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REBACIN® as a noninvasive clinical intervention for high-risk human papillomavirus persistent infection

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The development of highly sensitive HPV-genotyping tests has opened the possibility of treating HPV-infected women before high-grade lesions appear. The lack of efficient intervention for persistent high-risk HPV infection necessitates the need for development of novel therapeutic strategy. Here we demonstrate that REBACIN®, a proprietary antiviral biologics, has shown potent efficacy in the clearance of persistent HPV infections. Two independent parallel clinical studies were investigated, which a total of 199 patients were enrolled and randomly divided into a REBACIN®-test group and a control group without treatment. The viral clearance rates for the REBACIN® groups were 61.5% (24/39) and 62.5% (35/56), respectively, for the two independent parallel studies. In contrast, the nontreatment groups showed self-clearance rates at 20.0% (8/40) and 12.5% (8/64). We further found that REBACIN® was able to significantly repress the expression of HPV E6 and E7 oncogenes in TC-1 and Hela cells. The two viral genes are well known for the development of high-grade premalignancy lesion and cervical cancer. In a mouse model, REBACIN® was indicated to notably suppress E6/E7-induced tumor growth, suggesting E6 and E7 oncogenes as a potential target of REBACIN®. Taken together, our studies shed light into the development of a novel noninvasive therapeutic intervention for clearance of persistent HPV infection with significant efficacy.

Introduction

While over 80% of human papillomavirus (HPV) infections are transient and most of them can be self-cleared in the period of about 12 months, 10–20% existing infections will become persistent.[1–5] If viral DNA is integrated into the human genome during the period of persistent infection, it will be leading to high risk of HPV related disease as well as the possibility of invasive cancer.[6–9]

There are recently three prophylactic HPV vaccines, Gardasil®, Cervarix® and Gardasil®9 commercially available in China.[10,11] These HPV vaccines will be efficient for preventing the HPV infection by neutralizing antibodies to the HPV viral particles.[12] However, these preventive vaccines are not effective on eliminating preexisting infections.[13–16] Thus, the large amount of people who are already infected with the HPVs cannot get the benefit from these prophylactic HPV vaccines.

In China, according to survey statistics, the infection rate of high-risk HPV (hrHPV) in urban women is 15.2% while it is 15.7% in rural women. There are over 45 million Chinese

Key words: cervical cancer, human papillomavirus, HPV, REBACIN®, clearance

Abbreviations: CIN: cervical intraepithelial neoplasia; HPV: human papillomavirus; hrHPV: high-risk HPV; LEEP: loop electrosurgical excision procedure; SCID: severe combined immunodeficiency disease; TCT: thinprep cytologic test

Additional Supporting Information may be found in the online version of this article.

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What's new?

While most HPV infections clear up on their own, persistent infections can lead to cancer. No antiviral drugs are currently available to fight HPV infection. In this paper, the authors tested a proprietary biologic antiviral they developed, called REBACIN®. The drug successfully eliminated HPV infection in 60% of treated patients, while in the control group only 20% of infections cleared on their own. All patients began the trial with high-risk HPV infection lasting one year or more. The authors also found that REBACIN® inhibited mRNA transcription of HPV oncogenes E6/E7 and reduced E6/E7-stimulated tumor growth in mice.

women are infected by hrHPVs annually. Up to 19 million of them are at the stage of low-grade cervical lesions, 17 million at the stage of high-grade lesions and 99 thousand at the stage of cervical cancer.[17–21] Another investigation showed that more than 20% female patients are hrHPV-positive in hospital examination[22] and most of them belong to persistent infections. If it converted into integrated infection, it will be exposed under the high risk of invasive cancer. Therefore, the development of effective therapies, especially to make efficient drug targeting persistent infection remains very imperative and urgently needed.

There are currently no available antiviral drugs targeting HPV infection.[23] Recently, we generated an antiviral biologics termed REBACIN®, and it showed significant efficacy against HSVII viral infection in mouse (unpublished data). In this article, we investigated REBACIN® as a noninvasive clinical intervention and found its potential therapeutic efficacy against HPV persistent infection both *in vivo* and *in vitro* as well as in clinical trial.

