Bioorganic & Medicinal Chemistry Letters 23 (2013) 1310–1314

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and biological evaluation of nucleoside analogues than contain silatrane on the basis of the structure of acyclovir (ACV) as novel inhibitors of hepatitis B virus (HBV)

Anyue Han [a,†], Lingna Li [a,†], Kuiyou Qing [a], Xiaolu Qi [a], Leping Hou [a], Xintong Luo [a], Shaohua Shi [a], Faqing Ye [a,b,*]

[a] School of Pharmacy, Wenzhou Medical College, Wenzhou 325000, PR China
[b] Bioorganic & Medicinal Chemistry Research Center, Wenzhou Medical College, Wenzhou 325000, PR China

ARTICLE INFO

Article history:
Received 13 October 2012
Revised 25 December 2012
Accepted 28 December 2012
Available online 9 January 2013

Keywords:
Hepatitis B virus
Silatrane
ACV
HepG2.2.15 cell
HBV transgenic mouse

ABSTRACT

Hepatitis B virus (HBV) infection causes major public health problems worldwide. Acyclovir (ACV) is mainly used to inhibit herpes simplex virus (HSV) rather than HBV. In this study, we used the combination principle to design and synthesize nucleoside analogues that contain silatrane on the basis of the structure of ACV. We found that the compounds were effective inhibitors of HBV, both in vitro and in vivo. All of the compounds showed suppressive activity on the expression of HBV surface antigen (HBsAg) and HBV e antigen (HBeAg) in the HepG2.2.15 cell line with low cytotoxicity. One of compounds was studied in HBV transgenic mice model, and the test results showed its ability to reduce the levels of HBsAg, HBeAg and HBV DNA by ELASE and qPCR. Furthermore, significant improvement of T lymphocyte was observed after treatment, as evaluated by flow cytometry (FCM).

Infection with hepatitis B virus (HBV) leads to a wide spectrum of clinical presentations ranging from an asymptomatic carrier state to self-limited acute or fulminant hepatitis to chronic hepatitis with progression to cirrhosis, hepatocellular carcinoma and other complications.[1] The World Health Organization (WHO) estimated that one-third of the world's population has been infected by HBV. Chronic hepatitis B affects more than 350 million people worldwide and is associated with as many as 1 million deaths per year due to associated complications.[2] Nucleotide analogs, such as lamivudine (3TC), entecavir and adefovir, and interferon are approved drugs used for the treatment of HBV infections. However, unresolved critical issues, such as side effects, dose-dependent side effects, relatively low cure rate, and quick accumulation of drug-resistant mutants, have limited their use.[3] Therefore, alternative strategies and drugs are being sought to combat this disease, whether for monotherapy or combination therapy.

Acyclovir (ACV), 9-(2-hydroxyethoxymethyl)guanine, is used extensively to inhibit several herpes viruses, particularly HSV-1 and HSV-2.[4] In cells infected by herpes viruses, acyclovir can be selectively phosphorylated by virus-encoded thymidine kinase (TK) to its monophosphate and then transformed by cellular enzymes to the triphosphate, which is then used as a substrate by the viral DNA polymerase. Due to the lack of 3′-hydroxyl group, it functions as a chain terminator, thereby inhibiting the viral DNA synthesis.[5] However, acyclovir has been shown in animal models to be a modest inhibitor of HBV DNA polymerase for both in vitro and in vivo activities; however, it has demonstrated disappointing anti-HBV efficacy in human clinical trials.[6]

Silatrane derivatives are known for their extensive and enhanced biological activities, such as immunoregulation, anti-inflammatory effect, and anti-tumor effect.[7] Over the years, research has shown that silatrane derivatives are relatively safe due to their low toxicity and that they do not have any direct effect on cancer cells, working instead through the immune system.[8] In addition, it has been reported that 1-chloromethyl silatrane is effective in the treatment of rheumatoid arthritis due to a higher activity of T lymphocytes.[9]

