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Oxymatrine inhibits hepatitis B infection with an advantage of overcoming drug-resistance

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ABSTRACT

Oxymatrine (OMTR) is an anti-hepatitis drug used in China. Its mechanism of action is unknown. Recently, we found that OMTR inhibits hepatitis B virus (HBV) via down-regulating the expression of heat-stress cognate 70 (Hsc70), a host protein required for HBV DNA replication. Goal of this study was to assess the effect of OMTR on clinical HBV drug-resistance. OMTR monotherapy (oral, 12 months) reduced blood HBV DNA by 96% and HBeAg by 70% in the chronic hepatitis B (CHB) patients resistant to lamivudine ($n = 17$), equal to its efficacy in the naïve CHB cohort ($n = 20$). Liver biopsy study showed that OMTR treatment caused a decrease of Hcs70 mRNA in liver cells, parallel with a reduction of intracellular HBV DNA. Combination of lamivudine with OMTR ($n = 15$) (oral, 12 months) showed an enhanced anti-HBV effect as compared to lamivudine monotherapy ($n = 25$). The incidence of drug resistance against lamivudine in the combination group was significantly lower than that in the lamivudine group (1/15 vs 7/25; $p < 0.01$). The results were further confirmed *in vitro*. Treatment of HBV(+) HepH2215 cells with sub-optimal dose of OMTR for 8 months suppressed HBV replication without inducing drug resistance, whereas lamivudine monotherapy caused drug-resistant mutation in 3 months. Combination of OMTR with lamivudine prevented HBV from developing drug resistance.

1. Introduction

Reverse transcriptase (RT) inhibitors such as lamivudine, adefovir, entecavir, telbivudine and tenofovir directly target viral RT and are currently the first line antiviral drugs for chronic hepatitis B viral (HBV) infection. Although the RT inhibitors are potent against HBV, long-term use of these drugs in patients selects for HBV drug-resistant mutation (Zoulim et al., 2009; Yuan et al., 2009; Mohanty et al., 2006). The situation for other viral enzyme inhibitors, for instance protease inhibitors for HIV-1, is virtually identical (Zhang et al., 2010; Shafer, 2002). Drug-resistant mutation occurring in viral components represents a great challenge for antiviral chemotherapy, and an innovative antiviral strategy is highly needed.

Heat-stress cognate 70 (Hsc70) is a host protein which supports HBV DNA replication (Prange et al., 1999; Eddy, 1998; Wang et al., 2010). Our recent study has identified oxymatrine (OMTR) (MW: 264.31), an anti-hepatitis drug isolated from Kushen (*Sophora flavescens* Ait.) (Ling et al., 2007), to be a selective inhibitor of Hsc70 expression (Wang et al., 2010). OMTR significantly suppressed HBV *de novo* synthesis at the reverse transcription stage from pgRNA to DNA, and was active against either wild-type HBV or variants resistant to lamivudine, adefovir and entecavir (Wang et al., 2010). The anti-HBV effect of OMTR was mediated through destabilizing Hsc70 mRNA; Hsc70 mRNA 3′UTR sequence was the element responsible for the destabilization effect of OMTR (Wang et al., 2010). To further understand the therapeutic nature of OMTR, we have further extended our research into the clinic. Here, we show that the drug target for OMTR is a host protein. OMTR helps overcoming drug resistance.

2. Materials and methods

2.1. Compounds and cells

Oxymatrine (OMTR) and lamivudine with the purity over 98.5% were from the National Institute for the Control of Pharmaceutical and Biological Products, the State Federal Drug Administration (Beijing, China). The human HepG2 hepatocytes transfected with

Abbreviations: Hsc70, heat-stress cognate 70; HBV, hepatitis B virus; OMTR, oxymatrine; RT, reverse transcriptase; CHB, chronic hepatitis B.

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the full genome of HBV (HepG2215 cells) (Price et al., 1989) were used for *in vitro* short- or long-term anti-HBV therapy. The cells were cultivated in a MEM medium with 10% of fetal bovine serum.