Materials and Methods

Participants in clinical trial

This clinical trial was approved by the Ethic Committee on Clinical Trials of Drugs, Peking Union Medical College Hospital. Written informed consent was obtained from all patients prior to enrollment in the project. Seventy-nine volunteer patients were enrolled in a parallel study with sustained infections of hrHPV, but without a high level of cervical lesions (no more than grade one cervical intraepithelial neoplasia [CINI] according to the examination of colposcopy and biopsy) diagnosed in the Department of Obstetrics and Gynaecology at Peking Union Medical College Hospital. Similarly, 120 volunteer patients were enrolled in the Department of Obstetrics and Gynaecology at Tianjin Port Hospital. To evaluate the efficacy of REBACIN® on intervention of persistent hrHPV infections, the patients were randomly divided into a REBACIN® group and a control group by using a computer generated randomized numbering system. Inclusion criteria of participants: (*i*) women between 18 and 65 years old with sexual life, but nonpregnant or lactating; (*ii*) hrHPV infection for more than 12 months diagnosed by the Hybrid-Capture 2; (*iii*) no more than CIN1. Exclusion criteria of participants: (*i*) with a history of allergy, smoking, oral contraceptive and HPV vaccination; (*ii*), with severe heart, lung, liver and kidney dysfunction; (*iii*) women who cannot complete the designed drug administration (at least 20 days of drug application each month for consecutive 3 months) due to menstrual disorder. There is no significant difference ($p > 0.05$) in the age, HPV infection, cytological abnormality, and so on of the volunteers among different groups (Supporting Information Table S1).

Clinical trial design

REBACIN® is a biological drug, which is a compounding agent. The dose-finding, pharmacodynamics (PD) and toxicity studies of the REBACIN® were carried out both in the animal test and preclinical study, which defined the maximum tolerated dose (MTD) and recommended dose (RD) for subsequent trials. The standard dose-finding design as the "3 + 3 design" was applied for determining the MTD. The participants self-administered the dose. Before the first use, a trained research nurse on our study demonstrated how to apply the REBACIN® till the participants knowing the administration well. Detail instructions were also printed on the back of REBACIN® package.

For participants in the treatment group, REBACIN® (0.5 g compounding agent per dose) was administered intravaginally every other day for 3 months, except during the menstrual period. REBACIN® treatment was not applied in the non-treatment group (the control). For all participants, right before the trial and at all follow-ups, HPV-DNA test, cervical appearance (cervical erosion), cervical thinprep cytologic test (TCT), colposcopy and histopathological examination of cervix were performed; at the same time, physical examinations including blood routine test, liver function test and renal function test were also performed to evaluate potential adverse reaction.

The compliance of participants was guaranteed by following trial rule and strict follow-up control. First, the investigator provided all participants informed consent. After understanding the investigation well, and agreeing to obey the trial rule, the women signed the informed consent form and were enrolled successfully in the trial. Second, all participants had their own diary card, and had to record and report their detail administration, including application time, potential side effects, and any anomalous-phenomenon. By reading the diary card, the investigator could know the trial well and the compliance of participants. For participants whom could not obey the trial rule, they were removed from the trial list.

Third, all participants had their own follow-up card, and the investigators performed three visits according to scheduled date on the card. Meanwhile, beside regular follow-ups, the investigators and participants still actively communicated by each other. This close administration enabled the investigators to get to know patients well, and also enabled patients to obtain medical supervision and psychological support. All of the administration ensured the trial quality and participant's compliance.

HPV-DNA test and criterion of the judgment of REBACIN® efficacy

HPV-DNA from each cervical scrape of participants was detected by the Hybrid-Capture 2 (HC-2) in accordance with the manufacturer's instructions. HPV viral load expressed as relative light units/cut off (RLU/CO) values. Diagnostic criteria of HPV infection: if participant's RLU/CO value is more than 1, they were considered as positive of HPV infection; conversely, participants were considered as negative of HPV infection if their RLU/CO value is less than 1.

