Oxethazaine inhibits hepatitis B virus capsid assembly by blocking the cytosolic calcium-signalling pathway

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Chronic hepatitis B virus (HBV) infection is a serious public health problem and may progress to liver fibrosis, cirrhosis and hepatocellular carcinoma. It is currently treated with PEGylated IFN-α and nucleoside/nucleotide analogues (NAs). However, PEGylated IFN treatment has problems of high cost, low efficiency and side effects. Long-term administration of NAs is necessary to avoid virus relapse, which can cause drug resistance and side effects. New efforts are now being directed to develop novel anti-HBV drugs targeting either additional viral targets other than viral DNA polymerase or host targets to improve the treatment of chronic hepatitis B. In this study, we discovered that oxethazaine, approved for clinic use in a few countries such as Japan, India, South Africa and Brazil, can dose-dependently reduce the levels of HBV envelope antigen, extracellular HBV DNA in supernatants and intracellular HBV total DNA. However, the levels of HBV cccDNA and HBV RNAs were not affected by oxethazaine treatment. Further study confirmed that oxethazaine acts on the virus assembly stage of the HBV life cycle. A study of the mechanisms of oxethazaine suggested that this drug inhibits HBV replication and capsid assembly by blocking the cytosolic calcium-signalling pathway. Moreover, oxethazaine could inhibit the replication of lamivudine/entecavir-dual-resistant and adefovir-resistant HBV mutants. In conclusion, our study suggests that oxethazaine may serve as a promising drug, or could be used as a starting point for anti-HBV drug discovery.

INTRODUCTION

Human hepatitis B virus (HBV) is a small enveloped DNA virus that causes acute and chronic hepatitis B. Despite the availability of an effective preventive vaccine, chronic HBV infection is still a major public health burden affecting an estimated 350–400 million individuals worldwide, and carries a high risk for the development of chronic hepatitis B, cirrhosis and hepatocellular carcinoma (McMahon, 2005).

To date, two IFNs [IFN-α and PEGylated IFN (PEG-IFN-α)] and five nucleoside/nucleotide analogues [NAs; lamivudine (2′,3′-dideoxy-3′-thiacytidine, 3TC), adefovir (ADV), entecavir (ETV), telbivudine and tenofovir (TDF)] have been approved by the US Food and Drug Administration (FDA) for the treatment of chronic hepatitis B. At present, polyethylene glycol (PEG) is the agent most widely used to form multiple copies of IFN molecules, which degrade more slowly once injected. Therefore, PEG-IFN only needs to be injected once a week, which makes compliance with the treatment schedule more convenient and easier for patients (Liaw et al., 2012; Lok & McMahon, 2009). However, IFN is expensive, and IFN therapy yields low sustained virological responses and significant side effects (Janssen et al., 2005). Conversely, NAs are reverse transcriptase (RT) inhibitors and inhibit HBV replication by inhibiting RT activity to lower the HBV DNA level. However, HBV replication ability comes back quickly once the drugs are withdrawn. Therefore, the NAs need a long-term course to maintain inhibition. Although the second-generation NAs ETV and TDF have more potent inhibition ability and a higher barrier to drug resistance, the HBV envelope antigen (HBeAg) seroconversion rate in clinics is still low, and there is still a long way to go to cure chronic HBV infection (Chang et al., 2010; Marcellin et al., 2013).

Therefore, new drugs targeting viral targets other than the HBV polymerase (P protein) or targeting host factors are essential for future anti-HBV therapy. Recent significant progress includes the development of potent HBV entry inhibitors (Petersen et al., 2008), assembly inhibitors (Campagna et al., 2013; Wu et al., 2013; Yang et al., 2014) and covalently closed circular DNA (cccDNA) inhibitors (Cai et al., 2012), as well as efficient and safe technologies, such as the clustered regularly-interspaced short palindromic repeat (CRISPR)/Cas9 system and IFN-mediated degradation, which can be used to specifically degrade cccDNA (Lin et al., 2014; Lucifora...
et al., 2014). Moreover, many researchers are committed to identifying new cellular targets involved in HBV replication, and in innate and adaptive immune responses for anti-HBV therapy, and to developing related inhibitors such as Hsp90 inhibitors, Hsc70 inhibitors and TLR7 agonists (Hu et al., 2004; Lopatin et al., 2013). These newly identified compounds or therapeutic strategies are now under clinical evaluation or in pre-clinical development (Block et al., 2015). However, none of these drugs has been approved by the FDA for anti-HBV therapy so far. Thus, more novel anti-HBV drug candidates are needed for anti-HBV drug development.

In this study, we identified oxethazaine as having anti-HBV activity. Oxethazaine is a potent local anaesthetic approved in countries such as Japan, India, South Africa and Brazil. It is also administered orally (usually in combination with an antacid) for the relief of pain associated with peptic ulcer disease or oesophagitis (Seifter et al., 1974). We found that oxethazaine inhibited HBV capsid assembly by reducing the cytosolic Ca$^{2+}$ levels and blocking the cytosolic calcium-signalling pathway. Importantly, oxethazaine was confirmed to inhibit the replication of 3TC/ETV-dual-resistant and ADV-resistant HBV mutants. Our results suggest that oxethazaine may serve as a starting point for anti-HBV drug discovery.