2.2. Quantitative real-time RT-PCR and real-time PCR

RNA was isolated with TRIZOL® Reagent (Invitrogen, Carlsbad, CA) and genomic DNA was with QIAamp® DNA Mini Kit (Qiagen, Valencia, CA). HBV DNA as well as cccDNA was extracted using a method reported previously (Wang et al., 2010). Quantitative real-time PCR was done using the SYBR Green technique in the BIO-RAD iQ5 Multicolor Real-Time Detection System (Bio-Rad, Hercules, CA). Hsc70 mRNA assays were done with SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen) and HBV DNA was with Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen). Primers were designed with Primer 5.0 and tested for specificity using the BLASTN program. The mRNA expression of Hsc70 and GAPDH were determined using human primers (Hsc70, F: 5′-tgctgctgctattgcttacg-3′, R: 5′-tcaatagtgaggattgacacatca-3′; GAPDH, F: 5′-acccactcctccacctttg-3′, R: 5′-ctgtagccaaattcgtt gtcat-3′). HBV DNA, cccDNA and GAPDH DNA detection used human HBV DNA primers (F: 5′-ggctttcggaaaattcctatg-3′, R: 5′-agccctacgaaccactgaac-3′), cccDNA primers (F: 5′-ctccccgtctgtgccttct3′, R: 5′-gccccaaagccacccaag-3′), and GAPDH DNA primers (F: 5′-gactaaccctgcgctcctg-3′, R: 5′-catcaccc ggaggagaaat-3′), respectively. The Bio-Rad iQ5 software was used for data analysis.

2.3. Drug resistance in long-term treatment

The HepG2215 cells were exposed to a suboptimal dose of OMTR (30 μg/ml), or lamivudine (3 μg/ml), or their combination. Cells were washed with PBS every three days and then fed with fresh culture medium containing same concentration of the study drug. The cell passage was taken every 6–7 days followed by cultivation as described above. Treatment continued for 8 months. Intracellular HBV DNA was determined every 30 days, using quantitative real-time PCR mentioned above. Rebound of HBV DNA load was considered to be an indication of HBV drug-resistant mutation, which was further confirmed by analyzing HBV DNA sequence using a commercial technique.

2.4. Clinical study

(1) Treatment of lamivudine-resistant chronic hepatitis B (CHB) patients with OMTR. Thirty-seven CHB patients with positive readings for HBV DNA, HBsAg and HBeAg were enrolled in the Division of Viral Hepatitis, Nanjing Second Hospital (Nanjing, China). The patients entered into the study two weeks after cessation of the previous treatments. Among these patients 17 of them (age, 43 ± 12; M/F, 13/4) had YMDD drug-resistant mutation to lamivudine; the average HBV viral load of the patients before OMTR treatment was 1.7×10^7 per ml. The other 20 patients were naïve CHB patients (age, 31 ± 12; M/F, 11/9) with no experience of anti-HBV therapy; viral load for these patients before treatment was 1.9×10^7 per ml in average. All of the patients were treated with OMTR (Zhengda Tianqing Pharmaceutical Inc., Nanjing, China) orally, 0.2 g, bid for 12 months. Blood samples were taken before and after the therapy. HBV infection markers (HBsAg and HBeAg) were quantitatively measured with the commercial test kits from Abbott (Chicago, IL). Blood HBV DNA was quantified with real time PCR assay using kits from Kehua Biotech Inc. (Shanghai, China) and a fluorescence PCR reader (ABI-7000, ABI, CA). Briefly, 100 μl of serum sample was treated with DNA purification buffer followed by centrifugation. The DNA sample was then mixed with the amplification buffer containing HBV PCR reaction solution and Taq enzyme. The reaction was performed under the conditions recommended by the vendor. The final reaction product was measured in a fluorescent PCR quantitative reader and the result was expressed as HBV DNA copies per ml serum. The range of the measurement is 500–10^8 HBV DNA copies per ml serum. YMDD mutations in HBV reverse transcriptase gene were examined with commercially available BigDye Terminator V3.1 Cycle Sequencing Kit® using ABI PRISMTM 3730 XL DNA Analyzer and ABI PRISMTM 377XL DNA sequencer (Applied Biosystems, CA). Liver and kidney functions were examined in all subjects. The liver enzymes (alanine aminotransferase, ALT; aspartate aminotransferase, AST; and gamma glutamyl transpeptidase, γGT) of the patients were determined with the kits from Randox (England).