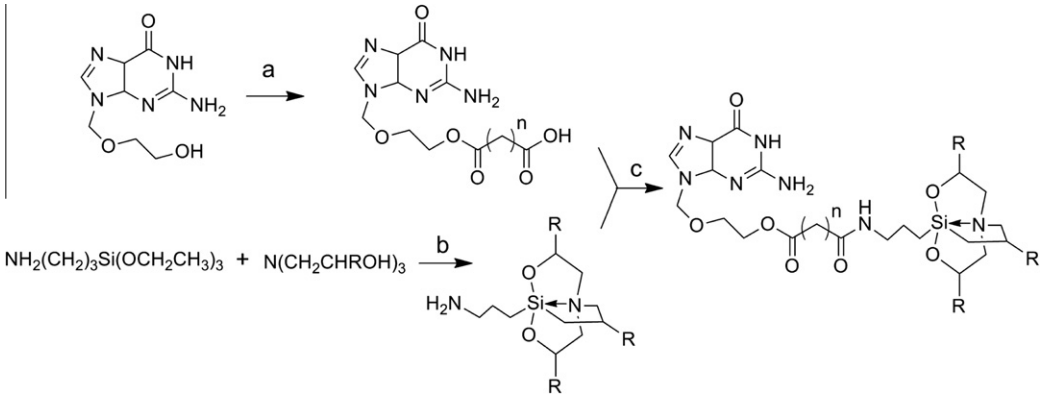
In this study, based on the combination principle, we designed and synthesized nucleoside analogues that contain silatrane. It was expected that these analogues could eliminate the virus from the

Abbreviations: HBV, hepatitis B virus; ACV, acyclovir; HSV, herpes simplex virus; HBsAg, hepatitis B virus surface antigen; HBeAg, hepatitis B e antigen; 3TC, lamivudine; MTT, methyl thiazolyl tetrazolium; ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FCM, flow cytometry.

* Corresponding author. Tel.: +86 0577 86689793; fax: +86 0577 86689983.
E-mail address: yfq664340@163.com (F. Ye).

† Both authors contributed equally to this work.

0960-894X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2012.12.097



Scheme 1. Reagents and conditions: (a) succinic anhydride or glutaric anhydride, pyridine, 120 °C, 10 h; (b) 130 °C, 2 h; (c) EDC·HCl, DMF, rt.

Table 1
Primer design for detection of target genes

Genes	Forward primer	Reverse primer
GAPDH	5′-GTTGTCTCCTGCGACTTCA-3′	5′-GGTGGTCCAGGGTTTCTTA-3′
IFN-γ	5′-TGCTCGAGACTTCAAGCTCTACAGC-3′	5′-CGGGTACCATGCTTTGACAGAAGGC-3′
IL-2	5′-TGCTCGAGACACACTGCATCTTGGC-3′	5′-CGGGTACCATGAGCTCATTGAATGC-3′
HBV-DNA	5′-ATAAAACGCCGCAGACACATC-3′	5′-AACCTCCAATCACTCACCAACC-3′

body by using silatrane to promote the activation and proliferation of T lymphocytes. Subsequently, we were pleasantly surprised and encouraged when our biological activity research indicated that some of our compounds showed very potent anti-HBV activity.

Starting from γ-aminopropyltriethoxysilane and triethanolamine, we prepared the silatrane in one step and with good yield.[10] Subsequently, acyclovir was reacted with anhydride in pyridine for 10 h with excellent yield.[11] Pyridine, which is an alkaline solvent, can promote reaction by the partial dissolution of the compounds. Compounds **L1**–**L4**, combinations of acyclovir derivatives and silatrane, were produced definitively by EDC·HCl coupling at room temperature.[12] The ultimate products were purified as a light-yellow solid by column chromatography and assayed by ^{1}H nuclear magnetic resonance, mass spectrometry, and infrared spectroscopy. The general procedure for their synthesis is depicted in Scheme 1.

The HepG2.2.15 cell (clonal cells derived from HepG2cells transfected with a plasmid containing HBV DNA), which was provided by Zhejiang University (China), was cultured routinely in Dulbecco's Modified Eagle Medium (DMEM, USA) supplemented with 10% (v/v) fetal bovine serum (Hyclone, USA) at 37 °C in an incubator at 5% CO_2. The cells were seeded in 24-well tissue culture plates at approximately 5×10^5/well. After incubation at 37 °C for 24 h, fresh culture media with a variety of concentrations of compounds, lamivudine (3TC) or acyclovir (ACV) was added and replaced every 2 days. After the treatment with the drugs for 6 days, the media were collected and used for Measurement of secreted HBsAg and HBeAg.[13] The expression levels of HBsAg and HBeAg were measured by enzyme-linked immunosorbent assay (ELISA) kits (Shanghai KeHua Bio-engineering Corporation, China) according to the manufacturer's instructions. The media samples were collected after 6 days of treatment, centrifuged at 5000 rpm for 10 min to eliminate cellular debris, and then used immediately for ELISA.