RESULTS

Oxethazaine inhibits HBV replication in HepAD38 cells

A screen of a drug library identified oxethazaine as having anti-HBV activity in HepAD38 cells. The chemical structure of oxethazaine is shown in Fig. 1(a). A cytotoxicity assay was performed in parallel with the antiviral assay. As shown in Fig. 1(b), the HepAD38 cells were treated with oxethazaine for 3 days, and the half maximal cytotoxicity concentration (CC$_{50}$) of oxethazaine was determined to be 54.74 mM. Oxethazaine at concentrations up to 30 mM was not cytotoxic to HepAD38 cells under the conditions used for the anti-HBV activity assay. To evaluate further the anti-HBV activity of oxethazaine, HepAD38 cells were treated with oxethazaine for 3 days, and ELISA and real-time PCR were used to quantify the levels of HBeAg, HBV surface antigen (HBsAg) and extracellular HBV DNA in the supernatants. As shown in Fig. 1(c), oxethazaine inhibited the secretion of HBeAg and did not significantly affect the levels of HBsAg. Oxethazaine significantly and dose-dependently inhibited the levels of extracellular HBV DNA in the supernatants (Fig. 1d) at a half maximal effect concentration (EC$_{50}$) of 1.25 mM and a selective index value of approximately 40.

The levels of intracellular HBV total DNA in HepAD38 cells treated with oxethazaine for 3 days were also measured using real-time PCR and Southern blot analyses. As shown in Fig. 1(e), real-time PCR showed that oxethazaine dose-dependently reduced the intracellular HBV DNA levels at an EC$_{50}$ of 2.5 mM. To further identify the species of HBV DNAs that were affected by oxethazaine, Southern blot analysis was performed. As shown in Fig. 1(f), oxethazaine reduced the production of both relaxed circular HBV DNA and single-stranded HBV DNA.

The anti-HBV activity of oxethazaine was also confirmed in another stable HBV-transfected cell line, HepG2.2.15, treated with oxethazaine for 3 days. As shown in Fig. 1(g), oxethazaine did not affect the levels of HBeAg and HBsAg in the supernatants. However, oxethazaine significantly and dose-dependently inhibited the levels of the intracellular and extracellular HBV DNA in the supernatants at a similar EC$_{50}$ of 5 mM (Fig. 1h).

Oxethazaine does not affect HBV cccDNA formation or degradation

As cccDNA plays a vital role in chronic HBV infection, the effect of oxethazaine on HBV cccDNA was determined using real-time PCR analysis. HepAD38 cells treated with oxethazaine for 6 days were lysed and the cccDNAs were extracted as described previously (Zhou et al., 2006). As shown in Fig. 2(a), the HepAD38 cells remained viable in response to treatment with non-toxic concentrations of up to 10 mM (left panel) for 6 days. Furthermore, oxethazaine at the concentrations tested did not affect the relative levels of HBV cccDNA (middle panel), whereas the relative levels of the intracellular HBV total DNA decreased dose-dependently compared with the untreated control (right panel). To demonstrate that the real-time PCR assay for cccDNA was actually detecting cccDNA, a negative control of HepAD38 cells treated with doxycycline (DOX) was included, and the level of cccDNA of this control was very low and almost undetectable. These results showed that oxethazaine does not affect HBV cccDNA formation or degradation.

Oxethazaine does not affect the levels of HBV RNAs

In order to illuminate the anti-HBV mechanism of oxethazaine, the effect of oxethazaine on the levels of HBV RNAs was determined. HBV RNAs, specifically precore mRNAs, pregenomic RNA (pgRNAs) and total RNA of HBV in HepAD38 cells treated with oxethazaine for 3 days, were reverse transcribed to cDNA. The levels of these cDNAs were determined by real-time PCR. As shown in Fig. 2(b), the relative levels of HBV precore mRNAs, pgRNAs and total RNA were not affected by oxethazaine treatment when compared with the untreated control. The results suggested that the anti-HBV activity of oxethazaine is not related to specific inhibition of the accumulation of HBV RNAs, which is regulated by both transcription and degradation.