Evaluation criteria for hrHPV infection

Judgment of efficacy	Decrease rate of hrHPV load (fluorescent reading) (%)
Significantly effective	›50%
Effective	25–50%
Invalid	No significant difference or ‹25%

Evaluation of REBACIN®-induced inhibition of E6/E7 transcription

TC-1 cells (ATCC® CRL-2785™) or Hela cells (ATCC® CCL-2™) stably expressing E6/E7 oncogenes were first grown on a six-well plate till 80% of logarithmic growth phase. Seventy-two hours after the treatment of different nontoxic concentration of REBACIN® (0–1,250 μg/ml), the cells were harvested using 0.25% trypsin and total RNA was extracted by RNeasy Mini Kit 74104 (Qiagen, Hilden, Germany). Cell total RNA was analyzed by agarose gel electrophoresis and spectrophotometer. To detect the E6/E7 and actin transcription, 1 μg of cell total RNA was first reverse-translated into cDNA by the Reverse Transcription System A3500 (Promega, Madison, WI). The total cDNA was then used as template to amplify the E6/E7 and actin gene by standard PCR using the following primers: the forward primer 5′-TAGAATTCATGCACC AAAAGA GAACTGCA-3′ and reverse primer 5′-TAGC GGCCGCTACAGCTGGGTTTCTCTACGT-3′ for the HPV16 E6; the forward primer 5′-TAGAATTCATGCATGGAGATAC ACCTACA-3′ and reverse primer 5′-TAGCGGCCGCTAT GGTTTCTGAGAACAGATG-3′ for the HPV16 E7; the forward primer 5′-AGGTGCCTGCGGTGCCAGAA-3′ and reverse primer 5′-CTGCGTCGTTGGAGTCGTTC-3′ for the HPV18 E6; the forward primer 5′-AATGAAATTCCGGTTGACCT-3′ and reverse primer 5′-ACGTTGTGGTTCGGCTCGTC-3′ for the HPV18 E7; in addition, the primer of 5′-GATGGTGGG TATGGGTCAGAAGGA-3′ and 5′-GCTCATTGCCGATA GTGATGACCT-3′ for actin used as a control. The RT-PCR products were analyzed using 1% agarose gel electrophoresis.

Assessment of REBACIN® effect on E6/E7-induced tumor growth

Preparation of tumor inducer. Cells were first grown on a six-well plate till 80% of logarithmic growth phase. After double wash with Hank's Balanced Salt solution (GIBCO, Carlsbad, CA), the cells were treated by different nontoxic concentration of REBACIN® (0–1,250 μg/ml) at normal culture condition for 72 hr. The cells were then harvested by 0.25% trypsin/PBS. After centrifugation, the cells were resuspended in PBS and adjusted to 5×10^5 cells per ml PBS. The cells were finally used as tumor inducer in mice.

E6/E7-induced tumor growth. Our study was approved by the Institutional Review Board at National Institute for Viral Disease Control and Prevention, Beijing, China. Male severe combined immunodeficiency (SCID) mice aged 6 weeks (Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences) were housed under controlled laboratory conditions (12-hr light/dark cycle, temperature $20 \pm 2°C$, humidity 50–60%). The mice were randomized, and injected at right axilla using the tumor inducer (100 μl each) on the 1st, 14th and 28th day. Twenty-four hours after the last injection, the mice were euthanized by cervical dislocation, and the tumor block was peeled away and weighted. The inhibition rate of tumor growth was evaluated as REBACIN® effect.

Thinprep cytologic test

According to the classification of the Bethesda System (TBS) 2001, cervical cytology was evaluated.

Statistical analysis

Clinical statistical analyses were performed by SPSS version 13.0. Data from the participants who were enrolled in the clinical trial were statistically analyzed. The qualitative data were analyzed by *t*-test while the counting data by the Chi-square test (χ^2). If *p*-value was ≤0.05, its related data were considered as statistically significant. The data from E6/E7 experiments were analyzed by GraphPad Prism 6.

Results

REBACIN® treatment prevents HPV infection and HPV-induce lesion

Recently, we generated an antiviral biologics termed REBACIN® significantly against HSVII viral infection (unpublished data), here we evaluated whether REBACIN® could be an antiviral drug on the clearance of persistent

Table 1. Efficacy of REBACIN® on HPV-clearance

Group	Before treatment		After treatment		
	HPV$^+$	HPV$^-$	HPV$^+$	HPV$^-$	Clearance rate
REBACIN®	39	0	15	24	61.54%[1]
Control	40	0	32	8	20.00%

[1]$p < 0.001$ *vs*. the control analyzed by the Chi-square test (χ^2).

