Original Article

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The supercritical CO_2 extract from the skin of *Bufo bufo gargarizans* Cantor blocks hepatitis B virus antigen secretion in HepG2.2.15 cells

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Summary **The skin of *Bufo bufo gargarizans* Cantor has long been used for the treatment of hepatitis B in China and supercritical carbon dioxide extraction (SC-CO_2) is widely used in extracting active ingredients from natural products. The aim of present study was to assess the anti-hepatitis B virus (HBV) effect of the supercritical CO_2 extract from the skin of *Bufo bufo gargarizans* Cantor (SCE-BC). Cytotoxicity of SCE-BC was analyzed using an MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] assay in HepG2.2.15 cells. The hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and hepatitis B core-related antigen (HBcrAg) concentrations in cell culture medium were determined by chemiluminescent enzyme immunoassay. HBV mRNA in cells was determined using real-time polymerase chain reaction. SCE-BC concentrations below 10^{-2} μg/mL had no significant toxicity to HepG2.2.15 cells. SCE-BC at 10^{-4} μg/mL effectively inhibited the secretion of HBeAg by 23.36% on day 6. It was more potent than the positive control lamivudine (100 μg/mL) in terms of the inhibition of HBeAg and HBcrAg secretion on day 6. Consistent with the HBV antigen reduction, HBV mRNA expression was markedly inhibited in comparison to the control when HepG2.2.15 cells were treated with SCE-BC. Moreover, SCE-BC had greater inhibitory activity with respect to HBeAg than to HBsAg. Since HBeAg promotes immune tolerance and persistent infection during HBV infection, the present results suggest that immune tolerance induced by HBeAg might be overcome by SCE-BC. Therefore, SCE-BC warrants further investigation.**

Keywords: Hepatitis B virus, traditional Chinese medicine, *Bufo bufo gargarizans* Cantor, HepG2.2.15 cells

1. Introduction

Hepatitis B virus (HBV), a member of the hepadnavirus (hepatotropic DNA viruses) family, infects approximate 400 million people, making it the most common chronic infectious disease worldwide (*1*). Chronic HBV infection can lead to cirrhosis, liver failure, and hepatocellular carcinoma, accounting for more than a million global deaths annually (*2,3*). Currently, only several antiviral drugs, including interferon-α and lamivudine (3TC), have been approved for the treatment of hepatitis B. However, the therapeutic effect of interferon-α and 3TC may be accompanied by adverse effects and drug resistance following prolonged administration (*4,5*). Therefore, there remains an urgent need for alternative drugs against HBV. Traditional Chinese medicines (TCMs), widely used to treat

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hepatitis B in China and many parts of the world (*6*), could provide a great opportunity for screening safe and more effective anti-HBV agents.

The venom and skin of toad *Bufo bufo gargarizans* Cantor has long been used in TCM (*7,8*). Chansu, a preparation of toad venom, has been used for the treatment of canker sores, toothache, sinusitis, and local inflammations in China for many years (*7*). Cinobufacini (Huachansu), the aqueous extract of the *Bufo bufo gargarizans* Cantor skin, is a Chinese medicine and has been extensively used in clinics to treat a number of diseases, such as malignant tumors, chronic hepatitis B, and systemic and local infection (*9-12*). It is reported the negative conversion rates of HBsAg and HBeAg increased significantly in patients treated with cinobufacini compared to the control group (patients untreated with cinobufacini) (*13*). Moreover, abnormal serum hepatic enzyme levels significantly decreased after treatment. In our previous study, cinobufacini at 1 μg/mL effectively inhibited the secretion of HBsAg and HBeAg by 29.58 and 32.87% in HepG2.2.15 cells on day 6. It was more potent than lamivudine (100 μg/mL), which served as a positive control (*14*).

