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Bicyclol attenuates pro-inflammatory cytokine and chemokine productions in CpG-DNA-stimulated L02 hepatocytes by inhibiting p65-NF- κ B and p38-MAPK activation

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Bicyclol, a novel synthetic anti-hepatitis drug, has a potent hepatocyte-protective effect and a mild anti-hepatitis virus function. However, its pharmaceutical effects and mechanism are still unclear. In the present study, we found that bicyclol pre-treatment could attenuate the production of the inflammatory cytokines (TNF- α and IL-18) and chemokines (MCP-1, MIP-1 α and Rantes) and inhibit the activation of the p65-NF- κ B and MAPK signaling pathways in CpG-ODN 2006-stimulated L02 hepatocytes in a dose-dependent manner. Collectively, these results suggest that bicyclol could exert its hepatocyte-protective effect through attenuating CpG-ODN 2006-stimulated inflammatory responses in L02 hepatocytes, which might be associated with the activations of NF- κ B and MAPK pathways.

1. Introduction

Viral hepatitis is a prevailing disease in China. To date, the incidence of viral hepatitis has always ranked first among infectious diseases. Therefore, scientists have been making every effort to search for therapeutic drugs with high potencies and low toxicities to treat viral hepatitis. One of the goals of treating hepatitis is to control liver damage in addition to promoting hepatocyte repair. Bicyclol, with a chemical name of 4,4'-dimethoxy-5,6,5',6'-bis(methylene-dioxy)-2-hydroxymethyl-2'-methoxycarbonyl biphenyl, is a novel schisandrin analogue synthesized by Chinese scientists. It is associated with drug patents in 16 countries and was marketed in 2002 (Liu 2009). Pharmacological studies indicate that this drug can inhibit hepatitis B and C virus replications in a duck hepatitis model and in the 2.2.15 cell line (Liu 2009). It can also protect hepatocytes from experimental liver injury in rats and mice induced by hepatotoxins, such as CCl₄ (Liu et al. 2005), acetaminophen (Li et al. 2001), ConA (Li and Liu 2004; Zhao and Liu 2001), D-galactosamine and lipopolysaccharide (LPS) (Wang and Li 2006). Preclinical studies indicate that bicyclol can protect liver cells from ConA, D-galactosamine or LPS induced injuries by inhibiting both the mitochondrial and Fas/FasL apoptotic pathways (Li et al. 2004; Zhao et al. 2001). Bicyclol can also decrease serum and liver TNF- α levels in ConA-induced liver injury and down-regulate IFN- γ mRNA expression in liver cells (Li et al. 2004). Furthermore, recent studies indicate that bicyclol can up-regulate expression of HSP27 and HSP70 to attenuate ConA-induced hepatocyte apoptosis and liver injury through suppression of NF- κ B in mice (Bao and Liu 2008, 2009). Although various studies have investigated the possible mechanisms for the protective effects of bicyclol on liver cells, there are still many avenues that remain to be studied. Specifically,

the mechanism by which bicyclol inhibits virus-induced liver inflammation and the manner in which its functions are associated with immunoregulatory effects need further investigation. Repeated unmethylated CpG dinucleotides are the smallest units to exert the immunostimulatory effects of bacterial and viral DNA (Bauer et al. 2001). At present, many oligodeoxynucleotides (ODNs) containing the proper CpG-DNA motif have been synthesized to mimic the immunostimulatory effects of bacterial and viral DNA. CpG-ODNs with different sequences are specific to lymphocytes of different species. The core sequences of CpG-ODNs that can best elicit an immune response in mouse and human lymphocytes are 5'-GACGTT-3' (Hartmann et al. 2000b; Krieg et al. 1995) and 5'-GTCGTT-3' (Hartmann and Krieg 2000a), respectively. The most frequently used CpG-ODN sequences to exert immune stimulatory effects in mouse and human are 5'-TCCATCACGTTCTGACGTT-3' (CpG1826) (Hartmann et al. 2000b) and 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3' (CpG2006) (Hartmann and Krieg 2000), respectively. Toll-like receptor (TLR) 9 is the internal receptor for CpG motifs, which elicits the innate immune response by specifically recognizing the CpG motifs of bacterial and viral DNA. It is primarily expressed by numerous immune cells, such as dendritic cells, B lymphocytes and natural killer (NK) cells (Hornung et al. 2002). Recent studies also indicate that several non-immune cells, including HepG2 and L02 hepatocytes, can express TLR 9 (Liu et al. 2002, 2009). Activation of the TLR9 signaling pathway can induce the production and secretion of pro-inflammatory cytokines and chemokines, which will elicit a Th1 type immune reaction and lead to a local inflammatory response (Krieg et al. 2000; Moser and Loetscher 2001). The CpG-ODN ligand for TLR9 may represent a major source

