Reagents

3-Hydroxyphthalic anhydride, bovine beta-lactoglobulin, trypsin–agarose beads, XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-(phenylamino) carbonyl-2*H*-tetrazolium hydroxide], and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma (St. Louis, MO), and *p*-Hydroxyphenylglyoxal (*p*-HPG) was purchased from Fisher Scientific Co. (Valley Park, VA). TZM-bl cells, which are HeLa-cell derivatives expressing high levels of CD4 and both co-receptors CXCR4 and CCR5, and are stably transduced carrying a LTR-driven firefly luciferase, and 293FT cells, a fast-growing variant of 293T cell line that contains the SV40 large T antigen, were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. VK2/E6E7 cells (an immortalized vaginal epithelial cell line) were purchased from American Type Culture Collection (ATCC) (Manassas, VA).

2.2. Preparation and identification of JB01

The JB01 protein was prepared based on a previously described method [10]. Briefly, bovine beta-lactoglobulin was dissolved in 0.1 M phosphate buffer (pH 8.5) to final concentration of 20 mg/ml. Then 3-hydroxyphthalic anhydride saturated in dimethylformamide was added slowly to this solution with gentle shaking. Five aliquots with the final concentrations of 3-hydroxyphthalic anhydride at 0, 10, 20, 40 and 60 mM, respectively (as indicated in Table 1), were prepared. The mixtures were kept for another 1 h at room temperature, extensively dialyzed against phosphate buffer saline (PBS), and filtered through 0.45 μm syringe filters (Acrodisc; Gelman Sciences, Ann Arbor, MI). Protein concentrations were determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL).

Table 1
Comparison of the anti-HPV activities and the percentages of modified residues of beta-lactoglobulin modified by 3-hydroxyphthalic anhydride (HP).

Concentration of HP (mM)	% of modified residues		IC_{50} (μM) for inhibiting infection by[a]	
	Lysine	Arginine	HPV6	HPV16
0	0	0	>20	>20
10	35.2	47.3	>20	10.432 ± 1.54
20	51.3	66.2	4.117 ± 0.531	1.321 ± 0.231
40	79.6	85.9	0.983 ± 0.125	0.125 ± 0.036
60	90.1	95.2	0.269 ± 0.073	0.027 ± 0.008

[a] Each sample was tested in triplicate, and the experiment was repeated twice. The data are presented in means ± SD of triplicate determinations from a representative experiment.

To quantify lysine residues in modified or unmodified proteins, a TNBS assay was used as previously described [15]. Briefly, 25 μl of anhydride-modified or -unmodified proteins (90 μM) were treated with 25 μl $Na_2B_4O_7$ (0.1 M) for 5 min at room temperature (RT). Then 10 μl TNBS were added to the mixture. After another 5 min, 100 μl stop solution (0.1 M NaH_2PO_4 and 1.5 mM Na_2SO_3) was added to terminate the reaction. The absorbance at 420 nm (A420) was measured using a microplate reader (Ultra 384; Tecan, Research Triangle Park, NC). The percentage of arginine residues modification was also detected using a previously described method [16]. In brief, 90 μl of anhydride-modified or -unmodified proteins (90 μM) in 0.1 M sodium phosphate (pH 9.0) were treated with 10 μl of 50 mM *p*-HPG for 90 min at RT in the dark. The absorbance at 340 nm (A340) was measured.

2.3. Detection of inhibitory activity of JB01 on HPV infection

The HPV pseudoviruses were generated by co-transfection of plasmid containing codon-modified HPV L1 and L2 genes into 293FT cells as described previously [17]. The pseudoviruses of HPV6, HPV16 and HPV18 at 100 $TCID_{50}$ (50% tissue culture infective doses) were incubated with JB01 or control proteins for 30 min at 37 °C. Then the mixture was added into 1×10^5/ml 293FT cells in DMEM medium containing 10% FBS overnight. The culture supernatants were removed, and fresh media were added the next day. The cells were harvested and lysed on the third day postinfection with 50 μl of lysing reagent. The luciferase activity was analyzed using a luciferase kit (Promega, Madison, WI) and a luminometer (Ultra 386; Tecan, Durham, NC) according to the manufacturer's instructions. The IC_{50}s of JB01 were calculated using the CalcuSyn software [18].

