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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

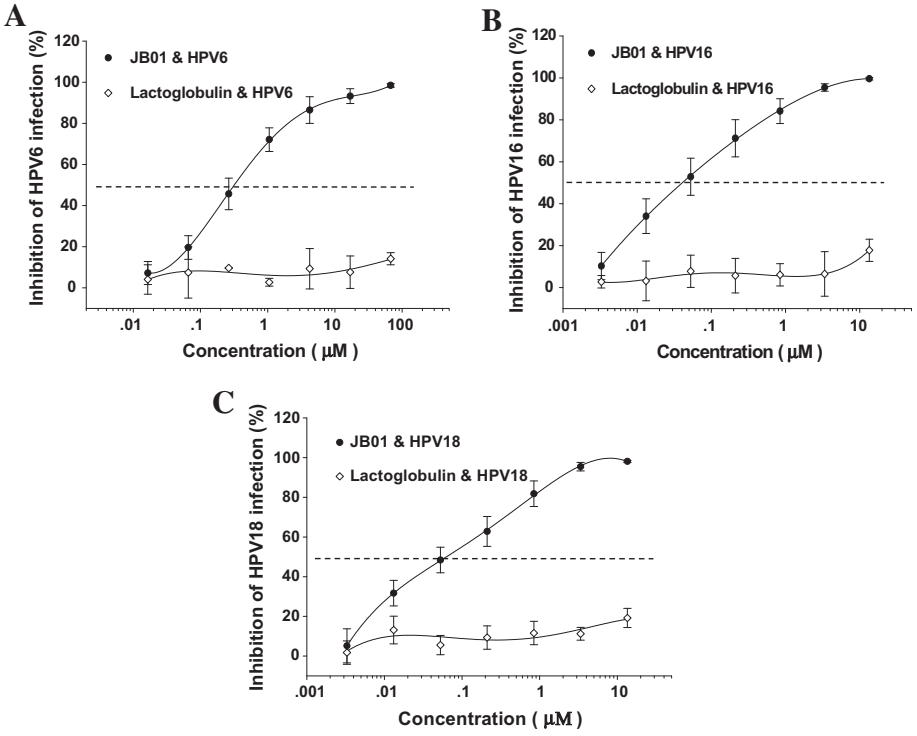

Fig. 1. The inhibitory activity of JB01 against infection by HPV, including viral HPV6 (A), HPV16 (B) and HPV18 (C). The measurements were performed in triplicate, and the experiment was repeated at least twice. The data are presented as the means ± SD of triplicate determinations from a representative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