of inflammatory damage to the hepatocytes (Klinman 2004). Sacher et al. (2002) found that CpG-ODN can directly act on the hepatocytes and up-regulate adhesion and costimulatory molecules in the hepatocytes that display an APC-like phenotype upon stimulation, resulting in inflammation and tissue damage. CpG-ODN-induced early hepatic injury is reported with *in vivo* models (Slotta et al. 2006). These findings demonstrate that hepatocytes also play an important role in the immune response within the liver. Although the details of the signaling pathways involved are still unclear, NF- κ B and MAPKs have been shown to play a key role in hepatocyte inflammatory response induced by CpG-ODNs (Lim et al. 2006, 2007; Osawa et al. 2006). In this study, we designed a CpG-ODN ligand to mimic virus DNA-induced activation of TLR9-associated intracellular signaling pathways and damage to L02 hepatocytes. Using this model, we investigated the effects of bicyclol on the activation of TLR9-associated signaling pathways and the production of pro-inflammatory cytokines and chemokines by hepatocytes. This study further explores the potential immuno-inhibitory effects of bicyclol on hepatitis and provides theoretical evidence for clinical applications.

2. Investigations and results

2.1. Bicyclol inhibits CpG-ODN-induced cytokine (TNF- α and IL-18) and chemokine (Rantes, MCP-1 and MIP-1 α) productions by L02 hepatocytes

The data show that CpG-ODN can stimulate TNF- α and IL-18 production by L02 hepatocytes. The secretion of these two cytokines are obviously increased in CpG-ODN-stimulated cells compared to the control group ($p < 0.01$). Pre-treatment with bicyclol inhibited TNF- α and IL-18 secretion in CpG-ODN-stimulated cells in a dose dependant manner ($r = 0.973$ and 0.937) (Fig. 1A). In addition, bicyclol shows a more potent inhibitory effect on IL-18 secretion. In the ODN + S3 group, 0.5 mM Bicyclol had the most potent inhibitory effects on CpG-ODN-induced TNF- α and IL-18 secretion, and IL-18 secretion was decreased to a level similar to that in the control groups (CTL and S3) ($p > 0.05$). TNF- α levels are less influenced by bicyclol compared to that of IL-18. In the ODN + S3 group, although the secretion of TNF- α is significantly inhibited, it is not reduced to the control levels apparent in the CTL and S3 groups ($p < 0.05$). Similarly, CpG-ODN can potentially induce the production of Rantes, MCP-1 and MIP-1 α in L02 hepatocytes, and pre-treatment with bicyclol inhibited the secretion of these cytokines into the culture media (Fig. 1B and C). Compared to that in the ODN-only group, 0.1 mM bicyclol significantly inhibited the secretion of both Rantes and MIP-1 α in a dose dependant manner (Rantes, $r = 0.973$; MIP-1 α , $r = 0.976$). Furthermore, 0.5 mM bicyclol shows more potent inhibitory effects on Rantes and MIP-1 α secretions but did not return their expressions to control levels. Among these three chemokines, MCP-1 secretion was most potentially inhibited by bicyclol, which, at the lowest dose of 0.01 mM, significantly inhibits MCP-1 secretion in CpG-ODN-stimulated L02 hepatocytes ($p < 0.05$). However, the inhibitory effect of Bicyclol on MCP-1 secretion is not dose dependent.