Oxethazaine inhibits HBV capsid assembly

Because almost all phases before and after capsid assembly in the HBV life cycle remained unaffected by oxethazaine, we investigated the ability of oxethazaine to inhibit HBV
Oxethazaine

(a)

(b)

CC\(_{50}\) = 54.74 μM

(c)

Relative levels of HBeAg/ HBsAg

(d)

Relative levels of HBV DNA

(e)

Relative levels of HBV intracellular DNA

(f)

Intracellular HBV DNA

(g)

Relative levels of HBV DNA in supernatants

(h)

Intracellular HBV DNA

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capsid assembly using a viral particle gel assay. As shown in Fig. 3(a), the levels of both HBV capsid protein and capsid-associated DNA dose-dependently decreased following oxethazaine treatment compared with the untreated control, whereas the levels of HBV core protein and cellular β-actin protein remained unchanged, suggesting that oxethazaine inhibits HBV capsid assembly. During HBV capsid assembly, pgRNA binds to the HBV P protein and forms the RNA–P protein (RNP) complex, which recruits HBV core protein dimers to form the HBV capsid. In this capsid, the interactions of core protein with the RNP complex and the core protein with core protein are all critical. In our study, oxethazaine inhibited HBV capsid assembly but did not affect the core protein

Fig. 1. Oxethazaine inhibits HBV replication in vitro. (a) Chemical structure of oxethazaine. (b) A cytotoxicity assay was performed using alamarBlue reagent to test the viability of HepAD38 cells treated with oxethazaine for 3 days. (c) The levels of HBeAg and HBsAg in the supernatants were determined by ELISA. (d) The relative levels of supernatant HBV total DNA were quantified by real-time PCR and normalized to the untreated control. (e) The relative levels of intracellular HBV total DNA were quantified by real-time PCR and normalized to the internal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) DNA control. (f) The relative levels of intracellular HBV replication intermediates were detected by Southern blotting hybridization using a digoxygenin-labelled HBV genomic fragment as a probe. RC, Relaxed circular HBV DNA; SS, single-stranded HBV DNA. 3TC was used as a positive control. (g) The levels of HBeAg and HBsAg in the supernatants of HepG2.2.15 cells treated with oxethazaine for 3 days were determined by ELISA. (h) The relative levels of supernatant HBV total DNA from HepG2.2.15 cells treated with oxethazaine for 3 days were quantified by real-time PCR and normalized to the untreated control. The relative levels of intracellular HBV total DNA were quantified by real-time PCR and normalized to the internal GAPDH DNA control. The results in (c–e), (g) and (h) are presented as the means ± SD (n=3). Ctr, Untreated control.

Fig. 2. Oxethazaine does not affect the levels of HBV cccDNA and RNAs in HepAD38 cells. (a) A cytotoxicity assay was performed to test the cell viabilities of HepAD38 cells treated with oxethazaine for 6 days (left panel); the relative levels of cccDNA (middle panel) and intracellular HBV total DNA (right panel) were quantified by real-time PCR and normalized to the internal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene DNA control. C, Untreated control; DOX, doxycycline. (b) HepAD38 cells were treated with oxethazaine for 72 h, and the treated cells were harvested and subjected to total RNA isolation. After the reverse transcription of the total RNA, the cDNA of the HBV precore mRNA (left panel), pgRNA (middle panel) and total RNA (right panel) were quantified by real-time PCR and normalized to the internal GAPDH RNA control. Ctr, Untreated control. The results in (a) and (b) are presented as means ± SD (n=3).
level. Furthermore, abnormal capsid formation was not observed, suggesting that oxethazaine did not affect the interaction of the RNP complex with core protein; interference with this interaction may produce an abnormal capsid that is smaller than the normal capsid (Yang et al., 2014). We speculated that oxethazaine might inhibit the interaction of HBV core proteins with the self-assembly process, leading to the inhibition of HBV capsid assembly. To test this hypothesis, Huh7 cells were transfected with the pFlag-Core plasmid expressing HBV core protein and treated with oxethazaine for 3 days. As shown in Fig. 3(b), core protein expression levels were not affected by oxethazaine treatment (upper panel), whereas the levels of self-assembled core proteins were dose-dependently reduced by oxethazaine treatment (lower panel). This finding suggested that oxethazaine can inhibit HBV assembly by disrupting the core–core interaction.

**Oxethazaine inhibits HBV replication by reducing the concentration of cytosolic Ca^{2+}**

Interestingly, Choi et al. (2005) showed that HBV core self-assembly in vitro is promoted by Ca^{2+}. Furthermore, treatment with BAPTA-AM and cyclosporine A, which reduce the cytosolic Ca^{2+} concentration, reduces HBV capsids in transfected HepG2 cells, and thapsigargin treatment, which increases the cytosolic Ca^{2+} concentration, increases HBV capsids in transfected HepG2 cells (Choi et al., 2005). Oxethazaine also reportedly affects the cytosolic Ca^{2+} concentration and has been suggested to be a specific blocker of L-type Ca^{2+} channels (Masuda et al., 2002). Because cytosolic calcium is essential for HBV replication (Boucharud et al., 2001), we speculated that oxethazaine may inhibit HBV capsid assembly and core protein self-assembly by reducing the concentration of cytosolic Ca^{2+}.