hrHPV infections in cervix. As shown in the Table 1, 3 months after the REBACIN® treatment, 61.54% of HPV-positive patients became HPV-negative compared to 20.00% in the control group ($p < 0.001$). In consistent with this finding, the total effective rate was significantly higher in treatment group in comparison with that in control group (89.74 and 52.50%, respectively, $p < 0.001$, Supporting Information Table S2). We further investigated whether REBACIN® treatment would lead to the regression of HPV-induce lesions in similar clinical treatment. During two follow-up visits (one at the end of administration and the other in 3 months later), TCT tests were carried out. Patients with negative TCT and normal cervical cells in both consecutive follow-ups were considered as regression of CIN. As shown in Table 2, patients displayed remarkable regression of HPV-induced CIN and lesions, and also similar viral clearance after REBACIN® treatment. In comparison to the 11% patients diagnosed with CIN1 lesions in control group, up to 50% patients diagnosed with CIN1 lesions in REBACIN® group were eliminated for CIN according to the histological evaluation of colposcopic-directed biopsy specimens. These studies demonstrate that REBACIN® has significant effect on the clearance of sustained hrHPV infection and HPV-induce lesions.

Table 2. Virological and clinical responses after REBACIN® treatment

Group	Before treatment		After treatment	
	HPV$^+$	CIN1$^+$	HPV$^-$	CIN1$^-$
REBACIN®	56	32	62.5% (35/56)*	50.0% (16/32)#
Control	64	35	12.5% (8/64)*	11.4% (4/35)#

Level *$p < 0.001$, #$p < 0.001$ *vs*. the control analyzed by the Chi-square test (χ^2).
Abbreviation: CIN1, cervical intraepithelial neoplasia grade 1.

REBACIN® inhibits the expression of oncogene E6 and E7

In recent decades, HPV studies on therapeutic strategies have proposed several options, including the role of HPV E6 and E7 oncogenes. Considering that the progression to cervical malignancy requires the overexpression of E6 and E7 genes of integrated HPV genome,[24–29] we further investigated whether this antiviral biologic inhibits the expression of E6 and E7 viral genes. Seventy-two hours after the application of different concentration of REBACIN® to TC-1 and Hela cells stably expression of HPV E6/E7, the transcription level of HPV