2.2. Bicyclol inhibits CpG-ODN-induced TLR-9 expression in L02 hepatocytes

As shown in Fig. 2, CpG-ODN (10 μ g/ml) treatment dramatically increases TLR9 expression in L02 hepatocytes compared to that of the blank control (CTL). This experiment was repeated three times, and the mean fluorescence intensities are not shown. The results indicate that pre-treatment with bicyclol inhibited CpG-ODN-induced TLR9 expression in a dose-dependent manner. The expression rate of TLR9 in the control group was

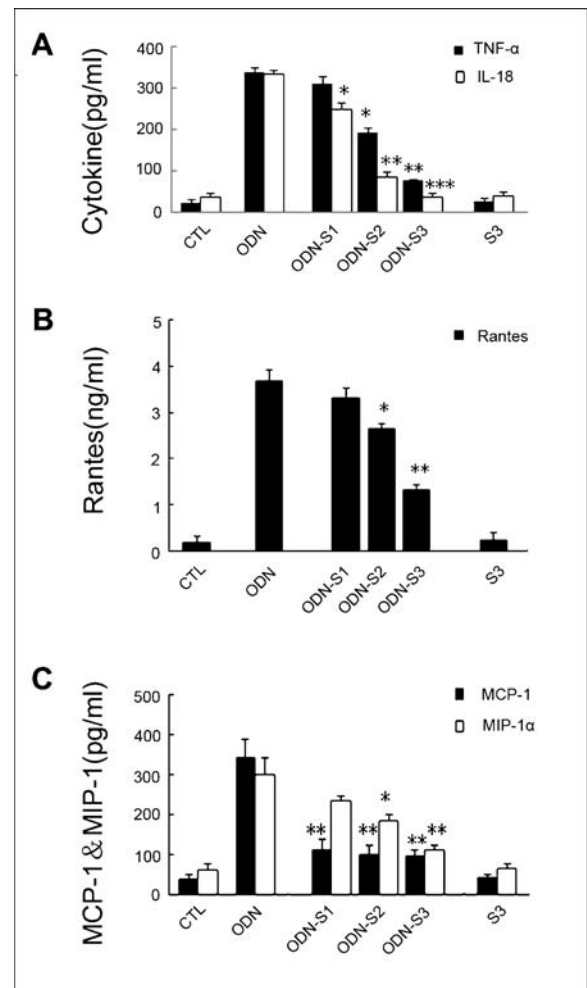


Fig. 1: Bicyclol inhibits CpG-ODN-induced cytokine and chemokine production by L02 hepatocytes.

L02 hepatocytes were pre-treated with a series of concentrations of bicyclol (0.01 mM, 0.1 mM and 0.5 mM) for 2 h, and co-incubated with 10 μ g/ml CpG-ODN (2006) for another 18 h. After the treatments, the levels of TNF- α , IL-18, MCP-1, MIP-1 α and Rantes in the culture media were analyzed with the ELISA method. CTL: Blank control group; S3: Bicyclol 0.5 mM; ODN: CpG-ODN (10 μ g/ml); ODN-S1: CpG-ODN (10 μ g/ml) + bicyclol (0.01 mM); ODN-S2: CpG-ODN (10 μ g/ml) + bicyclol (0.1 mM); ODN-S3: CpG-ODN (10 μ g/ml) + bicyclol (0.5 mM). (A) TNF- α and IL-18 productions in L02 (pg/ml); (B) Rantes productions in L02 (ng/ml); (C) MCP-1 and MIP-1 α productions in L02 (pg/ml). Bicyclol pretreatment inhibited the production of each cytokine or chemokine in a dose-dependent manner (* $p < 0.05$, ** $p < 0.01$)

$9.90 \pm 5.30\%$. After CpG-ODN stimulation, the expression rate of TLR9 is significantly increased to $80.97 \pm 6.97\%$, which is almost 10 times that in the control group. On the other hand, 0.5 mM bicyclol alone only slightly up-regulates TLR9 expression compared to the control group (an increase that is not statistically significant). All three doses of bicyclol inhibit TLR9 expression in ODN-stimulated L02 hepatocytes and do so in a dose dependant manner. In particular, the expression rate of TLR-9 is decreased by 43.9% in the 0.01 mM bicyclol (ODN-S1) group, by 73.9% in the 0.1 mM bicyclol (ODN-S2) group and by 83.9% in the 0.5 mM bicyclol (ODN-S3) group to a level similar to that in the S3 group.