2.4. Analysis of cytotoxicity

The potential cytotoxicity of JB01 on TZM-b1 and VK2/E6E7 cells was measured by using the colorimetric XTT assay as previously described [19,20]. Briefly, 100 μl of a compound at graded concentrations were added to equal

volumes of cells (10^5cells/ml) in wells of 96-well plates. After incubation at 37 °C for 4 days, 50 µl of XTT solution (1 mg/ml) containing 0.02 µM phenazinemethosulfate was added. After 4 h, the absorbance at 450 nm was measured with an ELISA reader, and the percentage of cytotoxicity was calculated.

3. Results

3.1. The percentage of positively charged lysine and arginine residues modified by 3-hydroxyphthalic anhydride is correlated with JB01's anti-HPV activity

Beta-lactoglobulin consists of 162 residues, with a molecular weight of 18.4 kDa and contains 18 positively charged residues, including 15 lysine residues and 3 arginine residues. We have previously demonstrated that the number of chemically modified lysine residues in JB01 is an important determinant of its anti-HIV-1 activity [21]. Here, we found that the number of both lysine and arginine residues is important for BJ01's anti-HPV activity. As shown in Table 1, with increased concentration of 3-hydroxyphthalic anhydride, more lysine and arginine residues were modified, resulting in an increased anti-HPV activity. When 60 mM of 3-hydroxyphthalic anhydride was used, about 90% and 95% of the lysine and arginine residues were modified, respectively, leading to the highest inhibition of HPV16 infection (IC_{50} = 0.027 µM). Therefore, we used this concentration of 3-hydroxyphthalic anhydride to modify beta-lactoglobulin in subsequent studies.

3.2. JB01 was effective in inhibiting infection by HPV6, HPV16 and HPV18

Subsequently, we tested the inhibitory activity of JB01 against infection by HPV6 that can cause benign genital warts, and HPV16 and HPV18, which are recognized as high-risk types causing cervical cancer [7,8]. As shown in Fig. 1, JB01 exhibited highly potent antiviral activity against all three HPV subtypes, HPV6, HPV16 and HPV18, with IC_{50} values of 0.33, 0.04, 0.065 µM, respectively. These results suggest that JB01 is effective against the major HPV subtypes that cause diseases in humans.

3.3. JB01 was stable and had low cytotoxicity

The stability and safety of an anti-HPV agent are important issues for its development as a clinically usable product. To study JB01's stability, we kept JB01 protein at room temperature (24 °C) and human body temperature (37 °C) for 12 weeks, followed by testing its anti-HPV activities at the week 1, 2, 3, 4, 6, and 12, respectively. The results showed that JB01 was highly stable since its anti-HPV activity showed no significant changes during the 12-week storage period (Fig. 2A).

Then, we further tested the cytotoxicity of JB01 to TZM-b1 cells, which were derived from a human cervical cell line (HeLa cells), and VK2/E6E7 cells (vaginal epithelial cells), using unmodified beta-lactoglobulin as a control. As shown in Fig. 2B, JB01 exhibited no significant cytotoxicity on these