(2) Liver biopsy study. Six otherwise healthy CHB patients (age, 31 ± 7; M/F, 5/1) with positive tests of HBsAg, HBeAg and HBV DNA were enrolled for the liver biopsy study. These patients did not use anti-HBV agents before the study. Their blood HBV DNA level before the treatment was 1.6×10^7 copies/ml in average. The patients received OMTR for 3 months (0.6 g/d, i.v., for the first month; 0.2 g, oral, bid for another 2 months) and liver biopsy was done before and after the 3-month therapy. Total RNA and genomic DNA from liver biopsy samples were extracted and examined for the Hsc70 mRNA expression and intracellular HBV DNA.

(3) Reduction of drug resistance. Forty CHB patients who were positive for HBsAg, HBeAg and HBV DNA, and had not been treated with anti-HBV drugs were enrolled in the Nanjing Second Hospital. Among these 40 patients, 25 of them (age, 30 ± 11; M/F, 13/12) were treated with lamivudine alone for 12 months (100 mg/d, oral; Glaxowelcome Pharmaceutical Inc., Shuzhou, China); and other 15 (age, 34 ± 10; M/F, 11/4) were treated with lamivudine (100 mg/d, oral) plus OMTR (0.2 g, bid, oral) for 12 months. The OMTR was from the Zhengda Tianqing Pharmaceutical Inc (Nanjing, China). Blood samples were taken before, between and after treatment. HBV markers (HBsAg, HBeAg and HBV DNA), drug-resistant mutations as well as liver functions were examined. In this study, HBV drug-resistance was clinically defined as a rebound of HBV DNA over 3 logs with liver enzymes back into abnormal range.

All of the clinical studies were conducted in the Nanjing Second Hospital, Nanjing, China, according to the principles expressed in the Declaration of Helsinki and approved by the Institution Committees of the Nanjing Second Hospital and the Institute of Medicinal Biotechnology. Informed consent was given by the patients.

3. Results and discussion

3.1. OMTR is effective in CHB patients with drug resistance to lamivudine

Our study *in vitro* has demonstrated that OMTR effectively inhibited *de novo* synthesis of the clinical HBV strains that were resistant to RT inhibitors (Wang et al., 2010). Next question is whether this is also true in patients. Previous reports showed that treatment of CHB patients with OMTR (600 mg/d, oral, 6 months) reduced HBV replication and did not cause severe side effects (Lu et al., 2002; Yu et al., 2001). About 2–5% of the patients had mild and tolerable stomach problem with no need to stop treatment (Wang, 2000; Cai et al., 1997). $T_{1/2}$ of OMTR in human blood was about 2.4 h (Wang et al., 2003). With these background information, anti-HBV effect of OMTR was studied in 17 CHB patients resistant to lamivudine.

Table 1
Anti-HBV effect of OMTR in lamivudine-resistant CHB patients.

Examinations (normal range)	OMTR[a] (naïve, $n=20$)		OMTR (resistant, $n=17$)	
	Before	After	Before	After
HBsAg (S/N, <2)	187.3 ± 60.6	$130.0 \pm 38.0^{**}$	198.9 ± 74.0	170.3 ± 67.5
HBeAg (PEIU/ml, <0.28)	2783.2 ± 1895.7	$796.7 \pm 1287.0^{***}$	2550.0 ± 2873.3	$773.9 \pm 1524.0^{*}$
HBV DNA (copies/ml, $<5 \times 10^2$)	$1.7 \pm 3.5 \times 10^7$	$1.1 \pm 3.0 \times 10^{6*}$	$1.9 \pm 3.8 \times 10^7$	$7.1 \pm 2.4 \times 10^{5*}$
ALT (IU/l, 0–40)	100.4 ± 24.3	$35.8 \pm 14.0^{***}$	130.8 ± 45.6	$40.5 \pm 15.0^{***}$
AST (IU/l, 0–40)	113.4 ± 76.2	$39.2 \pm 12.7^{***}$	120.4 ± 43.4	$41.4 \pm 13.9^{***}$
r-GT (IU/l, <50)	77.6 ± 33.1	$42.5 \pm 17.8^{**}$	111 ± 33.8	$54.2 \pm 15.4^{***}$