Cytotoxicity towards HepG2.2.15 was assessed by the MTT assay to determine the drug concentrations that did not affect cell viability. Briefly, confluent cultures of cells in 96-well flat-bottomed tissue culture plates were treated with different doses of the test compounds (six cultures for each of two dilutions) for 3 days. MTT (5 g/l) reagent was added 4 h before the end of the culture, and then the cells were dissolved with DMSO, OD values were read at 570 nm, and the percentage of cell death was calculated. These same drug concentrations were used in subsequent assays. The 50% cytotoxic concentration (CC_{50}), defined as the concentration that caused a 50% reduction in the number of viable cells or in the optical density, was calculated according to the Berkson logit method.[14] Lamivudine (3TC) and acyclovir (ACV) were used as positive controls.

The cytotoxicity of each compound, evaluated by using the MTT assay, was expressed as the concentration of compound required to kill 50% (CC_{50}) of the HepG2.2.15 cells. In addition, the anti-HBV activity of each compound was expressed as the concentration of compound that achieved 50% inhibition (IC_{50}) to the secretion of HBsAg and HBeAg using the ELISA method. As shown in Table 1, compounds **L1**–**L4** had low toxicity to HepG2.2.15 cells. According to the results derived from antiviral assays, **L2** had a better inhibitory effect on the secretion of HBsAg and HBeAg in the 2.2.15 cell line. Therefore, we chosen compound **L2** for further animal experiment to investigate its anti-HBV activities in vivo.

The HBV transgenic mice used in this study were obtained from Shanghai Research Centre for Model Organisms (Shanghai, China). The mice were initially produced on a C57BL/6 background and had 3.2 Kb of the HBV DNA integrated into the mouse genome, after which it were backcrossed to C57BL/6J for at least seven generations. A high level of HBsAg in the sera could be detected in the HBV transgenic mice. In all experiments, the mice were matched for age (8 weeks) with an equal number of males and females that had equal hepatitis B surface antigen (HBsAg) levels in their sera before experimental manipulations. All animals were kept under standard, pathogen-free conditions in the Laboratory Animal Centre of Wenzhou Medical College, and all animal experiments were performed according to the guidelines for the care and use of medical laboratory animals (Ministry of Health PR China, 1998) and the guidelines of the Laboratory Animal Ethical Commission of Wenzhou Medical College.

Mice were randomly divided into five group of ten in each group. The groups included the normal control group, model group, positive group, and drug-treatment group. The normal control

Table 2
Cellular toxicity and antigen secretion in HepG2.2.15 cells treated with compounds and 3TC

Compounds	*n*	R	CC_{50}[a] (μM)	IC_{50}[b] (μM)	
				HBsAg	HBeAg
L1	2	H	485 ± 67	79.7 ± 8.3	125.1 ± 35.7
L2	2	CH_3	467 ± 90	34.5 ± 3.7	89.8 ± 7.8
L3	3	H	570 ± 68	90.4 ± 3.6	160.2 ± 36.2
L4	3	CH_3	732 ± 87	140.9 ± 12.6	485.6 ± 68.3
3TC[c]	—	—	>1000	405.6 ± 98.4	>1000
ACV[d]	—	—	630 ± 40	>1000	>1000

All values are the mean of three independent experiments.
[a] CC_{50}, the cytotoxicity concentration of compound that reduced cell viability to 50%, denoted by means ± SD.
[b] IC_{50}, the concentrations of compound needed to inhibit HBsAg and HBeAg secretions to 50%, denoted by means ± SD.
[c] 3TC: lamivudine, an antiviral agent used as positive control.
[d] ACV: acyclovir, an antiviral agent used as positive control.

group was given only normal saline with no drugs, and DMSO solution (0.1%) was used as a model for the animal experiments. Positive groups were given lamivudine (3TC) and acyclovir (ACV). For administration to mice, the purified drugs, 3TC and ACV, solubilized by the aid of 0.1% DMSO, were given once daily orally by intraperitoneally administration with the same dose of 100 mg/kg for one month, respectively. After 30 days, the mice were euthanized, and their livers, spleens, blood, and sera were collected for the following detection.