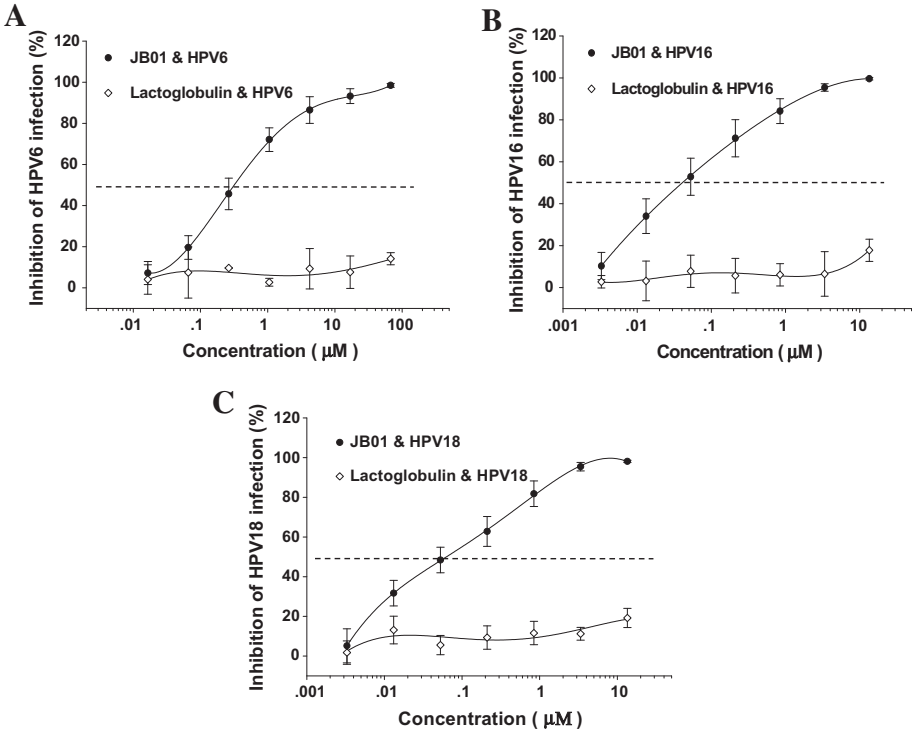

Fig. 1. The inhibitory activity of JB01 against infection by HPV, including viral HPV6 (A), HPV16 (B) and HPV18 (C). The measurements were performed in triplicate, and the experiment was repeated at least twice. The data are presented as the means ± SD of triplicate determinations from a representative experiment.