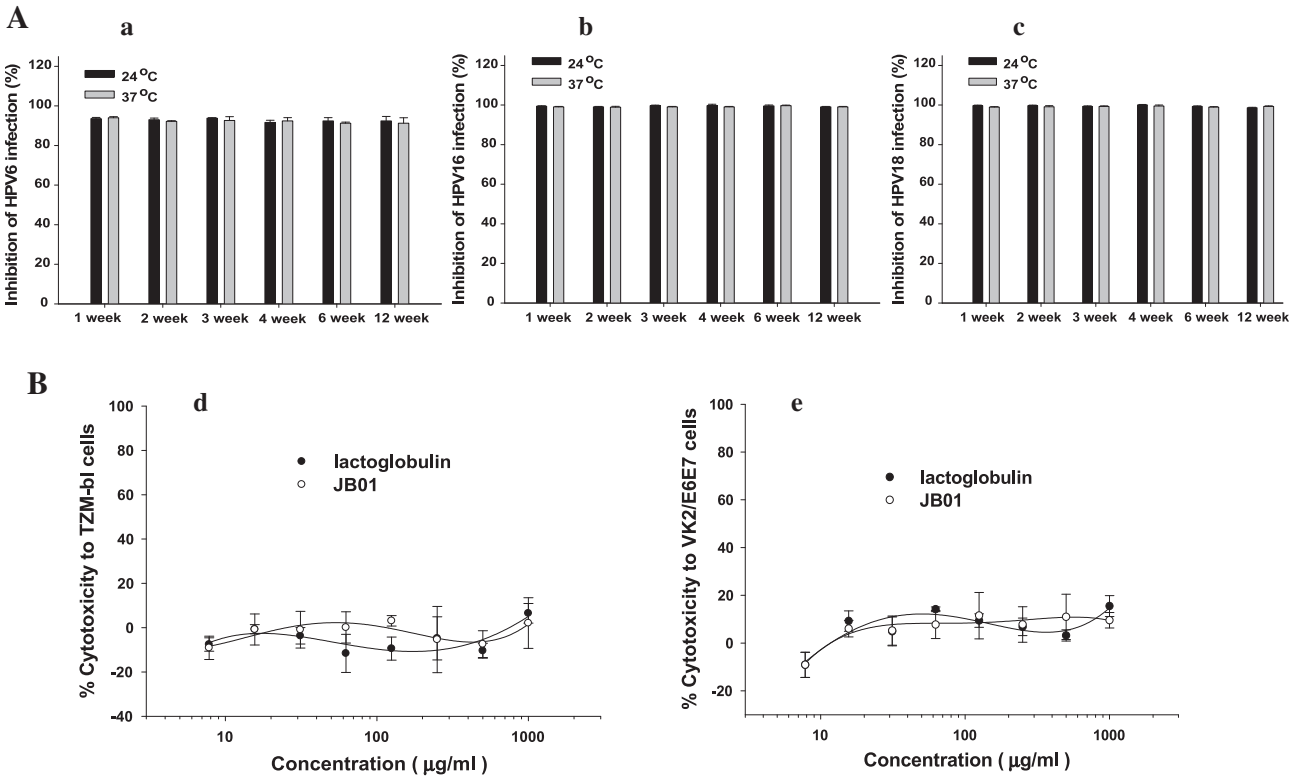

Fig. 2. The stability and cytotoxicity of JB01. (A) JB01's stability. JB01 was stored at 24 and 37 °C, respectively, for one to twelve weeks, and its antiviral activities against infection by HPV6 (a), HPV16 (b) and HIV18 (c), respectively, at the indicated time. (B) JB01's cytotoxicity. The potential 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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化学修饰牛 β-乳球蛋白可抑制人乳头瘤病毒感染

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1 这些作者同等贡献。

摘要

既往研究显示，3-羟基苯二甲酸酐-修饰牛 β-乳球蛋白有望成为新的抗-HIV 杀微生物剂。本研究发现，这一被命名为 JB01 的化学修饰蛋白在抗人乳头瘤病毒（HPV）感染中表现出高效的抗病毒活性，包括 HPV6、HPV16 和 HPV18 三种亚型。其抗-HPV 活性与 JB01 表面赖氨酸和精氨酸残基的被修饰比例紧密相关。JB01 蛋白在浓度为 1 mg/ml 时无任何细胞毒性，且在室温和 37℃条件下均保持不少于 12 周的高度稳定性。这些结果表明，JB01 极有望被开发成为一种安全、有效且低廉的抗病毒制剂，用于 HPV 感染的治疗和预防。

关键词：3-羟基苯二甲酸酐；化学修饰；牛 β-乳球蛋白；HPV 感染；子宫颈癌；抗-HPV 制剂

1. 引言

从全球而言，子宫颈癌是女性中第三大高发癌症，每年约有 274,000 例女性死于该病[1]。在发展中国家里，这一情况更为严重，子宫颈癌在 15-44 岁女性癌性死亡原因中名列第 2[2]。在中国，每年约有 135,000 例新发子宫颈癌女性患者，近 50,000 例患者死于该病，约占全球死亡总数的 18%[3]。特别是近 10 年来，子宫颈癌发病率迅速升高，严重威胁中国女性的健康。因此，亟需研发出一种安全、有效的生物制剂，用以预防子宫颈癌发病。