In recent years, some new techniques, including ultrasonic extraction and supercritical carbon dioxide extraction (SC-CO_2), are widely used in extracting active ingredients from traditional Chinese medicine. SC-CO_2 is a separation technique based on the enhanced solvating power of CO_2 above its critical point (*15*). This technology has advantage over traditional technology because of the non-toxic, non-flammable characteristics of CO_2 and its availability in high purity with low cost. Moreover, CO_2 has low critical temperature (31.1°C) and low critical pressure (73.8 bar). Therefore CO_2 can be treated as an ideal solvent for extraction of natural products (*16*). Our previous study indicates the aqueous extract of the *Bufo bufo gargarizans* Cantor skin cinobufacini possess significant activity against HBV (*15*). However, it is still unclear whether the supercritical CO_2 extract from the skin of *Bufo bufo gargarizans* Cantor (SCE-BC) possess the activity against HBV. In the present study, in order to search and develop more effective anti-HBV drug, SCE-BC was investigated for the inhibition of HBV replication and secretion of HBV antigen in HBV-infected hepatocytes.

2. Materials and Methods

2.1. *Drugs*

SCE-BC, which was prepared by extracting 10 g of the toad skin with dynamic extraction using 200 mL CO_2 at 40°C and 20 MPa followed by concentration to 1 mL, was obtained from Anhui China Resources Jinchan Pharmaceutical Co., Ltd. (Anhui, China). It was dissolved in dimethylsulfoxide (DMSO) as a 100 mg/mL stock solution and kept at 4°C. 3TC was from Moravek Biochemicals (Brea, CA, USA) and served as the positive control. Dilutions of the drugs were performed on the day of medium change. The final concentration of DMSO in the samples was less than 0.001% (v/v).

2.2. *Cell culture and treatment*

The human HBV-transfected cell line HepG2.2.15 (*17*) was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (both from Gibco-Invitrogen, Carlsbad, CA, USA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 200 μg/mL G418 (all from Sigma, St Louis, MO, USA) at 37°C in a humidified incubator with 5% CO_2. Cells were maintained for 24 h before treatment to reach confluence. The confluent HepG2.2.15 cells were treated with SCE-BC or 3TC at various concentrations in serum-free medium for 3 days or 6 days. The culture medium was replaced with a fresh one on day 3, with or without (negative control conditions) different concentrations of SCE-BC or 3TC during the 6-day experiment.

2.3. *Cytotoxicity assay*

HepG2.2.15 cells were seeded in 96-well plates at a density of 1.5×10^4 per well and treated with different concentrations (10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, or 10 μg/mL) of SCE-BC for 3 or 6 days. The cytotoxicity of SCE-BC was analyzed with an MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] assay using the Cell Proliferation Kit I (Roche, Mannheim, Germany) following the manufacturer's instructions. Each experiment was performed in triplicate. The cell viability was expressed as a percentage of the control.

2.4. *Measurement of HBV antigens*

HepG2.2.15 cells were seeded in 6-well plates at a density of 4.5×10^5 per well for measurement of HBV antigens. After incubation with various concentrations of SCE-BC or 3TC for 3 or 6 days, the culture medium was collected, cell debris was removed, and the result was stored at -70°C until analysis. Hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and hepatitis B core-related antigen (HBcrAg) in culture supernatants of HepG2.2.15 cells were respectively measured with a Lumipulse® II or I kit or HBcrAg kit (Fuji Rebio, Tokyo, Japan). These kits use a method of chemiluminescent enzyme immunoassay (CLEIA) based on the chemiluminescent capture of specific antigen–antibody reactions.

2.5. *Quantification of HBV DNA in the culture medium*

The HBV viral load in culture supernatants of HepG2.2.15 cells was quantified with a HBV DNA

quantitative kit (SRL, Tokyo, Japan). This kit is based on transcription-mediated amplification and hybridization protection assay (*18*). Briefly, a mixture of 10 μL of cell culture supernatants or amplification standards, Sample Diluent I, Sample Diluent II, and Primer Reagent and 50 μL of oil were placed in a reaction tube. The tube was heated at 95°C for 10 min and then incubated at 37°C for 5 min. Neutralization reagent and reconstituted amplification reagent solution were added and the reaction mixture was incubated at 37°C for 3 h. RNA amplicons were detected with a hybridization protection assay. The measurement range was 3.7-8.7 log genome equivalent/mL (LGE/mL).