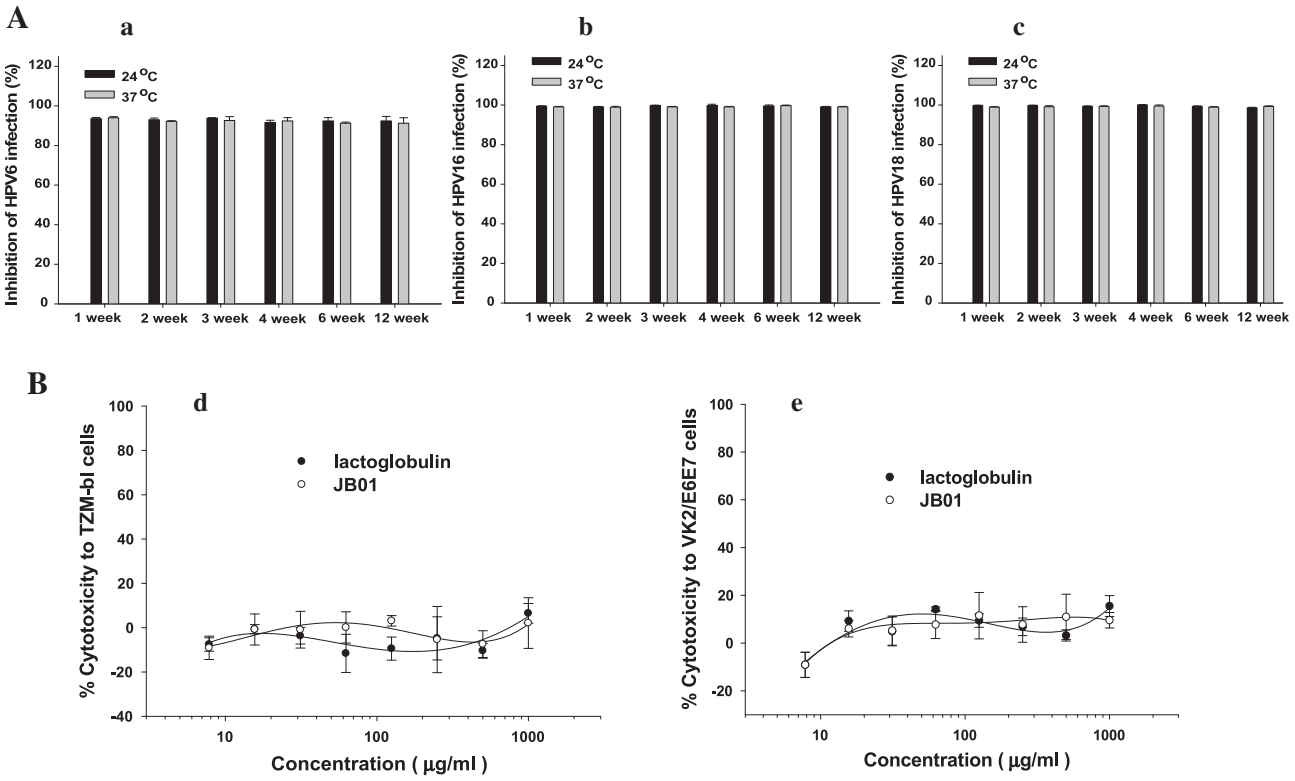

Fig. 2. The stability and cytotoxicity of JB01. (A) JB01's stability. JB01 was stored at 24 and 37 °C, respectively, for one to twelve weeks, and its antiviral activities against infection by HPV6 (a), HPV16 (b) and HIV18 (c), respectively, at the indicated time. (B) JB01's cytotoxicity. The potential toxic effect of JB01 on TZM-B1 cells (d) and VK2/E6E7 cells (e) was determined by a colorimetric XTT assay. Unmodified beta-lactoglobulin was included as a control. The measurements were performed in triplicate, and the experiment was repeated at least twice. The data are presented as the means ± SD of triplicate determinations from a representative experiment.

human cervical cells and vaginal epithelial cells, suggesting that it is as safe as the unmodified beta-lactoglobulin.

4. Discussion

HPV does not infect intact, but rather damaged epithelium of mucosa. Using its capsid proteins L1 and L2, it infects the basal cells of stratified epithelium via a unique mechanism. The L1 protein first binds to heparan sulfate proteoglycan (HSPG) on the basement membrane (BM), which is exposed after epithelial trauma, resulting in furin cleavage of the L2 protein and binding of L1 to an as yet undetermined receptor on surface of keratinocytes to cause a new replication cycle [22]. Therefore, a topical formulation containing anti-HPV agent that covers the wounds in genital mucosa is expected to stop HPV new infection, thus being useful for preventing and treating HPV infection.

Since the expression of L1 and L2 proteins requires cellular differentiation in the upper layers of the stratified squamous epithelial tissues [22], HPV cannot replicate in conventional monolayer cell cultures. However, the HPV-based pseudoviruses can be used for investigating the early phase of the HPV lifecycle [17]. Here we used a pseudotyped HPV for testing the inhibitory activity of JB01 on HPV infection in 293FT cells. We found that JB01 is highly effective in inhibiting infection by several types of HPV, including HPV6, HPV16 and HPV18. The percentage of modified lysine and arginine residues in JB01 was correlated with its anti-HPV activity, indicating that the net negative charges in JB01 play an important role in JB01-mediated inhibition of HPV infection. These results suggest that JB01 may block HPV entry into the target cell through the interactions between the negatively charged residues on beta-lactoglobulin and the positively charged residues on the L1 and/or L2 proteins. We speculate that the C-terminal region of L1 protein and the N-terminal region of L2 protein may be the target sites for JB01 since both regions contain the positively charged residues that are exposed on the surface of viral capsid [23,24]. Indeed, we found that JB01 could strongly bind to the positively charged peptides derived from the L1 and L2 proteins of HPV, while the unmodified beta-lactoglobulin exhibited no significant binding to these peptides (data not shown).