人乳头瘤病毒（Human papillomavirus，HPV）是目前公认的造成子宫颈癌的罪魁祸首[4]。HPV 是一种无包膜小病毒，大小约为 55 nm，内含双链 DNA。整个基因由三个区域组成。E 和 L 域分别控制早期和晚期病毒复制，而 LCR 域则负责调节基因功能[5]。到目前为止，已分离出超过 100 种不同亚型的 HPV，它们可以进一步被划分为高危组和低危组[6]。鉴于 HPV 6 和 11 主要会引发良性生殖器疣和轻度宫颈上皮坏死，故将其划分为低危型 HPV。相反， HPV16 和 HPV18 被列为高危组，因为它们引发的恶性肿瘤超过总数的 70%以上[7，8]。

HPV 通常会感染人皮肤和粘膜组织复层上皮的基底细胞，特别位于口、手、足和生殖器附近。病毒与人类细胞间的直接接触是病毒入侵的必要条件。当皮肤或粘膜受损时，游离的成熟 HPV 颗粒即有机会穿透轻微创伤部位，与组织细胞结合。一般而言，这即是 HPV 生命周期的开始，同时也是预防病毒感染的最佳阶段。抗 HPV 疫苗现已在 100 多个国家获批[9]。尽管如此，因其治疗费用高且不能有效抵抗所有类型 HPV，导致其在低收入、发展中国家应用受限。此外，目前还没有任何一种可局部抑制 HPV 感染的制剂，因此研发此类制剂势在必行。

我们的既往研究显示，3-羟基苯二甲酸酐-修饰牛 β-乳球蛋白、即本文中的

JB01，可有效抑制 HIV、HSV-1、HSV-2、以及某些衣原体[10-13]。本研究中针对 JB01 蛋白抑制 HPV 感染的活性进行了检测。结果证实，JB01 具有高效的抗病毒活性，可有效抑制 HPV6、HPV16 和 HPV18 感染。我们的既往研究还表明，该化学修饰蛋白不仅价格低廉，且在水溶液中高度稳定，易于制成外用凝胶制剂[10，14]。因此，我们相信，JB01 极有望被进一步开发成为一种安全、有效且价格低廉的外用生物制剂，用于 HPV 感染的预防和治疗。

2. 材料和方法

2.1. 试剂

自 Sigma 公司（圣路易斯，密苏里州，美国）购买 3-羟基苯二甲酸酐、牛 β-乳球蛋白、胰蛋白酶-琼脂糖珠、XTT [2,3-双（2-甲氧基-4-硝基-5-磺基苯）-5-[（苯胺基） 羧基]-2 氢-四唑氢氧化物]和 2,4,6-三硝基苯磺酸（trinitrobenzenesulfonic acid，TNBS），自 Fisher Scientific 公司（Valley Park，VA）购买对一水合羟苯基乙二醛。TZM-bl细胞，是人工改造的高度表达CD4及两种共受体CXCR4和CCR5的 HeLa-细胞系、同时还稳定转染了长末端重复序列（LTR）-驱动萤火虫荧光素酶；293FT 细胞，一种含 SV40 大 T 抗原可快速生长的 293T 细胞系变种，均由美国国立卫生研究院下属的艾滋病研究与相关试剂组提供。VK2/E6E7 细胞（一种永生化阴道上皮细胞系）购自美国菌种保藏中心（American Type Culture Collection，ATCC）（弗吉尼亚，马纳萨斯）。