Therefore, we measured the cytosolic Ca^{2+} concentration in Huh7 cells treated with oxethazaine using Fluo-3 AM, a fluorescence indicator of intracellular Ca^{2+}. As shown in Fig. 4(a, left panel), the cytosolic Ca^{2+} concentrations dose-dependently decreased in response to oxethazaine treatment compared with the untreated cells, suggesting that oxethazaine decreases the cytosolic Ca^{2+} concentration. To further confirm that this decrease in cytosolic Ca^{2+} concentration was mediated by oxethazaine treatment, the Huh7 cells were treated with the calcium-mobilizing agent thapsigargin along with oxethazaine to rescue the decrease in the cytosolic Ca^{2+} concentration. The results showed that treatment with 20 nM thapsigargin (non-cytotoxic concentration; data not shown) could rescue the decrease in the cytosolic Ca^{2+} concentration (Fig. 4a, right panel) caused by oxethazaine treatment, and could functionally rescue HBV replication (Fig. 4b, upper left panel), capsid assembly (Fig. 4b, upper right panel) and core self-assembly (Fig. 3b, lower panel). However, thapsigargin treatment alone did not significantly affect HBV replication, capsid assembly or core self-assembly. These results suggested that oxethazaine inhibits HBV replication by reducing the cytosolic Ca^{2+} concentration to inhibit HBV assembly.

HBV HBx protein is essential for HBV infection and replication (Chen et al., 1993; Zoulim et al., 1994), and activation of Src by HBx has also been shown to be important for HBV DNA replication (Benn & Schneider, 1994; Benn et al., 1996; Natoli et al., 1994). Moreover, Boucharud et al. (2001) reported that HBx activated cytosolic calcium-dependent proline-rich tyrosine kinase-2 (Pyk2), an Src
Fig. 4. Oxethazaine inhibits HBV replication by blocking the cytosolic calcium-signalling pathway. (a) Huh7 cells were washed three times with PBS and loaded with 5 µM Fluo-3 AM by incubation at 37 °C for 1 h. After three additional washes in PBS, the Huh7 cells were incubated with oxethazaine (OXZ) of various concentrations (2.5, 5, 10 and 20 µM) without (left panel) and with 20 nM (right panel) thapsigargin (Thap) or vehicle control (DMSO), and the fluorescence intensity of the cells was measured to quantify the relative levels of cytosolic Ca²⁺. (b) HepAD38 cells were treated with oxethazaine and the calcium-mobilizing agent thapsigargin, and the relative levels of intracellular HBV total DNA were quantified by real-time PCR and normalized to the internal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) DNA control. The results are presented as means ± SD (n=3). The cell lysates were prepared and analysed for HBV capsids by native agarose gel electrophoresis and Western blotting with the rabbit polyclonal HBcAb. (c) Huh7 cells were transfected with 1.1-fold HBV full genome containing plasmid pHBV1.1, and the transfected cells were treated with oxethazaine of various concentrations and 20 nM thapsigargin for 3 days. The cell lysates were loaded onto the SDS-PAGE gel to detect HBV core by Western blotting with antibodies against proline-rich tyrosine kinase-2 (Pyk2), phosphorylated Pyk2 [Pyk2-Y(P)402] and β-actin.
kinase activator, and that HBx activation of HBV DNA replication was blocked by inhibiting Pyk2 phosphorylation or calcium-signalling. Reagents that increased cytosolic Ca²⁺ substituted for HBx protein in HBV DNA replication (Bouchard et al., 2001). Thus, alteration of cytosolic Ca²⁺ is a fundamental requirement for HBV replication and is mediated by HBx protein (Bouchard et al., 2001). To assess the role of the cytosolic calcium-signalling pathway in inhibiting HBV capsid assembly caused by oxethazaine treatment in HBV-replicating cells, we detected the phosphorylation of Pyk2, which is the key step in activation of the calcium signal pathway and is critical for the stimulation of HBV replication (Bouchard et al., 2001). To this end, Huh7 cells were transfected with HBV-expressing plasmid that contained 1.1-fold HBV genome. These cells were cultured for 6 h and then treated with oxethazaine and/or thapsigargin for 3 days. As shown in Fig. 4(c), the levels of phosphorylated Pyk2 increased significantly in Huh7 cells transfected with the HBV-expressing plasmid, oxethazaine significantly reduced the levels of phosphorylated Pyk2, and the decreased phosphorylation of Pyk2 mediated by oxethazaine could be rescued by thapsigargin treatment. Notably, these treatments did not affect the expression levels of Pyk2. These results suggested that oxethazaine inhibits the activation of the cytosolic calcium-signalling pathway by impairing the phosphorylation of Pyk2 as a result of decreasing the cytosolic Ca²⁺ concentration.

**Oxethazaine inhibits the replication of 3TC/ETV-dual-resistant and ADV-resistant HBV mutants**

Long-term treatment with NAs can produce drug-resistant HBV mutants. Amino acid substitutions in the RT domains of the HBV P protein, which is associated with resistance to approved drugs, have been identified. Specifically, the major mutations associated with resistance to 3TC are rtM204I/V and rtL180M, the major mutations associated with resistance to ADV are rtN236T and rtA181S/V, and the major mutations associated with resistance to ETV are rtS202C/G/I, rtM204I/V and rtM250I/V/L. The above mutations are the most frequent mutations found in clinical isolates (Menéndez-Arias et al., 2014). Thus, two of the most common clinical drug-resistant HBV mutants, an ADV-resistant HBV mutant (rtN236T) and a 3TC/ETV-dual-resistant HBV mutant (L180M + M204V + S202G), were used to evaluate the anti-HBV activity of oxethazaine.