In conclusion, our study has demonstrated that JB01, a chemically modified beta-lactoglobulin with relatively increased surface negative charges (due to the chemical modification of the positively charged residues), inhibits HPV infection, possibly by targeting the early stage of viral replication, particularly the viral entry process. As an inexpensive, safe and stable anti-HPV agent, JB01 can be formulated in a topical gel formulation for prevention and treatment of HPV infection in genital mucosa, and consequently reduce the occurrence of cervical cancer.

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References

[1] J. Ferlay, H.R. Shin, F. Bray, D. Forman, C. Mathers, D.M. Parkin, GLOBOCAN 2008 v. 2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 (Internet), International Agency for Research on Cancer, Lyon, France, 2010.

[2] World Health Organization, Human Papillomavirus and HPV Vaccines: Technical Information for Policy-Makers and Health Professional (2007).

[3] W.Q. Chen, R.S. Zheng, H.M. Zeng, S.W. Zhang, P. Zhao, J. He, Trend analysis and projection of cancer incidence in China between 1989 and 2008, Zhonghua Zhong Liu Za Zhi 34 (2012) 517–524.

[4] J.M.M. Walboomers, M.V. Jacobs, M.M. Manos, F.X. Bosch, J.A. Kummer, K.V. Shah, P.J.F. Snijders, J. Peto, C.J.L.M. Meijer, N. Munoz, Human papillomavirus is a necessary cause of invasive cervical cancer worldwide, J. Pathol. 189 (1999) 12–19.

[5] R. Faridi, A. Zahra, K. Khan, M. Idrees, Oncogenic potential of Human Papillomavirus (HPV) and its relation with cervical cancer, Virol. J. 8 (2011) 269–276.

[6] S. Khan, N.N. Jaffer, M.N. Khan, M.A. Rai, M. Shafiq, A. Ali, S. Pervez, N. Khan, A. Aziz, S.H. Ali, Human papillomavirus subtype 16 is common in Pakistani women with cervical carcinoma, Int. J. Infect. Dis. 11 (2007) 313–317.

[7] C. Porras, C. Bennett, M. Safaeian, S. Coseo, A.C. Rodriguez, P. Gonzalez, M. Hutchinson, S. Jimenez, M.E. Sherman, S. Wacholder, D. Solomon, L.J. van Doorn, C. Bougelet, W. Quint, M. Schiffman, R. Herrero, A. Hildesheim, Determinants of seropositivity among HPV-16/18 DNA positive young women, BMC Infect. Dis. 10 (2010) 238–247.

[8] F.X. Bosch, A.N. Burchell, M. Schiffman, A.R. Giuliano, S. de Sanjose, L. Bruni, G. Tortolero-Luna, S.K. Kjaer, N. Munoz, Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia, Vaccine 26 (2008) K1–K16.

[9] L.L. Villa, R.L.R. Costa, C.A. Petta, R.P. Andrade, J. Paavonen, O.E. Iversen, S.E. Olsson, J. Hoye, M. Steinwall, G. Riis-Johannessen, A. Andersson-Ellstrom, K. Elfgren, G. von Krogh, M. Lehtinen, C. Malm, G.M. Tamms, K. Giacoletti, L. Lupinacci, R. Railkar, F.J. Taddeo, J. Bryan, M.T. Esser, H.L. Sings, A.J. Saah, E. Barr, High sustained efficacy of a prophylactic quadrivalent human papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up, Br. J. Cancer 95 (2006) 1459–1466.