2.2. JB01 制备和鉴别

利用先前描述的方法制备 JB01 蛋白[10]。简言之，即将牛 β-乳球蛋白溶于 0.1 M 磷酸盐缓冲液（pH 8.5），配制成最终浓度为 20 mg/ml 的溶液。之后将 3-羟基苯二甲酸酐二甲基甲酰胺饱合溶液缓慢加入该溶液内并轻轻摇动。将溶液分为 5 份，分别配制成 3-羟基苯二甲酸酐最终浓度为 0、10、20、40 和 60 mM 的溶液（如表 1 所示）。将混合物放在室温下静置 1 h，经磷酸盐缓冲液（phosphate buffer saline，PBS）充分透析后，利用 0.45 μm 注射器式过滤器（Acrodisc；Gelman Sciences，Ann Arbor，MI）进行过滤。采用 BCA 蛋白定量分析试剂盒（Pierce，Rockford，IL）测定蛋白浓度。

采用如前所述的 TNBS 分析法，定量测定修饰后或未修饰的蛋白中赖氨酸残基比例[15]。简言之，即在室温（room temperature，RT）下，用 25 μl $Na_2B_4O_7$（0.1 M）处理 25 μl 酐修饰或未修饰蛋白（90 μM）5 min。之后将 10 μl TNBS 加入混合物内。再过 5 min 后，加入 100 μl 终止液（0.1 MNaH_2PO_4 和 1.5 mM Na_2SO_3）终止反应。利用酶标仪（Ultra 384；Tecan，Research Triangle Park，NC）测定 420 nm（A420）处吸光度。另外，采用先前描述方法，检测精氨酸残基修饰比例[16]。简言之，即将 90 μl 酐修饰或未修饰蛋白（90 μM）溶于 0.1 M 磷酸钠溶液（pH 9.0），之后用 10 μl 50 mM p-HPG 在 RT、黑暗条件下处理 90 min。测定 340 nm（A340）处吸光度。

2.3. JB01 对 HPV 感染抑制活性的检测

采用先前所述方法，将含密码子-修饰 HPV L1 和 L2 基因共转染至 293FT 细胞内，生成 HPV 假病毒[17]。之后，在 37 °C 条件下用 JB01 或对照蛋白对 100 个 $TCID_{50}$（50%组织培养感染量）HPV6、HPV16 和 HPV18 假病毒进行 30 min 孵化。再将混合物加至含 10% FBS DMEM 培养基内的 1x 10^5/ml 293FT 细胞内，过夜。第 2 天，去除培养上清液，加入新鲜培养基。感染后第 3 天，收集细胞并 50 μl 裂解试剂进行裂解。利用荧光素酶试剂盒（Promega，Madison，WI）和光度计（Ultra 386；Tecan，Durham，NC），参照产品说明书，完成荧光素酶活性

分析测定。利用 CalcuSyn 软件计算 JB01 的 IC_{50}s[18]。

2.4. 细胞毒性分析

利用如前所述的 XTT 比色法，测定 JB01 对 TZM-b1 和 VK2/E6E7 细胞产生的细胞毒性[19，20]。简言之，即利用含等容积（10^5 个细胞/ml）细胞的 96-孔板，将各级别浓度 100 μl 复合物加入孔中的细胞内。在 37°C 条件下孵化 4 天后，加入含 0.02 μM 吩嗪硫酸甲酯的 50 μl XTT 溶液（1 mg/ml）。经过 4 h 后，利用酶标仪测定 450 nm 处吸光度，并计算细胞毒性比例。

3. 结果

3.1. 3-羟基苯二甲酸酐修饰带正电赖氨酸和精氨酸残基比例与 JB01 的抗-HPV 活性相关

β-乳球蛋白含有 162 个残基，分子量为 18.4 kDa，内含 18 个带正电残基，包括 15 个赖氨酸残基和 3 个精氨酸残基。我们的既往研究证明，JB01 上化学修饰赖氨酸残基数量是其抗-HIV-1 活性的重要决定因素[21]。本研究也证实，无论是赖氨酸还是精氨酸残基的比例，都对 JB01 的抗-HPV 活性有重要意义。如表 1 所示，随着 3-羟基苯二甲酸酐浓度的升高，赖氨酸和精氨酸残基被修饰的数量逐渐增加，JB01 抗-HPV 活性亦随之增强。采用 60 mM 3-羟基苯二甲酸酐处理时，分别约有 90%和 95%的赖氨酸和精氨酸残基被修饰，可产生最强的 HPV16 感染抑制作用（IC_{50} = 0.027 μM）。因此，后续的研究均采用这一浓度的 3-羟基苯二甲酸酐完成 β-乳球蛋白的修饰处理。