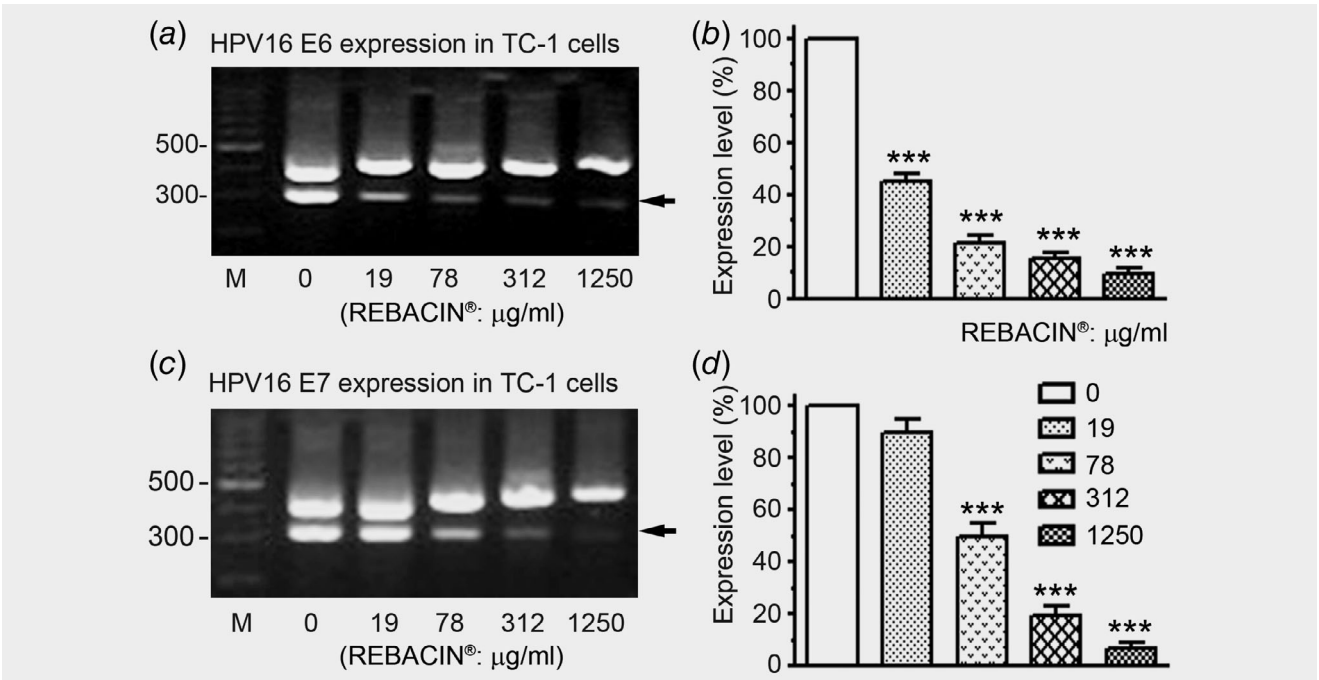

Figure 1. REBACIN® inhibits HPV16 E6/E7 oncogene transcription. TC-1 cells stably expressing HPV16 E6/E7 were treated by different concentration of REBACIN® for 72 hr, transcription of the E6 and E7 was then evaluated by RT-PCR. Low concentration of REBACIN® markedly inhibited both HPV16 E6 (*a*, *b*) and E7 expression (*c*, *d*). The results in (*a*) and (*c*) were quantified and shown in (*b*) and (*d*), respectively. Histograms show mean ± SEM of three independent experiments performed in duplicate. ***$p < 0.001$ *vs*. control (0 μg/ml REBACIN®) analyzed by one-way analysis of variance followed by Bonferroni test.

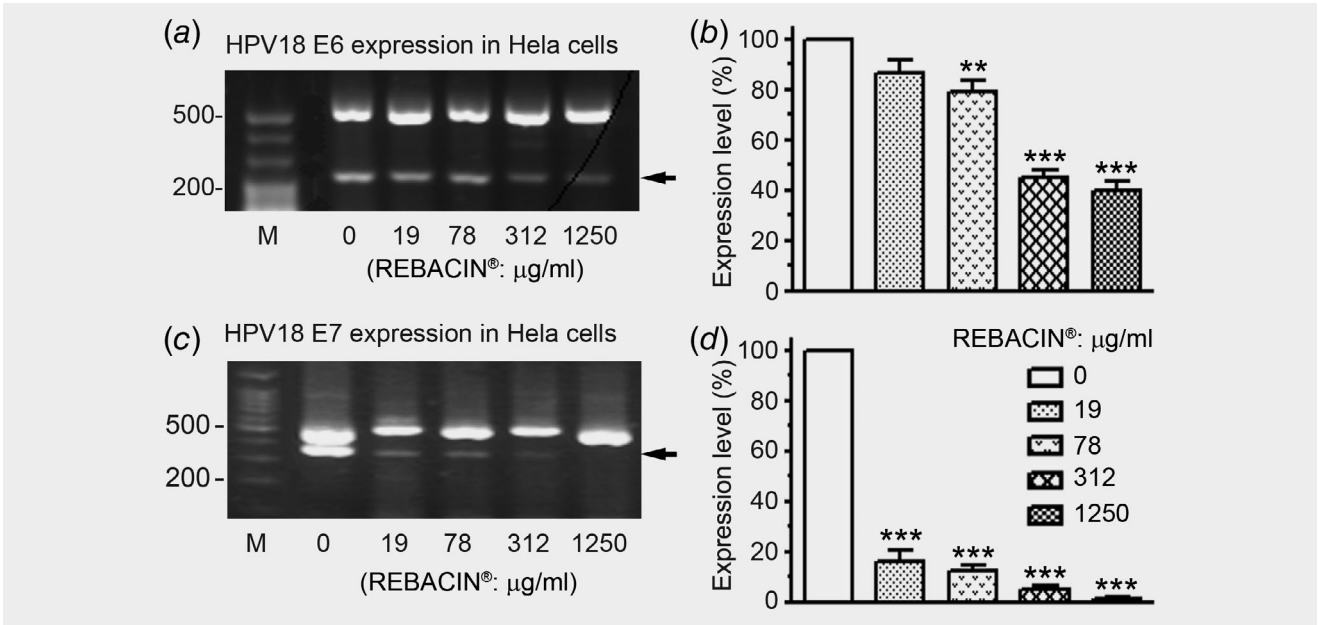

Figure 2. REBACIN® inhibits HPV18 E6/E7 oncogene transcription. Hela cells stably expressing HPV18 E6/E7 were treated by different concentration of REBACIN® for 72 hr, transcription of the E6 and E7 was then evaluated by RT-PCR. Low concentration of REBACIN® markedly inhibited both HPV18 E6 (*a*, *b*) and E7 expression (*c*, *d*). The results in (*a*) and (*c*) were quantified and shown in (*b*) and (*d*), respectively. Histograms show mean ± SEM of three independent experiments performed in duplicate. $^{**}p < 0.01$; $^{***}p < 0.001$ *vs*. control (0 μg/ml REBACIN®) analyzed by one-way analysis of variance followed by Bonferroni test.