To test the ability of oxethazaine to inhibit NA-resistant HBV mutants, Huh7 cells were transfected with 3TC/ETV-dual-resistant and ADV-resistant HBV mutant-expressing plasmids. The transfected cells were treated with ADV, 3TC, ETV or oxethazaine, and a control group remained untreated. As shown in Fig. 5, Southern blot analysis of the HBV capsid-associated DNA revealed that synthesis of HBV DNA in the cells was efficiently inhibited by oxethazaine, 3TC, ADV and ETV. Conversely, treatment with ADV significantly attenuated the inhibition of HBV DNA synthesis in the cells transfected with ADV-resistant mutant-expressing plasmids. The same effect was also observed in cells transfected with the 3TV/ETV-dual-resistant mutant-expressing plasmid when the cells were treated with 3TV or ETV, which suggested that the two drug-resistant mutants were significantly resistant to the corresponding NAs but not to oxethazaine. These findings suggested that oxethazaine can efficiently inhibit the synthesis of WT, ADV-resistant mutant and 3TV/ETV-dual-resistant mutant HBV DNA.

**DISCUSSION**

In this study, oxethazaine, a drug in clinical use (usually in combination with an antacid) orally for the relief of pain associated with peptic ulcer disease or oesophagitis, was identified as an anti-HBV agent that acts by lowering the cytosolic Ca²⁺ concentration to block capsid assembly. Moreover, we excluded the possibility that oxethazaine inhibits HBV replication by reducing cccDNA, HBV RNAs or HBV transcription. Further study suggested that oxethazaine can inhibit HBV capsid assembly and core protein self-assembly without affecting the expression of HBV core protein. A study of the mechanism of action revealed that oxethazaine dose-dependently reduced the cytosolic Ca²⁺ concentration, and that increases in the cytosolic Ca²⁺ concentration mediated by thapsigargin eliminated the anti-HBV effect of oxethazaine. Moreover, we showed that oxethazaine significantly inhibited Pyk2 phosphorylation, which is critical to the activation of the cytosolic calcium-signalling pathway.

The major classes of HBV capsid inhibitors that are under active clinical development can be divided into two classes by the mechanism of inhibiting HBV capsid assembly: the first class of inhibitors, such as Bay 41-4109, inhibit capsid formation by reducing the level of core protein, whereas the second class of inhibitors, such as AT-61, prevent the encapsidation of viral pgRNA into nucleocapsids without affecting self-assembly of the core. Recently, three inhibitors of L-type Ca²⁺ channels (lomerizine, cilnidipine and nifedipine) have been reported to inhibit HBV replication (van de Klundert et al., 2016). However, the mechanism of action was not studied. Our results demonstrate that oxethazaine, an L-type Ca²⁺ channel inhibitor, inhibits HBV capsid assembly through blocking self-assembly of the HBV core without reducing the level of HBV core, and that a reduction of cytosolic Ca²⁺ concentration is involved in this inhibition effect. This mechanism of inhibiting HBV capsid assembly is new and may represent a third class of HBV capsid assembly inhibitors.

In our study, HBeAg secretion declined on oxethazaine treatment in HepAD38 cells but not HepG2.2.15 cells. The difference in HBeAg secretion contributed to the difference in HBV replication in the two cell lines. In HepAD38 cells, the precore mRNA could only be transcribed from cccDNA. Three days after DOX withdrawal, the level of cccDNA in HepAD38 cells is very low (Zhou et al., 2006). Compared with the untreated control, oxethazaine could reduce the relaxed circular
Fig. 5. Oxethazaine inhibits the replication of 3TC/ETV-dual-resistant and ADV-resistant HBV mutants. pHBV1.1 plasmids containing WT, ADV-resistant (rtN236T) or 3TC/ETV-dual-resistant (L180M+M204V+S202G) 1.1-fold HBV genome were transiently transfected into Huh7 cells. Six hours after transfection, the cells were treated with serially diluted oxethazaine (a), 3TC (b), ADV (c) or ETV (d) for 3 days; control cells (Ctr) remained untreated. HBV capsid-associated DNA was analysed by Southern blotting 3 days after NA treatment.
HBV DNA level by inhibiting capsid assembly and thereby the accumulation of cccDNA, resulting in the decline in HBeAg. In contrast, in HepG2.2.15 cells, the mRNAs encoding HBeAg could be transcribed from the transfected HBV genomes (Sells et al., 1987), and this action could not be inhibited by oxethazaine, and therefore HBeAg secretion remained unchanged following oxethazaine treatment.