3.2. JB01 可有效抑制 HPV6、HPV 16 和 HPV18 感染

接下来的研究检测了 JB01 对可导致生殖器疣的 HPV6、及公认的可导致子宫颈癌的高危型 HPV16 和 HPV18 的抑制活性[7，8]。如图 1 所示，对于所有这三种 HPV 亚型，即 HPV6、HPV16 和 HPV18，JB01 都表现出了高效的抗病毒活性，IC_{50} 分别为 0.33、0.04、和 0.065 μM。这些结果表明，JB01 可有效抑制导致人类疾病的主要的 HPV 亚型。

3.3. JB01 稳定且细胞毒性低

作为可应用于临床的产品，抗-HPV 制剂的稳定性和安全性是其研发中要解决的重要问题。在评价 JB01 稳定性时，我们分别将 JB01 蛋白在室温（24℃）及人体温（37℃）温度条件下放置 12 周，并于第 1、2、3、4、6、和 12 周时测定其抗-HPV 活性。结果显示，12-周贮藏期内，其抗-HPV 活性无显著变化，即证明 JB01 高度稳定（图 2A）。

之后，研究又利用未修饰 β-乳球蛋白作为对照组，进一步评价了 JB01 对人宫颈癌细胞系（HeLa 细胞）衍生 TZM-b1 细胞和 VK2/E6E7 细胞（阴道上皮细胞）的细胞毒性。如图 2B 所示，JB01 对这些人宫颈细胞和阴道上皮细胞无明显细胞毒性，这意味着它和未修饰 β-乳球蛋白一样安全。

4. 讨论

虽然 HPV 并不会感染未受损粘膜上皮细胞，但可感染受损细胞。在其衣壳蛋白 L1 和 L2 作用下，该病毒可通过独特机制感染复层上皮的基底细胞。首先，L1 蛋白与上皮损伤后暴露基底膜（basement membrane，BM）上的硫酸乙酰肝素蛋白多糖（heparan sulfate proteoglycan，HSPG）结合，引发弗林蛋白酶切割 L2 蛋白，并与 L1 结合后在角质化细胞表面形成尚未明确的受体，进而引发新的复制循环[22]。因此，采用含抗-HPV 成分外用制剂涂抹生殖器粘膜表面这一方法有望阻断新的 HPV 感染，籍此有效预防和治疗 HPV 感染。

由于 L1 和 L2 蛋白表达要求复层鳞状上皮组织的上层出现细胞分化[22]，因

此 HPV 无法在常规单层细胞培养中复制。但可利用基于 HPV 的假病毒对 HPV 生命周期的早期进行观察[17]。本研究采用 HPV 假病毒系统评价了 JB01 对 293FT 细胞 HPV 感染的抑制活性。研究发现，JB01 可高效抑制多种类型 HPV 感染，包括 HPV6、HPV16 和 HPV18。JB01 内修饰赖氨酸和精氨酸残基的比例与其抗-HPV 活性密切相关，这意味着 JB01 的净负电荷在 JB01 介导 HPV 感染抑制效应中具有重要作用。这些结果表明，JB01 可通过 β-乳球蛋白上带负电残基与 L1 和/或 L2 蛋白上带正电残基的交互作用，阻止 HPV 进入靶细胞内。据此我们推测，L1 蛋白的 C 末端缘结构域与 L2 蛋白的 N-末端结构域有可能是 JB01 的作用靶点，因为这两个结构域都含有暴露于病毒衣壳表面的带正电残基[23,24]。事实上，研究亦发现，JB01 可与 HPV L1 和 L2 蛋白上的带正电残基牢固结合，而未修饰 β-乳球蛋白与这些肽间未观察到明显的结合作用（未提供数据）。