[a] OMTR was given 0.2 g, bid, orally for 1 year.
[*] $p < 0.05$, as compared to that before therapy using paired Student's t-test.
[**] $p < 0.01$, as compared to that before therapy using paired Student's t-test.
[***] $p < 0.001$, as compared to that before therapy using paired Student's t-test.

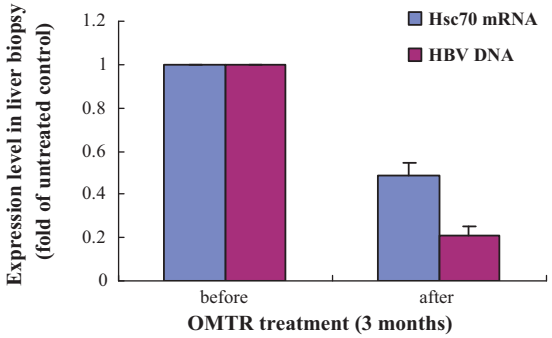

Fig. 1. Liver biopsy study in CHB patients treated with OMTR. Six hepatitis B patients were treated with OMTR for 3 months (see Section 2). The liver biopsy samples taken before and after the 3-month treatment were examined for Hsc70 mRNA and intracellular HBV DNA. Presented are mean and SD of the 6 CHB patients. Untreated control, values before treatment.

As shown in Table 1, OMTR monotherapy (0.2 g, oral, bid, for 12 months) significantly reduced HBV DNA by 96% and HBeAg by 70%. Patients' blood HBsAg declined but to a lesser extent. Accordingly, the liver function of the patients improved with a significant reduction of the liver aminotransferases ($p < 0.001$ for AST, ALT and γGT). The anti-HBV efficacy of OMTR in the lamivudine-resistant cohort was almost identical to that in the naïve CHB patient cohort ($n = 20$). Significant side-effect was not observed. Out of the total 37 patients treated with OMTR there was one individual complained of mild stomach problem after 8 months on therapy; but the treatment was continued as the side-effect was transient. This observation was consistent with the previous results in naïve CHB patients independently conducted by Lu et al. (2003) and Yu et al. (2001).

OMTR inhibits Hsc70 expression in human liver cell lines (Wang et al., 2010). To investigate whether Hsc70 mRNA in patient liver is truly down-regulated by OMTR, liver tissue biopsy was performed in another 6 CHB patients who had no experience of receiving anti-HBV treatment. As shown in Fig. 1, OMTR treatment for 3 months (0.6 g/d, i.v., for the first month; 0.2 g, oral, bid for another 2 months) reduced Hsc70 mRNA in liver biopsy samples by 50% at average; accordingly the intracellular HBV DNA went down by 80%. The results support the Hsc70-targeted anti-HBV action of OMTR, and provide a direct link between Hsc70 expression and HBV replication in CHB patients.