Choi et al. (2005) showed that HBV core self-assembly in vitro is promoted by Ca\(^{2+}\). Treatment with BAPTA-AM and cyclosporine A, which reduce the cytosolic Ca\(^{2+}\) concentration, reduced HBV capsids in the transfected HepG2 cells, while thapsigargin treatment, which increases the cytosolic Ca\(^{2+}\) concentration, increased HBV capsids in the transfected HepG2 cells. Furthermore, oxethazaine also reportedly affects the cytosolic Ca\(^{2+}\) concentration and has been suggested to be a specific blocker of L-type Ca\(^{2+}\) channels (Masuda et al., 2002). Taken together, we concluded that oxethazaine inhibits HBV assembly by reducing the concentration of cytosolic Ca\(^{2+}\) and possibly by blocking the cytosolic calcium-signalling pathway. Cytosolic Ca\(^{2+}\) has been shown to be essential for HBV replication (Bouchard et al., 2001), so the oxethazaine is likely to interact with the Ca\(^{2+}\) channels, most likely L-type Ca\(^{2+}\) channels to reduce the concentration of cytosolic Ca\(^{2+}\). Interestingly, van de Klundert et al. (2016) recently reported that L-type Ca\(^{2+}\) channel blockers (lomerizine, cilnidipine and nifedipine) all efficiently suppressed HBV replication in HepG2.2.15 cells. Notably, L-type Ca\(^{2+}\) channel blockers are available orally, do not have severe side effects and can be administered daily for more than a year (Dubiel et al., 1990; Imai et al., 2007). This may suggest that the L-type Ca\(^{2+}\) channel is a potential anti-HBV target.

HBV replication requires the multifunctional HBx protein (Chen et al., 1993; Zoulim et al., 1994). In the cytoplasm, HBx stimulates Ras, Src and JNK signal transduction pathways (Benn & Schneider, 1994; Benn et al., 1996; Natoli et al., 1994). HBx activation of Src promotes reverse transcription in the virus and DNA replication (Klein & Schneider, 1997; Klein et al., 1999; Natoli et al., 1994). Pyk2 is a cytoplasmic calcium-activated kinase that activates Src kinases (Lev et al., 1995) and downstream effectors, such as JNKs (Zhao et al., 2000). Increased cytosolic Ca\(^{2+}\) activates Pyk2, leading to its autophasorylation at Tyr-402 (Y402), which creates a binding site for Src kinases and activates them. HBx has also been shown to activate Pyk2 and induce phosphorylation of Pyk2 at Y402, while HBx activation of Pyk2 involves alteration of cytosolic Ca\(^{2+}\) (Bouchard et al., 2001). In addition to this, HBx activation of Pyk2 activates downstream Src kinases, and HBx specifically promotes HBV DNA replication in a Pyk2-dependent manner (Bouchard et al., 2001). HBx activation of viral reverse transcription and DNA replication can be replaced by agents that mobilize cytosolic Ca\(^{2+}\). HBx acts on cytosolic stored Ca\(^{2+}\) to stimulate Pyk2–Src kinase signal transduction pathways that activate HBV reverse transcription and DNA replication (Bouchard et al., 2001). In this case, in our study, oxethazaine probably reduced the cytosolic stored Ca\(^{2+}\) to inhibit Pyk2–Src kinase signal transduction pathways, which may reduce the phosphorylation of HBV core protein to inhibit HBV assembly and replication.

The HBV core protein self-assembly was suggested to be inhibited by oxethazaine in our study. Oxethazaine also can block the activation of the cytosolic calcium-signalling pathway, which is suggested to be important for HBV replication by increasing the phosphorylation of HBV core protein and capsid (Bouchard et al., 2001). Mutational analyses indicate that phosphorylation of the core protein C-terminal domain is important for RNA packaging and DNA synthesis (Jung et al., 2014; Lan et al., 1999; Lewellyn & Loeb, 2011). Several kinases have been reported to phosphorylate the core protein in vitro, including protein kinase C (Kann & Gerlich, 1994; Kann et al., 1999) and the serine–arginine protein kinases 1 and 2 (SRPK1 and SRPK2) (Daub et al., 2002), but the specific sites of the core protein or C-terminal domain that are phosphorylated by these kinases in vivo remain unknown. The putative cellular phosphatase(s) that mediates the dephosphorylation of mature capsid remains to be identified. We speculate that the phosphorylation of core protein may be inhibited as a result of the inhibition of the phosphorylation of Pyk2 caused by oxethazaine treatment. Therefore, identifying the cellular phosphatase(s) and the phosphorylation sites on HBV core protein associated with HBV capsid assembly may elucidate the mechanism by which oxethazaine inhibits HBV and the detailed role of cytosolic Ca\(^{2+}\) in HBV capsid assembly.