总之，本研究证明，作为一种表面负电荷量相对增多（对带正电残基进行化学修饰的结果）的化学修饰 β-乳球蛋白，JB01 可有效抑制 HPV 感染，其机制很可能是靶向作用于病毒复制早期、特别是病毒进入过程。作为一种廉价、安全且稳定的抗-HPV 剂，可将 JB01 制成外用凝胶制剂，用于生殖粘膜 HPV 感染的预防和治疗，进而减少子宫颈癌的发病。

致谢

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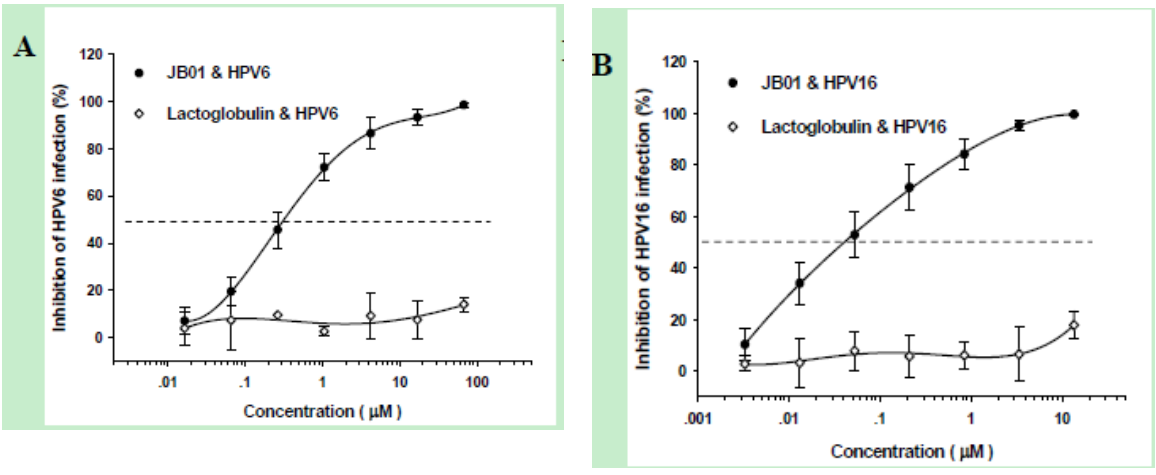
参考文献

图释



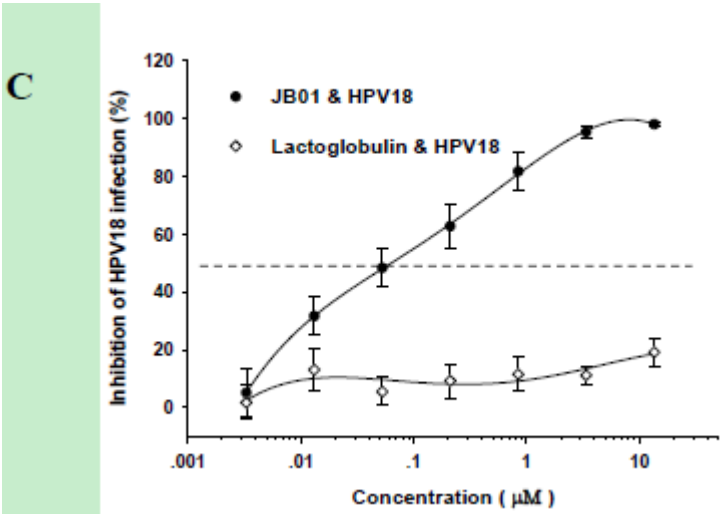


图 1. JB01 对 HPV 感染的抑制活性，包括 HPV6（A）、HPV16（B）和 HPV18（C）病毒。将标本分成将标本分成 3 份完成测定，并重复实验至少 2 次。图中数据为代表性实验三份测定值的均数 ± SD。