3.2. OMTR reduces drug resistance to lamivudine in CHB patients

Then, we investigated whether long-term treatment of CHB patients with OMTR prevents drug-resistance against RT inhibitors. Forty naïve CHB were enrolled in this study. Twenty-five of them were assigned randomly for lamivudine treatment (100 mg/d, oral) and another fifteen were on a combination regimen using lamivudine (100 mg/d, oral) plus OMTR (0.2 g, bid, oral, given at same time). The treatment continued for 12 months. As shown in Table 2, by the end of the 12-month treatment the combination therapy had a higher anti-HBV efficacy (−3.35 log of HBV DNA in average) as compared with the lamivudine monotherapy (−1.18 log of HBV DNA in average). The difference seemed to result from the increased anti-HBV efficacy in the combination regimen as well as the drug-resistance occurred in the lamivudine monotherapy group. After 12 months on therapy, 7 out of the 25 patients in the lamivudine group developed drug-resistance, showing HBV DNA equal or above 10^5 copies per ml serum; genotyping showed that HBV YMDD mutations of 180M and 204I were detectable in these patients. In the group combining lamivudine with OMTR ($n = 15$), drug resistance developed in only one patient (Table 2), whose HBV DNA rebounded at 9 months and went back to about 6 logs level at 12 months. The 204I mutation was detected in this patient, validating resistance to lamivudine. The incidence of drug resistance was significantly higher in the lamivudine monotherapy (7/25) as compared with that in the combination therapy (1/15; $p < 0.01$ by Fisher's exact test). HBV DNA viral load rebound curve in these drug-resistant patients during the treatment is shown in Fig. 2. Six of them were from the lamivudine group, and one from the combination group; there was one drug-resistant patient of the lamivudine group who lost his month-3 and month-6 testing results; therefore, his results were not included in Fig. 2. It appeared that OMTR prevented the development of HBV drug resistance. There could be at least two explanations. First, as OMTR works through destabilization of host

Table 2
OMTR reduces drug-resistance to lamivudine in CHB patients.

Treatment[a] (12 months)	HBV DNA (copies/ml)[b]		Drug-resistance to lamivudine	
	Before treatment	After treatment	Viral load rebound[c]	YMDD
Lamivudine ($n = 25$)	$1.5 \pm 0.13 \times 10^7$	$1.0 \pm 0.1 \times 10^6$	7/25	180M or 204I
Lamivudine + OMTR ($n = 15$)	$1.5 \pm 0.2 \times 10^8$	$6.7 \pm 1.7 \times 10^4$	1/15[**]	204I

[a] Lamivudine: 100 mg/d, oral, 12 months; Lamivudine + OMTR: lamivudine, 100 mg/d, oral, plus OMTR, 0.2 g, bid, orally, for 1 year.
[b] Mean and SE.
[c] Number of patients developed lamivudine-resistance/number of patients in the group. HBV drug-resistance was clinically defined as a rebound of HBV DNA over 3 logs and liver enzymes went back to abnormal levels.
[**] $p < 0.01$, as compared to those treated with lamivudine alone, by the Fisher's exact test.

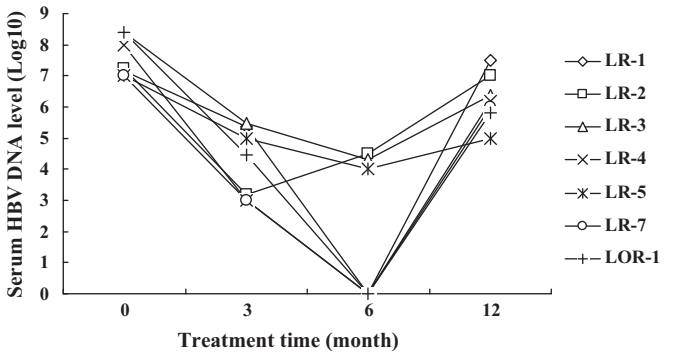

Fig. 2. Development of drug-resistance in treated hepatitis B patients. The figure shows HBV DNA load rebound curves of the 7 hepatitis B patients who developed lamivudine-resistance (LR) or lamivudine/OMTR-resistance (LOR) after 12-month on treatment. LR-1, 2, 3, 4, 5, 7 as well as LOR-1 are patient code. Data of the patient LR-6 was not completed and is not included in the figure.