Oxethazaine is a drug with low toxicity that potently inhibits HBV capsid assembly. Notably, oxethazaine can inhibit the replication of 3TC/ETV-dual-resistant and ADV-resistant HBV mutants, which demonstrates its future value in the clinical treatment of hepatitis caused by NA-resistant HBV. It could be a good lead to design more anti-HBV drugs with higher selective indexes of anti-HBV effect (lower toxicity and higher efficiency), and a longer half-life in vivo. Moreover, it could be also used as a tool drug in the study of the mechanism of HBV capsid assembly and the exact role of the cytosolic calcium-signalling pathway in HBV replication. The further development of oxethazaine or its derivatives and additional antiviral compounds to target virus-encoded proteins other than HBV P protein is likely to result in the establishment of combination therapies that will limit the appearance of virus resistance during chronic infection by acting on multiple targets.

**METHODS**

**Drugs.** 3TC was purchased from GlaxoSmithKline. ADV and ETV were purchased from Melone Pharma Corp. G418, DOX, oxethazaine
and thapsigargin were purchased from Sigma-Aldrich. Penicillin and streptomycin were purchased from Shandong Lukang Pharmaceutical Co., Ltd. The purities of the drugs were all >98 %.

**Cell and cultures.** The HBV-producing cell line HepAD38 was maintained in Dulbecco’s modified Eagle’s medium/F-12 (DMEM/F-12; Gibco) supplemented with 10 % FBS, 100 U penicillin ml⁻¹, 100 U streptomycin ml⁻¹, 200 µg G418 ml⁻¹ and 1 µg DOX ml⁻¹. In the presence of DOX, the HBV replication cycle was strongly suppressed. In the absence of DOX, the HBV replication cycle was robust under the control of the native HBV enhancers and promoters, unless an inhibitor of HBV replication was present. The HepAD38 cell line was a generous gift from Christoph Seeger (Fox Chase Cancer Center, PA, USA) (Lader et al., 1997; Zhou et al., 2006).

The HepG2.2.15 cell line is a HepG2 cell line transfected with a plasmid containing two head-to-tail dimers of the HBV genome. The Huh7 cell line is a well-differentiated, hepatocyte-derived cellular line. Huh7 and HepG2.2.15 cells were both maintained in DMEM supplemented with 10 % FBS, 100 U penicillin ml⁻¹, 100 U streptomycin ml⁻¹. Both cell lines were a generous gift from Xinwen Chen (Wuhan Institute of Virology, Chinese Academy of Sciences, China).

**Plasmids and constructs.** The pFlag-GAPDH and pFlag-Core plasmids are human cytomembranovirus (CMV) promoter-controlled, Flag-tagged GAPDH or HBV core expression plasmids, respectively. To construct these two plasmids, the GAPDH or HBV core gene was cloned into the pFlag-CMV vector using BamHI and EcoRI.

The WT and two drug-resistant mutant HBV-expressing plasmids, pHBV1.1-wt, pHBV1.1-N236T and pHBV1.1-L180M + M204V + S202G, were generous gifts from Xinwen Chen. The N236T mutation confers resistance to ADV, whereas the L180M S202G, were generous gifts from Xinwen Chen. The N236T mutation confers resistance to 3TC and ETV.

**Cytotoxicity assay.** HepAD38 cells were seeded into 96-well plates at a density of 1 × 10⁴ cells per well and cultured in DMEM/F-12. After drug treatment, the supernatants were removed and the cells were incubated in 100 µl diluted alamarBlue reagent per well at 37 °C for 1 h. Subsequently, the fluorescence was measured at an excitation wavelength of 530–560 nm and an emission wavelength of 590 nm using a Perkin Elmer Envision Multi-label Plate Reader.

**Quantification of HBsAg and HBeAg.** HepAD38 cells were cultured without DOX for 2 days and then seeded into 96-well plates at a density of 1 × 10⁴ cells per well in DMEM/F-12. After drug treatment, the levels of HBsAg and HBeAg in the supernatants were detected using an ELISA (Kehua Bio-engineering Corp.) according to the manufacturer’s recommendations.

**Quantification of HBV DNA by real-time PCR analysis.** HepAD38 cells cultured without DOX for 2 days were seeded into 24-well plates at a density of 5 × 10⁴ cells per well and cultured in DMEM/F-12. After drug treatment, the cccDNA, intracellular HBV total DNA, and HBV virion-associated DNA in the supernatant were extracted, purified and quantified using real-time PCR as described previously (Guo et al., 2005; Hirt, 1967; Zhang et al., 2011). The forward primer for the quantification of the intracellular HBV total DNA and HBV virion-associated DNA in the supernatant was 5'-ACTCACAACCACCTTGTCCCT-3'; the reverse primer was 5'-GACAAAGGCGAAGATACCT-3'; the forward primer for the quantification of cccDNA was 5'-CTCCCGGCTCGTTGCTCCT-3'; and the reverse primer was 5'-CCCGCAATCAGCCTCATCCT-3'; the forward primer for internal control GAPDH gene quantification was 5'-GAAGGTGAAGGTCGGAGTC-3' and the reverse primer was 5'-GACAGTGTTATGGAGATTTC-3'. PCRs were performed using Bio-Rad iQ Universal SYBR Green PCR Master Mix and the primer pair with the following programme: initial denaturation at 95 °C for 5 min, followed by 39 cycles of amplification at 95 °C for 15 s and annealing/extension + plate read at 60 °C for 30 s.