E6/E7 mRNA was examined via RT-PCR. REBACIN® treatment in the nontoxic concentration (no more than 1,250 μg/ml) did not significantly influence the total RNA expression of the cells (Supporting Information Table S3). However, the mRNA transcription of HPV E6/E7 in both cell lines was markedly inhibited by the application of REBACIN® (Figs. 1 and 2). In TC-1 cells, a low concentration of 19 μg/ml REBACIN® significantly inhibited the E6 expression, while the 78 μg/ml REBACIN® has clear effect on the inhibition of the E7 expression (Fig. 1). Similarly, in Hela cells, the 78 μg/ml REBACIN® markedly prevented the E6 expression, while the 19 μg/ml REBACIN® decreased the E7 expression (Fig. 2). These findings indicate that nontoxic concentration of REBACIN® is significantly effective in the inhibition of HPV E6 and E7 expression.

HPV E6/E7-induced tumor growth was inhibited after REBACIN® administration

Subcutaneous injection of the cells expressing HPV E6/E7 into the SCID mice induces tumor development.[30,31] As demonstrated above that the application of REBACIN® inhibits the transcription of HPV E6 and E7, we therefore examined whether REBACIN®-mediated inhibition of HPV E6/E7 expression influences the E6/E7-stimulated tumor growth using the SCID animal model. After the treatment of different concentration of REBACIN®, TC-1 or Hela cells stably expressing HPV E6/E7 were inoculated into the SCID mice through subcutaneous injection at the right armpit of the mice. As shown in Figures 3 and 4, in the SCID model, REBACIN® treatment significantly inhibited E6/E7-stimulated tumor growth after three time injections (on Day 1, 14 and 28). Consistent with the finding that REBACIN® inhibits HPV E6/E7 expression (Figs. 1 and 2), TC-1 cells with inhibition of E6/E7 expression after REBACIN® treatment (as low as 19 μg/ml) were significantly poor to induce the tumor growth (Fig. 3). Similarly, after inhibition of the E6/E7 expression, the Hela cells also poorly induced tumor growth (Fig. 4). These studies demonstrate that the serious level of either TC-1 or Hela cells induced malignant transformation is depended on the expression level of E6/E7, suggesting a potential role of REBACIN® in the prevention of E6/E7-stimulated cervical carcinogenesis.

No adverse reaction in the trial

Clinical observation plus relevant auxiliary examination has shown that, for all participants, there was no significant side effect during 6-month follow-ups in the trial, including 3-month observation after the administration. Moreover, routine physical examinations in all participants before and after the treatment indicated that the participant's blood routine, liver function (ALT and AST), kidney function (BUN and Cr) and other tests were in the normal range. However, REBACIN®-induced mild vaginal itch was occasionally observed.