Hsc70 mRNA, there is no or little chemotherapy pressure on HBV; and second, combination of drugs targeting at different targets is known to reduce drug-resistance (Delfraissy et al., 2008; Larder et al., 1995). If confirmed in large-scale clinical studies, OMTR could make a significant contribution to anti-HBV regimens. Lamivudine was used as a reference in this study, because it would be easy for us to learn whether OMTR could prevent drug-resistance. New generation of HBV RT inhibitors, such as tenofovir and entecavir, have less chance of inducing drug-resistance in respect to lamivudine, and therefore combination of OMTR with these drugs might yield improved results.

3.3. Long-term treatment of HBV with OMTR in vitro

To validate the clinical observation presented in Table 2, we tested *in vitro* whether long-term treatment with OMTR would cause HBV mutation for drug-resistance. In this *in vitro* study, a suboptimal dose of OMTR was used to treat the HepG2215 cells, with lamivudine as a reference. As shown in Fig. 3a, HBV DNA declined in all of the drug treatment groups in the first 2 months, indicating the therapeutic response of HBV to the study agents; however, the viral load rebounded in the lamivudine group after 3-month monotherapy. Sequence analysis for the subdomain of C and B in the pol/rt domain showed a mutation of rtL180M in the DNA polymerase gene of the HBV viral sample taken after 3 months on lamivudine therapy. Lamivudine-resistant mutation of rtM204I/V/S, rtL80V/I and rtV173L were not detected. The results were confirmed with the second round sequencing. Increasing concentration of lamivudine by 10-fold, or more, efficiently inhibited the replication of this drug-resistant HBV (Fig. 3b). The result verified the occurrence of drug resistance against lamivudine in HepG2215 cells, and hinted that the mutant might represent a small portion of HBV in the cells. In contrast, HBV DNA load remained low for 8 months in either OMTR monotherapy or the group using lamivudine in combination with OMTR. No drug-resistant mutation was detected in these two groups.

These results show for the first time that drugs inhibiting HBV replication through an effect on host Hsc70 have no or reduced chance of inducing drug resistance. We have repeatedly detected cccDNA in the HepG2215 cells used in this study, and the results were consistent with other reports (Bock et al., 1994; Zhao et al., 2005). In fact, after treating HepG2215 cells with OMTR for 6 days, we detected a reduction of HBV DNA paralleled with a decrease in cccDNA; however, HBsAg declined only slightly (Fig. 4), consistent with the clinical observation in Table 1. The presence of cccDNA in the HepG2215 cells might account at least in part for the drug-resistant mutation in the cells.