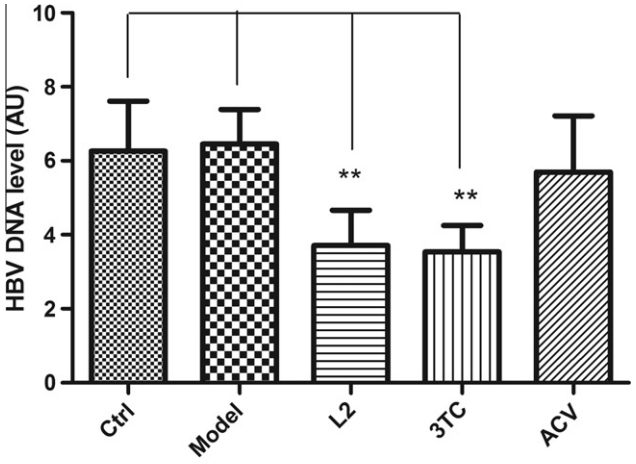


Figure 1. HBV DNA in liver was extracted and analyzed by the qPCR method using an HBV DNA PCR kit. Lamivudine (3TC), an antiviral agent, was used as the positive control. The data were the mean ± SD from 10 transgenic mice.

Liver tissues were dissolved in Trizol Reagent (Invitrogen, Carlsbad, CA, USA) for mRNA and HBV DNA extraction. Fluorescent quantitative polymerase chain reaction (qPCR) was performed using the commercial PCR kit (Fosun High Technology Co. Ltd, Shanghai, China), following the manufacturer's directions. Gene-specific primers for mouse IL-2, IFN-γ, and HBV were designed (Table 2) (synthesized by Sangon Biological Engineering Technology, Shanghai, China). Fluorescence signals were measured after 40 PCR cycles, and all samples were normalized to GAPDH housekeeping gene levels. All samples were run in duplicate.

To further evaluate the anti-HBV activity of the drug in HBV transgenic mice, we detected the expression level of HBV DNA in liver tissue by using qPCR techniques. As shown in Figure 1, the results demonstrated that the titer of HBV DNA in liver was reduced markedly in the mice that received the drug compared with those that received 0.1% DMSO and normal saline (p <0.01). In addition, the titer of HBV DNA was analogous to the positive group that was administered 3TC. The results demonstrated that compound **L2** inhibited HBV DNA replication and that it was effective at doing so than 3TC.

The two mRNA levels of cytokines were assayed by PCR, and the results were shown in Figure 2. The mRNA levels of cytokines IFN-γ and IL-2 were elevated in the drug groups, but they were low in the control. In addition, liver IFN-γ mRNA levels were significantly higher in the treatment group than in the positive group. In these assays, mRNA for GAPDH was used as an internal control. The results indicated that the drug treatment could genuinely and efficaciously enhance the expression of IFN-γ and IL-2 mRNA.

There were numerous HBsAg and HBeAg in the sera of the HBV transgenic mice, which are an important animal model for studying the immunotherapeutic effects of antiviral drugs or vaccines. To investigate the effect of anti-HBV activity elicited by the drugs in HBV transgenic mice, serum HBsAg and HBeAg levels were detected by ELISA using the HBsAg and HBeAg ELISA kit (Shanghai KeHua Bio-engineering Corporation, China). As shown in Figure 3, serum HBsAg and HBeAg levels from the mice that received drug **L2** decreased markedly compared to the normal control group (normal saline) and the model group (0.1% DMSO) (p <0.01). In addition, the HBsAg and HBeAg levels of the treatment group undoubtedly were lower than the levels obtained in the mice with lamivudine control. These results indicated that the treatment drug inhibited both HBsAg and HBeAg of HBV effectively and simultaneously in vivo.