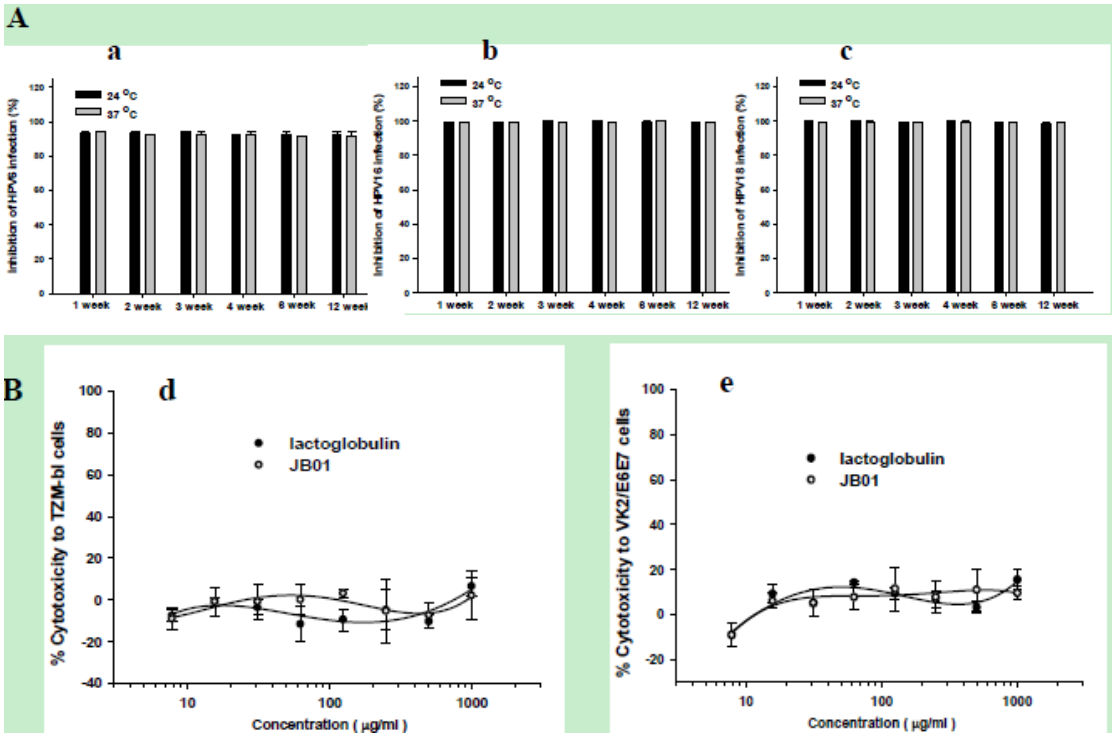


图 2. JB01 的稳定性和细胞毒性。（A）JB01 的稳定性。分别将 JB01 放置在 24 和 37℃条件下贮存 1-12 周，各指示时点其抗 HPV6（a）、HPV16（b）和 HIV18（c）感染的抗病毒活性如图所示。（B）JB01 的细胞毒性。采用 XTT 比色法测定 JB01 对 TZM-B1 细胞（d）和 VK2/E6E7 细胞（e）的可能毒性反应。利用未修饰 β-乳球蛋白作为对照。将标本分成 3 份完成测定，并重复实验至少 2 次。图中数据为代表性实验三份测定值的均数 ± SD。

表 1. 3-羟基苯二甲酸酐（HP）修饰 β-乳球蛋白抗-HPV 活性与其修饰残基比例的对照

	修饰残基（%）		抑制下述病毒感染的 IC_{50}（μM）	
HP（mM）浓度	赖氨酸	精氨酸	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 每份样本都分成三份进行测定，实验重复两次。表中所示数据为代表性实验三份测定值的均数 ± SD。