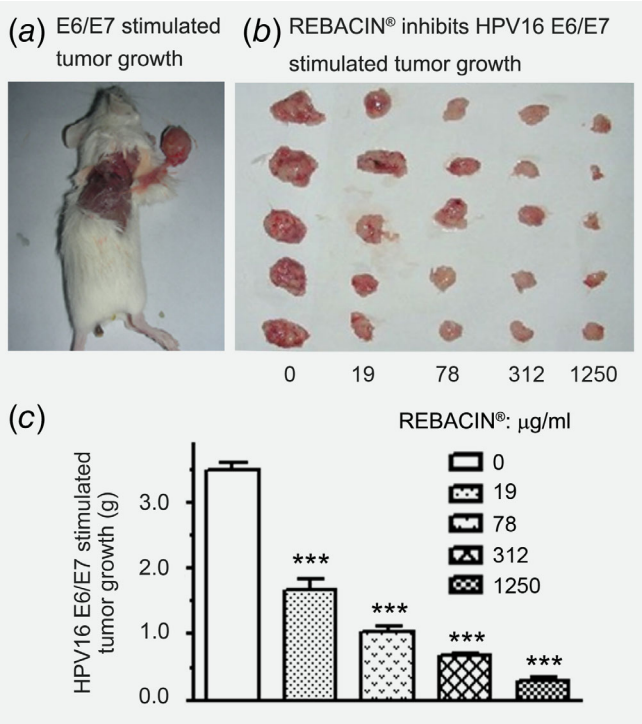

Figure 3. REBACIN®-mediated inhibition of HPV16 E6/E7 expression in TC-1 cells leads to the inhibition of tumor growth in SCID mice. (*a*) Subcutaneous injection of TC-1 cells stably expressing HPV16 E6/E7 into SCID mice induced tumor development well. (*b*) The TC-1 cells were first treated by different concentration of REBACIN® for 72 hr, and then the cells were harvested and used as tumor inducer. At the end of tumor induction, the SCID mice were euthanized by cervical dislocation and the tumor blocks were peeled away. (*c*) Quantification of REBACIN® effect on SCID tumor growth in (*b*). The tumor blocks in (*b*) were weighted and analyzed. Histograms show mean ± SEM of five independent experiments performed in triple repeat. ***$p < 0.001$ *vs*. the control (0 μg/ml REBACIN®) analyzed by one-way analysis of variance followed by Bonferroni test.

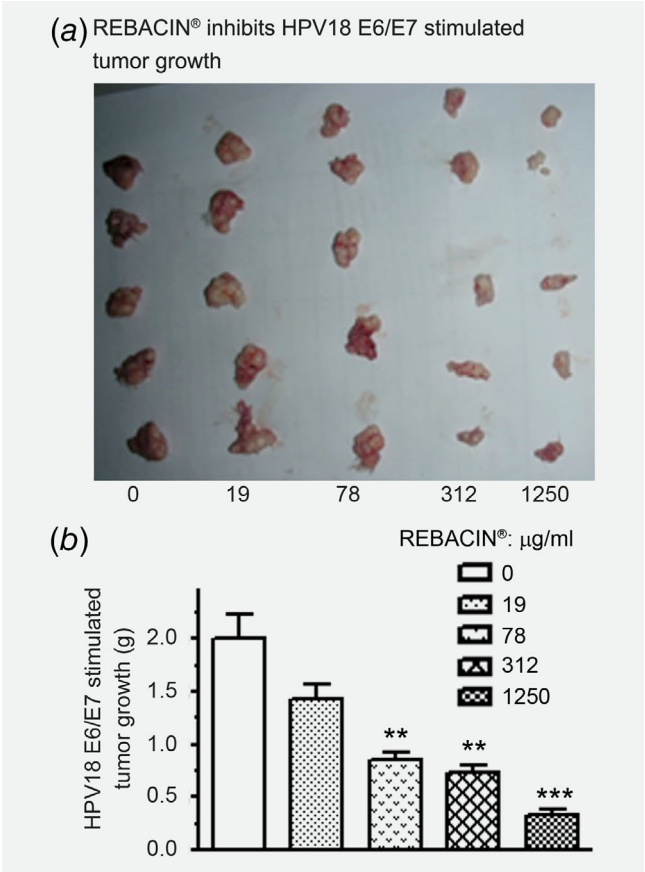

Figure 4. REBACIN®-mediated inhibition of HPV18 E6/E7 expression in Hela cells leads to the inhibition of tumor growth in SCID mice. (*a*) The Hella cells were treated by different concentration of REBACIN® for 72 hr, and then the cells were harvested and used as tumor inducer. At the end of tumor induction, the SCID mice were euthanized by cervical dislocation and the tumor blocks were peeled away. (*b*) Quantification of REBACIN® effect on SCID tumor growth. The tumor blocks in (*a*) were weighted and analyzed. Histograms show mean ± SEM of five independent experiments performed in triple repeat. **$p < 0.01$; ***$p < 0.001$ *vs*. control (0 μg/ml REBACIN®) analyzed by one-way analysis of variance followed by Bonferroni test.