Th1 cells produce IFN-γ, IL-2, and they play a critical role in directing cell-mediated immune responses, which are important for clearance of intracellular pathogens. Serum levels of IFN-γ, IL-2 were detected by ELISA (Shanghai KeHua Bio-engineering Corporation, China) to assess our drug, and the results are shown in Figure 4. Our data, which were generated from ELISA analyses,

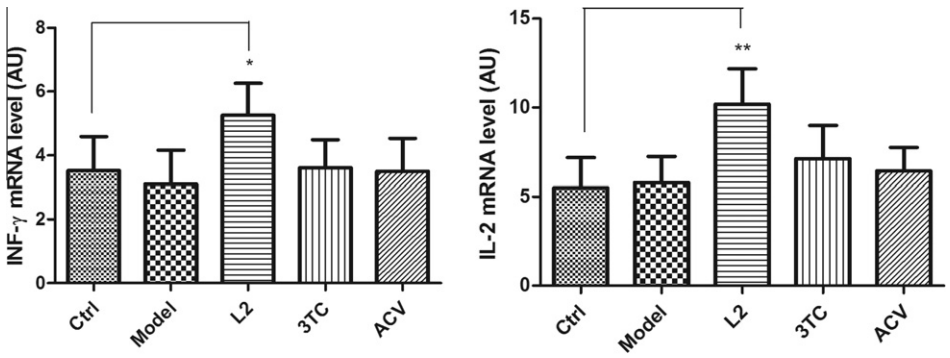


Figure 2. The level of IFN-γ mRNA and IL-2 mRNA in liver was extracted and analyzed by quantitative PCR method using PCR kit. The 3TC, an antiviral agent, was used as positive control. The data were the mean ± SD from 10 transgenic mice.

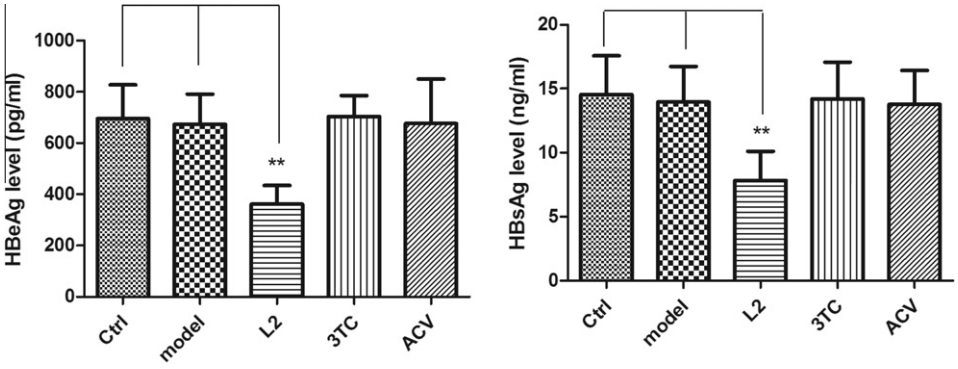


Figure 3. Serum levels of HBsAg and HBeAg in HBV transgenic mice were analyzed by ELISA. Data are presented as mean values ± SD from 10 transgenic mice.

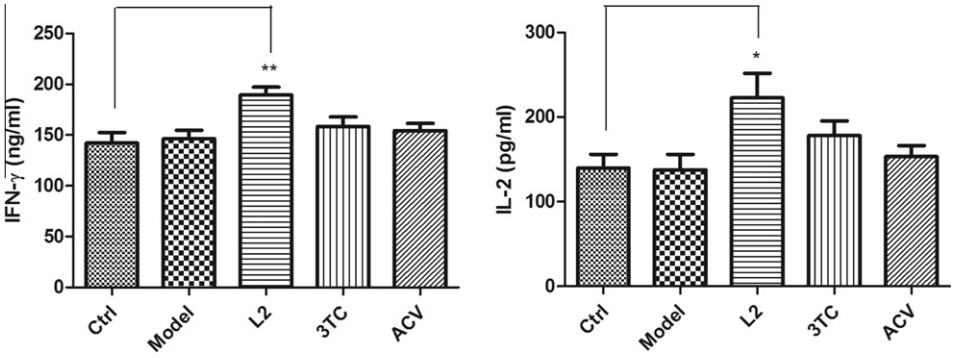


Figure 4. The serum levels of cytokine (IFN-γ and IL-2) were extracted and examined by the ELISA technique. The results were the mean ± SD from 10 transgenic mice.

showed elevated serum concentration of IFN-γ and IL-2 of HBV transgenic mice in comparison to the model group and the normal control group (p <0.05).