2.6. *Determination of HBV RNA*

Total RNA was isolated from HepG2.2.15 cells using Trizol reagent (Invitrogen) in accordance with the manufacturer's instructions. The mRNA was then reverse-transcribed to make cDNA using a QuantiTect Rev. Transcription Kit (Qiagen GmbH, Hilden, Germany) and oligo dT primer following the manufacturer's instructions. The cDNA was quantified using the Thermal Cycler Dice™ Real Time System (Takara, Shiga, Japan). The polymerase chain reaction (PCR) was performed using primers (synthesized by Invitrogen): HBV surface region F 5`-GCCAAAATTCGCAGTCC-3` and R 5`-ACGGGCAACATACCTT-3`; HBV core region F 5`-AGACCACCAAATGCCCCTAT-3` and R 5`-GATCTTCTGCGACGCGGCGA-3`; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (internal control) F (h) 5`-AGGGGTCATTgATGGCAACAATATCCA-3` and R (h) 5`-TTTACCAGAGTTAAAAGCAGCCCT GGTG-3`. Controls included water blanks and RNA extracts that were not subjected to reverse transcription. A series of dilutions of Topo-HBV plasmid containing HBV genes and Topo-GAPDH plasmid containing GAPDH cDNA were used to create standard curves for quantifying HBV and GAPDH mRNA levels, respectively. These plasmid concentrations were as follows (copy/μl): 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8. Taq readymix with SYBR green (Sigma, St Louis, MO, USA) was used to amplify and detect DNA during the reaction. Thermal cycling parameters for the HBV core region and GAPDH consisted of a hot start for 2 min at 94°C followed by 40 cycles of 9 °C for 15 s, 60°C for 30 s, and then 72°C for 40 s. Thermal cycling parameters for the HBV surface region consisted of a hot start for 2 min at 94°C followed by 45 cycles of 94°C for 15 s, 58°C for 30 s, and then 72°C for 30 s. Specificity of the polymerase chain reaction products was verified by melting curve analysis and agarose gel electrophoresis.

2.7. *Measurement of active components resibufogenin and cinobufagin using HPLC*

To compare active components resibufogenin and cinobufagin in cinobufacini and SCE-BC, we performed HPLC. HPLC conditions: reversed-phase COSMOSIL Cholester C-18 column (Waters, 4.6 mm × 150 mm, 5 μm); mobile phase, 45% acetonitrile; flow rate, 1 mL/min; detection wavelength, 200-400 nm.

2.8. *Statistical analysis*

All the items determined in this study were repeated at least three times, and the results were expressed as mean ± S.D. Statistical significance was determined using analysis of variance (ANOVA) or a rank sum test and an independent-samples *t* test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Cytotoxic effect of SCE-BC on HepG2.2.15 cell viability*

The viabilities of the HepG2.2.15 cells in the presence of different concentrations of SCE-BC were examined with a MTT assay. The results showed that SCE-BC concentrations below 10^{-2} μg/mL had no significant toxicity to HepG2.2.15 cells (Figure 1). SCE-BC significantly inhibited the growth of HepG2.2.15 cells at concentrations above 10^{-2} μg/mL. The cytotoxicity of SCE-BC was examined to determine the treatment concentrations in the following HepG2 2.2.15 cell culture experiments.

3.2. *Effects of SCE-BC on HBV antigens and DNA*

In this anti-HBV assay, HepG2.2.15 cells were treated with different concentrations of SCE-BC for 3 or 6 days. On day 3, SCE-BC at concentrations of 10^{-4} and 10^{-2} μg/mL had no action on HBsAg secretion in the culture medium (Table 1). On day 6, SCE-BC at concentrations of 10^{-4} μg/mL decreased HBsAg secretion by 8.08%

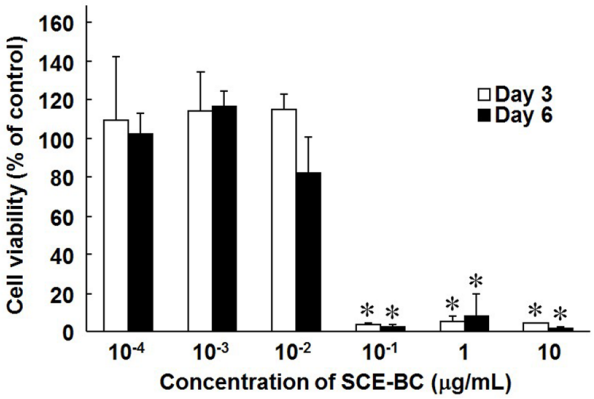

Figure 1. MTT cytotoxicity assay results of SCE-BC. The cell viability was expressed as a percentage of the control. Data shown represent the mean values (± S.D.) based on three independent experiments. Symbols represent statistical significance. * $p < 0.05$ *vs*. control using a rank sum test.