**Southern blot analysis.** HepAD38 cells cultured without DOX for 2 days were seeded into six-well plates at a density of 2 × 10⁴ cells per well and cultured in DMEM/F-12. Twenty-four hours after seeding, the cells were treated with oxethazaine for 3 days. The cells were collected, and the HBV capsid DNAs were prepared, followed by Southern blot analysis as described previously (Pugh et al., 1988; Yang et al., 2014). Briefly, HBV DNAs were separated by electrophoresis using 1 % agarose and transferred to a nylon membrane (Hybond-N+ membrane; GE Healthcare) using the capillary transfer method with 20 × SSC buffer overnight at room temperature. HBV DNAs were fixed to the membrane by baking for 30 min at 120 °C and then detected using a digoxigenin-labelled 1.3 kb HBV probe.

**Quantification of HBV RNA by real-time PCR analysis.** HepAD38 cells cultured without DOX for 2 days were seeded into 24-well plates at a density of 5 × 10⁴ cells per well and cultured in DMEM/F-12. Twenty-four hours after seeding, the cells were treated with oxethazaine for 3 days. Total RNAs were extracted from the treated cells using Trizol isolation buffer (Invitrogen), according to the manufacturer’s protocol. After the RNA had been reverse transcribed into cDNA, the cDNAs of HBV total RNA, precore mRNA and pgRNA were quantified by real-time PCR as previously (Yu et al., 2013). The forward primer for HBV total RNA quantification was 5'-CCGTCGTCCCTCTCTCCTGC-3' and the reverse primer was 5'-ACAAATTATGGCCTACAGCCTC-3'; the forward primer for HBV precore mRNA was 5'-TCTTGCGACACAGCAGCACTGACAC-3' and the reverse primer was 5'-GGGAATTACGGAGGATGCC-3'; the forward primer for HBV pgRNA was 5'-GCTGCTGATTGTAAATTGC-3' and the reverse primer was 5'-TAAGGTTAGGGAGATC-3'; the forward primer for the control GAPDH RNA was 5'-GAAAGTGAAGGATCCAGAT-3' and the reverse primer was 5'-GAAGATGTTATGGAGATTTC-3'. PCRs were performed using Bio-Rad iQ Universal SYBR Green PCR Master Mix and the primer pair with the following programme: initial denaturation at 95 °C for 5 min, followed by 39 cycles of amplification at 95 °C for 15 s and annealing/extension + plate read at 55 °C for 30 s.

**Western blot analysis.** After treatment, the cells were harvested and lysed in RIPA buffer, and equal amounts of proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked with 5 % non-fat milk or BSA for 1 h before being incubated with diluted primary antibodies against HBV core protein (HBcAb; Dako), GAPDH (ZSGB-BIO), β-actin (ZSGB-BIO), Flap (Sigma), Pγ2 (Abcam) or phospho-Y02 Pγ2 (Abcam) overnight at 4 °C. Bound primary antibodies were detected with the corresponding HRP-tagged secondary antibody and visualized using an enhanced chemiluminescence system (AlphaEase FluorChem System; Alpha Innotech Corp.) and the ECL Western blotting reagents (Advanta).

**Viral particle gel assay.** Briefly, cells were collected and lysed with lysis buffer [10 mM Tris/HCl (pH 7.6), 0.1 % NP-40, 100 mM NaCl, 1 mM EDTA] for 30 min at room temperature. Cell debris was removed by centrifugation at 5000 g for 10 min. The clarified cell lysate was fractionated by electrophoresis on a 1 % native agarose gel at 70 V in TAE buffer and transferred to an Amersham Hybond-N+ membrane (GE Healthcare) using the capillary transfer method with 10 × SSC buffer overnight. HBV capsids were detected using HBcAb and a corresponding secondary antibody, followed by detection with chemiluminescence (Yang et al., 2014). To detect capsid-associated HBV DNA, the membranes were treated with a denaturing solution for 5 min, followed by neutralization for 5 min. The capsid-associated HBV DNA was detected by Southern blot analysis as described pre-
viously (Campagna et al., 2013; Yang et al., 2014). The assembled core particles in the lysate were detected using the same method as used to detect the HBV capsid particles.

**Measurement of the cytosolic calcium.** Briefly, HuH7 cells were seeded in 96-well plates at 8 × 10^3 cells per well and cultured in DMEM at 37 °C for 48 h. The cells were then washed three times with PBS and incubated with 30 μl diluted Fluo-3 AM (working concentration 5 μM) at 37 °C for 1 h, followed by another three washes with PBS. The cells were then incubated with serially diluted drugs to measure the cytosolic Ca^{2+} level for 400 s based on the fluorescence (excitation wavelength 480 nm, emission wavelength 525 nm) on a Perkin Elmer Enspire multi-label detection system (Zhang et al., 2014).

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**REFERENCES**