T lymphocyte and their subpopulations are the key compositions of cellular immune system. $CD4^+$ and $CD8^+$ cells are the major functional subgroups of T lymphocyte and play an important role in response to HBV infection, which can reflect the cellular immune function and immunoregulation and usually regarded as a valuable index.[15] Heparinized blood samples were collected, and erythrocytes of blood were lysed in NH_4Cl buffer. All samples were analyzed with flow cytometry (FCM) by using anti-mouse monoclonal antibodies CD3-PE-CY5/CD4-FITC/CD8-PE (Sigma). For each sample, detection was conducted using CellQuest software (Coulter). The results were expressed as the percentages of $CD3^+/CD4^+$ (short for $CD4^+$ below) and $CD3^+/CD8^+$ (short for $CD8^+$ below) cells found to be positive for the marker antigen in the total T-cell population. The handling procedures were performed in strict accordance with the instructions provided on the insert in the reagent kit package. As shown in Table 3, $CD3^+$, $CD4^+$, and $CD8^+$ subpopulations and the $CD4^+/CD8^+$ ratio of peripheral blood increased in the treatment group in comparison to other groups. The results demonstrated that the impaired balance of T-cell subsets was improved significantly by our drug.

In this Letter, we examined the anti-HBV activities of our compounds both in vitro and in vivo. For the in vitro study, we took advantage of the HepG2.2.15 cells, which contain HBV genomes integrated into the host DNA, and thus produce and secrete HBsAg and HBeAg. This cell line is widely accepted as a standard model for the evaluation of anti-HBV activities in vitro.[16] Prior to the investigation of anti-HBV activity for these compounds, the cytotoxicity against HepG2.2.15 cells was determined. The corresponding CC_{50} values of [**L1**–**L4**] on HepG2.2.15 cells were all similar to that of lamivudine, which was considered as non-cytotoxic to the cell line.

Table 3
Peripheral blood T lymphocyte subgroups in transgenic mice treated with compounds, 3TC or ACV

Groups	$CD3^+$ (%)	$CD4^+$ (%)	$CD8^+$ (%)	$CD4^+/CD8^+$ (%)
Control	29.8 ± 1.7	14.1 ± 1.4	14.7 ± 1.8	0.9 ± 0.1
Model	30.1 ± 2.9	13.6 ± 2.7	14.1 ± 1.4	0.9 ± 0.2
L2	48.3 ± 3.4	33.0 ± 3.2	20.6 ± 2.3	1.6 ± 0.3
3TC	34.1 ± 2.1	19.3 ± 4.8	16.7 ± 1.7	1.2 ± 0.3
ACV	31.2 ± 4.7	18.7 ± 2.5	15.1 ± 2.0	1.2 ± 0.2

Heparinized blood without erythrocyte was analyzed with FCM by suing anti-mouse monoclonal antibodies. All values are the mean ± SD from 10 transgenic mice.

Based on the experimental outcomes discussed above, it could be suggested that these compounds possess the bioactivity of suppressing the expression of HBsAg and HBeAg in the culture medium. Especially, compound **L2** seemed to be more potential than the other compounds against HBV in HepG2.2.15 cells. This indicated that the compounds may show protective effects via a different mechanism to 3TC, and they may have the potential to work synergistically with other antiviral agents. Therefore, additional investigation of compound **L2** in vivo to clarify its anti-HBV activities was required.

Although the anti-HBV nucleoside drugs mainly exhibit the early virological response and target the viral polymerase activity, the long-term effects of most antiviral drugs are unsatisfactory.[17] According to previous reports, in HBV infection, efficient therapy of anti-HBV drugs results in sustained inhibition of HBV replication and a significant enhancement of cellular immune responses.[18]

However, chronic HBV infection, like many other chronic viral diseases and cancers, is associated with T-cell hyporesponsiveness or tolerance. The exact molecular mechanisms associated with this phenomenon are not entirely clear, but negative selection,

peripheral anergy, and imbalances in lymphokine production all appear to contribute to maintaining the hyporesponsive state of the host that is exposed chronically to viral or cancer antigens. T-cell failure was associated significantly with viral replication level, and viral clearance requires the coordinated action of the immune response.[19] $CD4^+$ cells as T-helpers are essential for the control of HBV infection by facilitating the induction and maintenance of the $CD8^+$ T-cell response. Many studies show that lysis of infected hepatocytes is unlikely to be the only mechanism by which $CD8^+$ T-cells exert their anti-viral effect and that the control of viral replication in the transgenic mouse models also may be an important mechanism.[20] The role of antivirals in the setting of chronic infection is to decrease viral production and also to restore the specific $CD4^-$ and $CD8^-$ mediated immune response following the decrease in viral load in the infected host.[18a,21]