and the positive control 3TC (100 μg/mL) decreased HBsAg secretion by 21.88%. SCE-BC at concentrations of 10^{-4} and 10^{-2} μg/mL inhibited HBeAg secretion on day 3 and 6 (Table 2). Moreover, on day 6 SCE-BC at a concentration of 10^{-4} exhibited more potent activity than the positive control 3TC (100 μg/mL) in terms of the inhibition of HBeAg secretion. SCE-BC at a concentration of 10^{-4} decreased HBeAg secretion by 23.36% while 3TC at 100 μg/mL reduced the secretion of this antigen by 20.81%. Based on this analysis, SCE-BC has greater inhibitory activity with respect to HBeAg than to HBsAg. SCE-BC at concentrations of 10^{-4} and 10^{-2} μg/mL had no action on HBcrAg secretion in the culture medium on day 3 (Table 3). However, on day 6 SCE-BC exhibited more potent activity than the positive control 3TC (100 μg/mL) in terms of the inhibition of HBcrAg secretion. There were no significant differences between SCE-BC treatment and the control in terms of the HBV DNA levels in the culture medium (Table 4).

3.3. *Effect of SCE-BC on HBV mRNA expression*

To determine if the effects of SCE-BC on HBV antigen expression were induced by decreases in HBV RNA

Table 1. Inhibitory effect of SCE-BC on HBsAg secretion in HepG2.2.15 cells

Group	Dose (μg/mL)	Day 3		Day 6	
		(COI) × 10^2	Inhibition (%)	(COI) × 10^2	Inhibition (%)
Control	0	7.09 ± 0.09	–	12.86 ± 0.22	–
3TC	100	6.49 ± 0.18*	8.34	10.05 ± 0.22*	21.88
SCE-BC	10^{-2}	7.16 ± 0.18	–	13.31 ± 0.11	–
	10^{-4}	7.46 ± 0.16	–	11.82 ± 0.10*	8.08

COI: cut-off index. Data shown represent the mean values (± S.D.) based on three independent experiments. Symbols represent statistical significance. * $p < 0.05$ *vs*. control.

Table 2. Inhibitory effect of SCE-BC on HBeAg secretion in HepG2.2.15 cells

Group	Dose (μg/mL)	Day 3		Day 6	
		(COI) × 10^2	Inhibition (%)	(COI) × 10^2	Inhibition (%)
Control	0	1.06 ± 0.25	–	1.73 ± 0.32	–
3TC	100	0.98 ± 0.29	7.71	1.37 ± 0.17*	20.81
SCE-BC	10^{-2}	0.89 ± 0.06	16.18	1.49 ± 0.08	13.91
	10^{-4}	0.99 ± 0.09	6.02	1.32 ± 0.05*	23.36

COI: cut-off index. Data shown represent the mean values (± S.D.) based on three independent experiments. Symbols represent statistical significance. * $p < 0.05$ *vs*. control.

Table 3. Inhibitory effect of SCE-BC on HBcrAg secretion in HepG2.2.15 cells

Group	Dose (μg/mL)	Day 3		Day 6	
		(kU/mL) × 10^2	Inhibition (%)	(kU/mL) × 10^2	Inhibition (%)
Control	0	18.46 ± 0.24	–	58.46 ± 0.60	–
3TC	100	20.22 ± 0.05	–	48.30 ± 0.40*	17.38
SCE-BC	10^{-2}	19.34 ± 0.05	–	45.89 ± 0.10*,#	21.51
	10^{-4}	19.33 ± 0.15	–	40.37 ± 0.12*,#	30.94

Data shown represent the mean values (± S.D.) based on three independent experiments. Symbols represent statistical significance. * $p < 0.05$ *vs*. control. # $p < 0.05$ *vs*. 3TC (100 μg/mL).