Discussion

HPVs persistent infection is the causative cause of cervical cancer.[32–35] Some of the hrHPV persistent infection will be followed by viral DNA integration with human genome, resulting in over expression of two viral oncogene E6 and E7.[36–40] These oncoproteins bind to the tumor suppressor genes p53 and the retinoblastoma protein (pRB), destroy their function, leading to dysplasia and invasive cancer.[41–43] Therefore, the inhibition of E6 and E7 expression will stop or regress the development of high-grade CIN and thus reduces invasive cancer. These studies demonstrated that REBACIN® drastically inhibited the expression of E6 and E7 oncogenes with the relations of dose-effect *in vitro* in the absence of cellular toxicity. In mouse, REBACIN® significantly suppress the growth of tumors induced by expression of E6 and E7 oncogenes of either HPV16 or HPV18 by up to 91.84 and 83.75%, respectively ($p < 0.001$) and showed the dose dependent. The finding strongly supports that the REBACIN® is a novel effective anti-HPV factor that is able to suppress E6 and E7 gene expression *in vitro* and suppresses tumors induced by HPV16 and HPV18 *in vivo*. Based on the experiment, REBACIN® inhibits HPV E6/E7 oncogene transcription both in Hela and in TC-1 cells, suggesting that REBACIN®-mediated E6/E7 suppression is probably involved in clearance of hrHPV persistent infection via a change of crosstalk of transcriptional regulation, which a mechanism is worth further investigation.

To evaluate the clinical effect of REBACIN® on patients with hrHPV persistent infection, a parallel study of 199 patients were subject to this clinical investigation with one trial of 79 patients and 120 patents for another experiment, the patients were randomly divided into two groups, the experimental groups treated with REBACIN® via vaginal administration, and a control group that received no drug

treatment. The efficacy against HPV infection was evaluated by analysis of HPV clearance using the Hybrid-Capture 2 tests at the month after the drug treatment ceased. The two parallel clinical treatments got a similar result that the clearance rates of HPV in REBACIN®-treated group were 61.5% (24/39) and 62.5% (35/56) while the self-clearance rates in the control group of patients receiving no drug interferences were 20.0% (8/40) and 12.5% (8/64), respectively. These results have demonstrated that REBACIN® is significantly effective on the clearance of hrHPV persistent infection with notable virus negative conversion rate. Furthermore, two or three courses of the treatment could lead up to 75% clearance rate (Supporting Information Table S4). Three months after the REBACIN® treatment, 61.54% of HPV-positive patients became HPV-negative (Table 1). However, the total effective rate was 89.74% (Supporting Information Table S1). This finding indicated that although some patients still showed HPV positive after the treatment, their viral load was reduced. The unsuccessful clearance of persistent HPV infection probably is due to their viral genome integration or poor individual immune system, which could influence the clearance. Therefore, prolonged treatment of REBACIN® will be necessary for some patients.

It is well noted that the expression of viral oncogenes E6/E7 in HPV-infected premalignant and malignant cells plays an important role in high-grade CIN formation and tumorigenesis.[44–48] Consistent with those opinions, our studies revealed that REBACIN® treatment led to significant regression of HPV-induced CIN1; moreover, our preliminary data indicated that REBACIN® treatment also had strong effect on cervical lesion regression in CIN2 patients. REBACIN® showed notable 86.7% (13/15) efficacy in regression of high-grade cervical lesion, and 93.3% (14/15) efficacy in HPV infection clearance after one course treatment (Supporting Information Table S5). These findings are in line with our previous preliminary investigations that loop electrosurgical excision procedure (LEEP) combination with REBACIN® treatment was much more effective in controlling the development of CIN. In this combination, the healing rate (HPV clearance and CIN elimination) and recurrence rate were 96.8 and 3.8% whereas 73.5 and 26.5% in the LEEP alone, respectively. These studies are also consistent with our current preliminary investigations that the treatment of 5-aminolevulinic acid-photodynamic therapy (ALA-PDT) with REBACIN® showed a potent efficacy both in regression of CIN and in HPV infection clearance. In this combination, the recovery and effective rate were 71.6 and 86.6% for CIN1 patients, 55.2 and 74.0% for CIN2 patients, while 22.5 and 62.5% for CIN3 patients, respectively.[49,50]

Taken together, these studies strongly support REBACIN® as a noninvasive intervention therapy with high clinical efficacy not only in the clearance of persistent HPV infection but also in playing a crucial role in the prevention of cervical premalignancy development.

Acknowledgements

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