In this study, we observed major changes of T lymphocyte subgroups in the peripheral blood from HBV transgenic mice with **L2** treatment. Concomitantly with a quantitative reduction in viral replication, the frequency of $CD8^+$ T-cells increased during effective therapy. The disappearance of most of the HBV DNA from the liver was followed by increased expression of T-cell markers, maximal $CD4^+$ and $CD8^+$ T-cell responses in the blood, and seroconversion to HBeAg- and HBsAg-specific antibodies. And the impaired balance of T-lymphocyte subsets was improved significantly.

The production of type 1 cytokines, such as interferon (IFN)-y and interleukin (IL)-2, favors cellular immune responses and is associated with protective antiviral immune responses.[22] It has been suggested that the alteration of serum and intrahepatic IFN-γ and IL-2 cytokine play an important role in viral persistence, host immune response and liver damage in chronic viral hepatitis. Our study detected the molecular level of IFN-γ and IL-2 in serum and their mRNA levels. The results all showed that the compound enhanced the secretion of two cytokines, which was due to the activation of T-cells.

In conclusion, the results of our study indicated that an acyclovir derivative that contains silatrane reduced the production of HBV-specific antigens, reduced the level of HBV DNA, and activated immunoregulation. Although further studies are required to elucidate the molecular mechanisms, these results suggest that this kind of compound may play an important role in the therapy associated with hepatitis B virus infections.

Acknowledgments

This work was financed in part by Zhejiang Science & Technology Funding (Y2080697 to F.Q.Y.; Y204089 to F.Q.Y.). We are grateful to Professor Mifang Liang for the assistance in this work.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.12.097.