Table 4. Inhibitory effect of SCE-BC on HBV DNA release in HepG2.2.15 cells

Group	Dose (μg/mL)	Day 3		Day 6	
		LGE × 10^2	Inhibition (%)	LGE × 10^2	Inhibition (%)
Control	0	5.4	–	5.9	–
3TC	100	5.2	36.90	5.1	84.15
SCE-BC	10^{-2}	5.4	–	6.0	–
	10^{-4}	5.5	–	5.9	–

LGE/mL: log genome equivalent/mL.

levels, real-time PCR analysis was performed using total RNA isolated from HepG2.2.15 cells. When treated with SCE-BC at a concentration of 10^{-4} μg/mL for 6 days, inhibition of HBV mRNA was observed with a 33.64% reduction in the HBV surface/GAPDH mRNA ratio and 61.06% reduction in the HBV core/GAPDH mRNA ratio in comparison to controls (Figure 2). These results showed that the HBV mRNA expression was markedly inhibited in comparison to the control when HepG2.2.15 cells were treated with SCE-BC at a lower concentration. This finding revealed that the inhibitory effects of SCE-BC on HBV antigens would be induced by the specific inhibition of HBV mRNA expression.

3.4. *Content of active components resibufogenin and cinobufagin*

The levels of resibufogenin and cinobufagin were measured using HPLC (Figure 3). The concentrations of resibufogenin and cinobufagin in cinobufacini were respectively 3.20 and 1.56 μg/g of the toad skin, while the concentrations of resibufogenin and cinobufagin in SCE-BC were respectively 121.17 and 33.43 μg/g of the toad skin. The levels of these two active components in SCE-BC were obviously higher than those in cinobufacini.

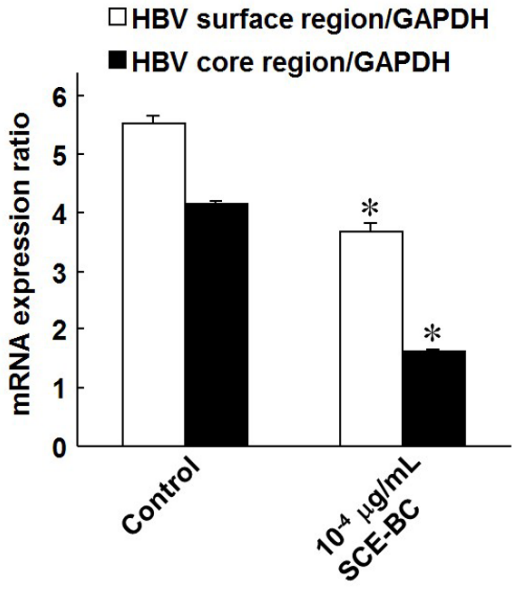

Figure 2. Effect of SCE-BC at a concentration of 10^{-4} μg/mL on the mRNA levels of HBV from the surface and core regions in HepG2.2.15 cells on day 6. Data shown represent the mean values (± S.D.) based on three independent experiments. Symbols represent statistical significance. * $p < 0.05$ *vs*. control using an independent-samples *t* test.

4. Discussion

The skin of toad *Bufo bufo gargarizans* Cantor (Bufonidae) has long been used in Chinese medicine for the treatment of hepatitis B in China (*11,13,19*) and its extract has also been considered to have the efficacy in the management of HBV infection (*20*). Our previous study indicates the aqueous extract of the *Bufo bufo gargarizans* Cantor skin, cinobufacini possess significant activity against HBV (*14*). In the present study, the anti-HBV activity of the supercritical CO_2 extract from the toad skin was evaluated in the HepG2.2.15 cell line. SCE-BC at a concentration of 10^{-4} μg/mL exhibited more potent activity than the positive control 3TC (100 μg/mL) in terms of the inhibition of HBeAg and HBcrAg secretion after treatment of HepG2.2.15 cells for 6 days. This effect of 3TC was consistent with that noted in a previous report (*21*). Moreover, no cytotoxicity was observed with SCE-BC at 10^{-4} μg/mL. These results clearly revealed that SCE-BC had anti-HBV properties and that its inhibitory activity was not induced by cytotoxicity. Although SCE-BC at 10^{-4} μg/mL exhibited lower activity than cinobufacini at 1 μg/mL in terms of the inhibition of HBsAg and HBeAg secretion (*14*), the concentration of SCE-BC was much lower than the concentration of cinobufacini used to treat HepG2.2.15 cells. Moreover, the levels of two active components resibufogenin and cinobufagin in SCE-BC were obviously higher than those in cinobufacini. The supercritical CO_2 extract from the toad skin produced 37-fold and 20-fold increase of the levels of resibufogenin and cinobufagin than the aqueous extract. Therefore, SCE-BC may have better anti-HBV effects than cinobufacini.