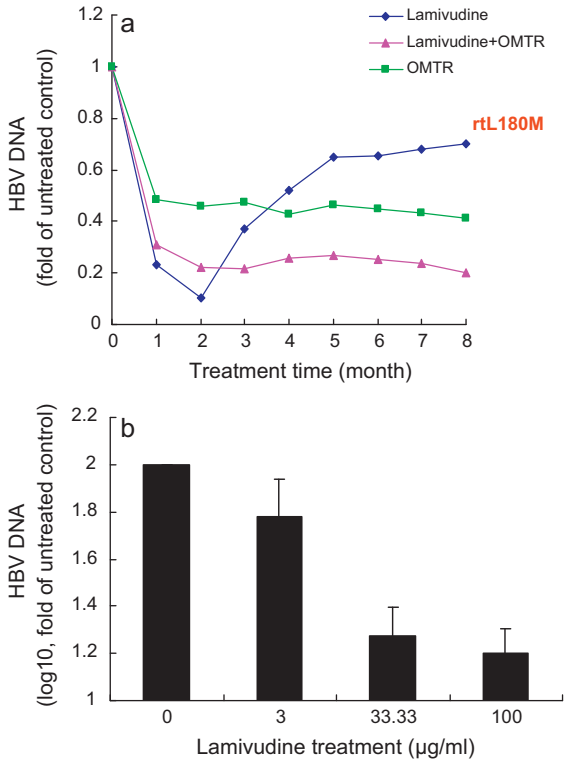

Fig. 3. OMTR caused no or little HBV drug-resistance *in vitro*. (a) The HepG2215 cells were continuously exposed to a suboptimal dose of OMTR (0.03 mg/ml), or lamivudine (3 μg/ml) or OMTR (0.03 mg/ml) plus lamivudine (3 μg/ml) for 8 months, respectively. HBV DNA level was determined every 30 days. The HBV samples were collected from each group for HBV RT gene sequence analysis. Drug-resistant mutation was detected only in the lamivudine group (180M) but not in the OMTR and combination groups. Gene sequence analysis was done twice. HBV DNA of the untreated flasks at each time point was defined as 1, the amount of HBV DNA of the treated cells at this time point was plotted relative to that value. The result for each time point represents an average of 2 flasks. (b) The 2215 cells with lamivudine-resistant HBV (180M) were taken from the experiment described in Fig. 3a, and treated with increased concentrations of lamivudine. Lamivudine at 33.33 μg/ml or up showed a significant inhibition of the virus after 3 days treatment. The amount of HBV DNA in the untreated cells was defined as 100 (2 log), and the level of HBV DNA in the lamivudine-treated cells was plotted on a logarithmic scale.

Non-RT inhibitor natural agents are options in China to treat CHB patients (Chen et al., 2005; Yao et al., 2002), although their mechanisms often remain unclear. As their antiviral activity is not as potent as that of RT inhibitors, their clinical use has been restricted in mono- as well as combination therapy. We have used these agents as chemical probes to discover novel mode of action against

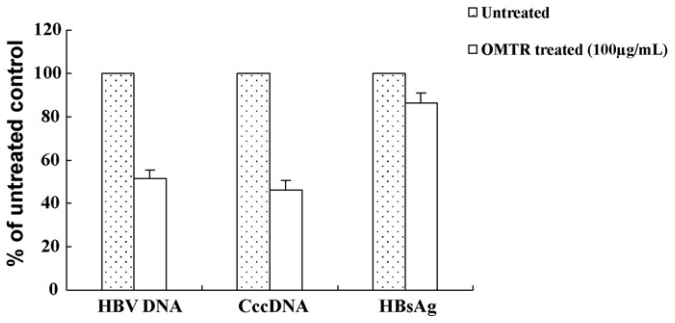

Fig. 4. Anti-HBV effect of OMTR in HepG2215 cells. The HBV transfected human liver cells were incubated in the absence of presence of OMTR (100 μg/ml) for 6 days. HBV DNA and cccDNA were extracted and measured as previously described (Bock et al., 1994; Wang et al., 2010). HBsAg level in the supernatant was also measured. Presented are the mean and SD of 3 tests.

HBV, especially the host-based antiviral strategies. The goal is to identify drugs (or drug candidates) that are able to create an intracellular environment which is not supportive for viruses. As the therapeutic pressure of these agents is not on viruses but on the host, it might have an advantage of overcoming drug resistance.

OMTR is one of these agents. Host Hsc70 was identified to be the target for the anti-HBV effect of OMTR (Wang et al., 2010). Additionally, Hsc70 is also a host supportive factor for HCV life cycle (Parent et al., 2009; Chen et al., 2010), and inhibition of the expression of Hsc70 by OMTR analogs suppressed HCV replication (Peng et al., 2010). As a continuation of our study on Hsc70, this clinical research showed that (1) the target for OMTR in the liver of CHB patients appeared to be Hsc70, down-regulation of which correlated with a reduction of intracellular HBV viral load; (2) OMTR effectively reduced HBV viral load in lamivudine-resistant CHB patients, at a potency equal to that in naïve CHB patients with wild-type HBV; and (3) combination of OMTR with lamivudine prevented CHB patients from developing drug-resistance against lamivudine. The results in CHB patients were further confirmed with the experiments in cell culture. It should be mentioned here that as the anti-HBV effect of OMTR is mediated through Hsc70 down-regulation (an indirect effect), it is less potent as compared with RT inhibitors. In conclusion, as a host mechanism based anti-HBV agent, OMTR seems to be effective in treating CHB patients with drug resistance against lamivudine; combination of OMTR with RT inhibitors could reduce the chance of developing drug resistance.

Acknowledgments

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