Reference and notes

1. Baumert, T. F.; Rosler, C.; Malim, M. H.; von Weizsacker, F. *J. Hepatol.* **2007**, *46*, 682.
2. (a) Lee, W. M. *N. Engl. J. Med.* **1997**, *337*, 1733; (b) Hoofnagle, J. H. *Digestion* **1998**, *59*, 563; (c) Robinson, W. S.; Klote, L.; Aoki, N. *J. Med. Virol.* **1990**, *31*, 18; (d) Beasley, R. P. *Cancer* **1988**, *61*, 1942.
3. (a) Lai, C. L.; Chien, R. N.; Leung, N. W.; Chang, T. T.; Guan, R.; Tai, D. I.; Ng, K. Y.; Wu, P. C.; Dent, J. C.; Barber, J.; Stephenson, S. L.; Gray, D. F. *N. Engl. J. Med.* **1998**, *339*, 61; (b) Dona, M.; Dell'Aica, I.; Calabrese, F.; Benelli, R.; Morini, M.; Albini, A.; Garbisa, S. *J. Immunol.* **2003**, *170*, 4335; (c) Fattovich, G.; Brollo, L.; Alberti, A.; Pontisso, P.; Giustina, G.; Realdi, G. *J. Hepatol.* **1988**, *8*, 1651.
4. (a) Elion, G. B.; Furman, P. A.; Fyfe, J. A.; de Miranda, P.; Beauchamp, L.; Schaeffer, H. J. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5716; (b) Schaeffer, H. J.; Beauchamp, L.; de Miranda, P.; Elion, G. B.; Bauer, D. J.; Collins, P. *Nature* **1978**, *272*, 583.
5. Hong, J. H.; Choi, Y.; Chun, B. K.; Lee, K.; Chu, C. K. *Arch. Pharm. Res.* **1998**, *21*, 89.
6. Minuk, G. Y.; German, G. B.; Bernstein, C.; Benarroch, A.; Gauthier, T.; Sekla, L. *Clin. Invest. Med.* **1992**, *15*, 506.
7. (a) Watanabe, T.; Sorensen, E. M.; Naito, A.; Schott, M.; Kim, S.; Ahlquist, P. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 10205; (b) Kozlowski, H.; Radecki, A.; Lukasiak, J.; Hrabowska, M. *Exp. Pathol.* **1985**, *28*, 215.
8. Puri, J. K.; Singh, R.; Chahal, V. K. *Chem. Soc. Rev.* **2011**, *40*, 1791.
9. (a) Shirinskii, V. S.; Zhuk, E. A.; Baryshok, V. P. *Eksp. Klin. Farmakol.* **1993**, *56*, 43; (b) Shirinskii, V. S.; Zhuk, E. A.; Domnina, E. S.; Baikalova, L. A. *Eksp. Klin. Farmakol.* **1993**, *56*, 33.
10. Synthesis of silatrane was carried out by placing 0.1 mol of γ-aminopropyltriethoxysilane and 0.1 mol of triethanolamine into a 100-ml reaction flask equipped with a reflux condenser and a dry pipe with anhydrous calcium chloride. The mixture was stirred magnetically and heated at 130 °C for 2 h. During the course of the reaction, a large quantity of ethanol was created, and it was removed by refluxing for 4 h. The remaining liquid was distilled under reduced pressure, and the fraction that boiled between 164 and 166 °C at 66.6 Pa was collected. After cooling, the resulting yellow solid, that is, silatrane, was filtered out of the solution and washed. The yield was 81%.
11. A mixture of acyclovir (10 mmol) and anhydride (20 mmol) in pyridine (20 ml) was stirred and heated in an oil bath at 120 °C for 10 h. The completion of the reaction was monitored by thin layer chromatography (TLC). After the mixture was cooled to 0–5 °C, the crystalline solid that separated out was collected by filtration, thoroughly washed with acetone, and dried in vacuo at 40 °C. An analytical sample was obtained by crystallization from methanol as a white solid in 76% yield.
12. A solution of silatrane (10 mmol) in chloroform (15 ml) was added dropwise to a stirred solution of acyclovir esters (10 mmol) and EDC·HCl (20 mmol) in DMF (15 ml). The resulting mixture was stirred at room temperature for 10 h. After removing the solvents under reduced pressure, the crude residue was purified by chromatography on silica ($CHCl_3$/CH_3OH 10:1), which produced the combination of silatrane and acyclovir esters as a light yellow solid (39–58% yield).
13. Xu, J.; Wang, J.; Deng, F.; Hu, Z.; Wang, H. *Antiviral Res.* **2008**, *78*, 242.
14. Berkson, J. *Biometrics* **1968**, *24*, 75.
15. (a) Baumert, T. F.; Thimme, R.; von Weizsacker, F. *World J. Gastroenterol.* **2007**, *13*, 82; (b) Bertoletti, A.; Gehring, A. J. *J. Gen. Virol.* **2006**, *87*, 1439.
16. Sells, M. A.; Chen, M. L.; Acs, G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1005.
17. Lok, A. S.; McMahon, B. J. *Hepatology* **2004**, *39*, 857.
18. (a) Boni, C.; Penna, A.; Ogg, G. S.; Bertoletti, A.; Pilli, M.; Cavallo, C.; Cavalli, A.; Urbani, S.; Boehme, R.; Panebianco, R.; Fiaccadori, F.; Ferrari, C. *Hepatology* **2001**, *33*, 963; (b) Rico, M. A.; Quiroga, J. A.; Subira, D.; Castanon, S.; Esteban, J. M.; Pardo, M.; Carreno, V. *Hepatology* **2001**, *33*, 295; (c) Tsai, S. L.; Sheen, I. S.; Chien, R. N.; Chu, C. M.; Huang, H. C.; Chuang, Y. L.; Lee, T. H.; Liao, S. K.; Lin, C. L.; Kuo, G. C.; Liaw, Y. F. *J. Biomed. Sci.* **2003**, *10*, 120.
19. Bertoletti, A.; Naoumov, N. V. *J. Hepatol.* **2003**, *39*, 115.
20. Guidotti, L. G.; Ishikawa, T.; Hobbs, M. V.; Matzke, B.; Schreiber, R.; Chisari, F. V. *Immunity* **1996**, *4*, 25.
21. Boni, C.; Bertoletti, A.; Penna, A.; Cavalli, A.; Pilli, M.; Urbani, S.; Scognamiglio, P.; Boehme, R.; Panebianco, R.; Fiaccadori, F.; Ferrari, C. *J. Clin. Invest.* **1998**, *102*, 968.
22. Dumoulin, F. L.; Bach, A.; Leifeld, L.; El-Bakri, M.; Fischer, H. P.; Sauerbruch, T.; Spengler, U. *J. Infect. Dis.* **1997**, *175*, 681.