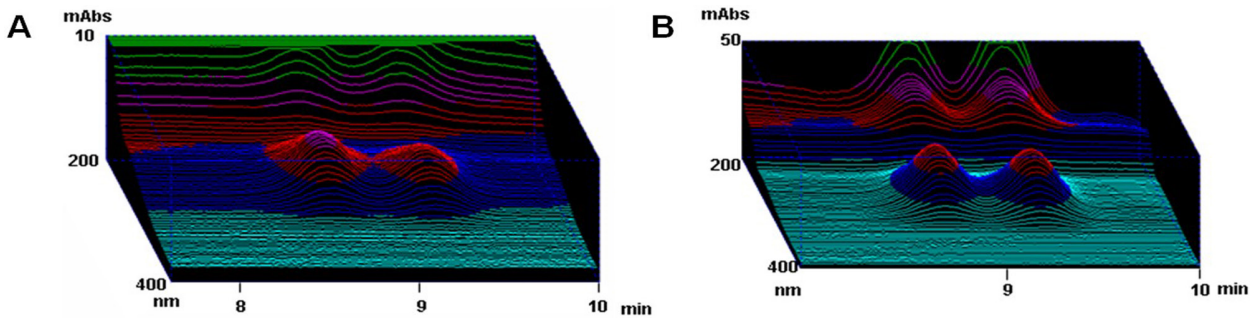

Figure 3. HPLC 3D chromatographic graph of cinobufacini and SCE-BC. (A) The concentrations of resibufogenin and cinobufagin in cinobufacini were respectively 3.20 and 1.56 μg/g of the toad skin. **(B)** The concentrations of resibufogenin and cinobufagin in SCE-BC were respectively 121.17 and 33.43 μg/g of the toad skin.

The effects of SCE-BC at a lower concentration (10^{-4} μg/mL) were more potent than those of this drug at a higher concentration (10^{-2} μg/mL) in terms of inhibiting HBV antigen secretion on day 6. This result could be explained by 18.30% of toxic effect induced by SCE-BC at 10^{-2} μg/mL on day 6. The impairment of cells might affect the release of HBV particles, which were increased.

In the present study, SCE-BC inhibited HBV antigen secretion. This role may take place at the transcription level (*22*). HBV enhancers, the transcriptional regulatory DNA fragments, may mediate the transcriptional suppression by binding several transcription factors. Alternatively, host's general transcription regulatory proteins, such as histone modification enzymes, epigenetic readers and transcription co-activators, are targeted to alter the transcription of a broad spectrum of host and viral genes (*23*). Therefore, the HBV mRNA level was determined in HepG2.2.15 cells in this study. Data demonstrated that in comparison to the control HBV mRNA levels were significantly decreased by SCE-BC. These results revealed that HBV antigen inhibition by SCE-BC was attributed to the specific inhibition of HBV mRNA expression. More work is needed to determine the mechanism of the transcriptional suppression.

In conclusion, the present study demonstrated that SCE-BC blocked HBV antigen secretion *in vitro*. This role of SCE-BC might take place at the transcription level. Moreover, SCE-BC had greater inhibitory activity with respect to HBeAg than to HBsAg. Since HBeAg promotes immune tolerance and persistent infection during HBV infection (*24*), the present results suggest that immune tolerance induced by HBeAg might be overcome by SCE-BC. Therefore, SCE-BC warrants further investigation.

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