[10] A.R. Neurath, A.K. Debnath, N. Strick, Y.Y. Li, K. Lin, S. Jiang, Blocking of CD4 cell receptors for the human immunodeficiency virus type 1 (HIV-1) by chemically modified bovine milk proteins: potential for AIDS prophylaxis, J. Mol. Recognit. 8 (1995) 304–316.

[11] A.R. Neurath, Y.Y. Li, N. Strick, S. Jiang, A herpesvirus inhibitor from bovine whey, Lancet 347 (1996) 1703–1704.

[12] A.R. Neurath, N. Strick, Y.Y. Li, 3-Hydroxyphthaloyl beta-lactoglobulin. III. Antiviral activity against herpesviruses, Antivir. Chem. Chemother. 9 (1998) 177–184.

[13] H. Kokuba, L. Aurelian, A.R. Neurath, 3-Hydroxyphthaloyl beta-lactoglobulin. IV. Antiviral activity in the mouse model of genital herpesvirus infection, Antivir. Chem. Chemother. 9 (1998) 353–357.

[14] S. Jiang, K. Lin, N. Strick, Y.Y. Li, A.R. Neurath, Chemically modified bovine beta-lactoglobulin blocks uptake of HIV-1 by colon- and cervix-derived epithelial cell lines, J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 13 (1996) 461–462.

[15] L. Li, L.L. He, S.Y. Tan, X.H. Guo, H. Lu, Z. Qi, C. Pan, X.L. An, S. Jiang, S.W. Liu, 3-Hydroxyphthalic anhydride-modified chicken ovalbumin exhibits potent and broad anti-HIV-1 activity: a potential microbicide for preventing sexual transmission of HIV-1, Antimicrob. Agents Chemother. 54 (2010) 1700–1711.

[16] D. Elton, L. Medcalf, K. Bishop, D. Harrison, P. Digard, Identification of amino acid residues of influenza virus nucleoprotein essential for RNA binding, J. Virol. 73 (1999) 7357–7367.

[17] C.B. Buck, D.V. Pastrana, D.R. Lowy, J.T. Schiller, Efficient intracellular assembly of papillomaviral vectors, J. Virol. 78 (2004) 751–757.

[18] T.C. Chou, P. Talalay, Quantitative-analysis of dose–effect relationships – the combined effects of multiple-drugs or enzyme-inhibitors, Adv. Enzyme Regul. 22 (1984) 27–55.

[19] Y. Zhu, L. Lu, L. Xu, H. Yang, S. Jiang, Y.H. Chen, Identification of a gp41 core-binding molecule with homologous sequence of human TNNI3K-like protein as a novel human immunodeficiency virus type 1 entry inhibitor, J. Virol. 84 (2010) 9359–9368.

[20] S. Jiang, K. Lin, A.R. Neurath, Enhancement of human-immunodeficiency-virus type-1 infection by antisera to peptides from the envelope glycoproteins gp120/gp41, J. Exp. Med. 174 (1991) 1557–1563.

[21] A.R. Neurath, S. Jiang, N. Strick, K. Lin, Y.Y. Li, A.K. Debnath, Bovine beta-lactoglobulin modified by 3-hydroxyphthalic anhydride blocks the CD4 cell receptor for HIV, Nat. Med. 2 (1996) 230–234.

[22] J. Doorbar, The papillomavirus life cycle, J. Clin. Virol. 32 (2005) S7–S15.

[23] L. Bousarghin, A. Touze, A.L. Combita-Rojas, P. Coursaget, Positively charged sequences of human papillomavirus type 16 capsid proteins are sufficient to mediate gene transfer into target cells via the heparan sulfate receptor, J. Gen. Virol. 84 (2003) 157–164.

[24] C.B. Buck, N. Cheng, C.D. Thompson, D.R. Lowy, A.C. Steven, J.T. Schiller, B.L. Trus, Arrangement of L2 within the papillomavirus capsid, J. Virol. 82 (2008) 5190–5197.