2.3. Bicyclol inhibits the activation of the MAPK signaling pathway in CpG-ODN-stimulated L02 hepatocytes

Western-blot analyses indicate that CpG-ODN-stimulation and bicyclol treatment do not have any effect on MAPK-p38 and

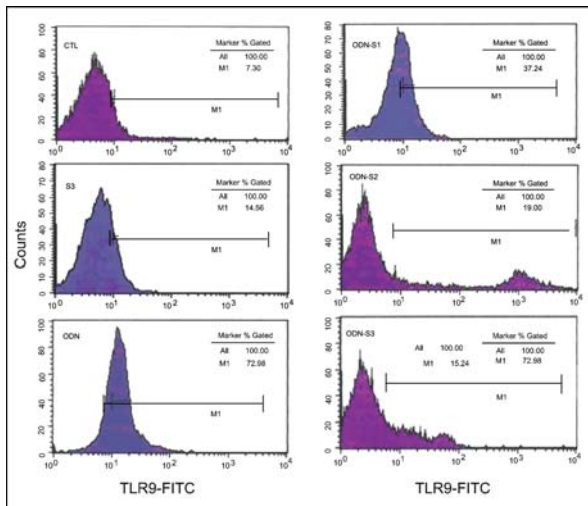


Fig. 2: Flow cytometry analysis of TLR9 expressions in L02 cells. L02 hepatocytes were incubated with CpG ODN (2006) and/or Bicyclol as described. TLR9 was marked with a FITC-labeled anti-TLR9 antibody for flow cytometry analysis of TLR9 expression. To evaluate the efficacy of the experiment, an isotype-matched control was used. At least 3000 cells were analyzed for each sample. CTL: Blank control group; S3: Bicyclol 0.5 mM; ODN: CpG-ODN (10 μ g/ml); ODN-S1: CpG-ODN (10 μ g/ml) + Bicyclol (0.01 mM); ODN-S2: CpG-ODN (10 μ g/ml) + Bicyclol (0.1 mM); ODN-S3: CpG-ODN (10 μ g/ml) + Bicyclol (0.5 mM)

ERK1/2 protein levels. Nevertheless, CpG-ODN-stimulation dramatically increases the phosphorylation levels of both proteins (Fig. 3). In addition, western-blot results show that bicyclol down-regulates the phospho-p38 level elevated by CpG-ODN stimulation. The phospho-p38 level in the high-dosage bicyclol (ODN-S3) group is decreased to a level similar to that in the control groups. ERK1/2 proteins show very little phosphorylation in normal L02 hepatocytes in the CTL and S3 control groups. However, upon CpG-ODN stimulation, phospho-ERK1/2 levels are greatly up-regulated and are also inhibited by bicyclol in a dose-dependent manner (Fig. 3A).

FACE analysis indicates that, upon CpG-ODN stimulation, the ratio of phosphorylated (activated) MAPK-p38 (phospho-p38/p38 %) was increased to $89.95 \pm 5.87\%$, which is 6.988 and 7.591 times that in the control group's CTL and S3, respectively. These differences show great statistical significance ($p < 0.01$). In addition, all three doses of bicyclol can inhibit p38 activation in CpG-ODN-stimulated L02 hepatocytes. Compared to the ODN group, median and high doses of bicyclol inhibited the phospho-p38/p38 ratio to $37.64 \pm 1.29\%$ and $23.82 \pm 4.03\%$, respectively ($p < 0.05$) (Fig. 3B). The FACE result is consistent with that of the western-blot analysis, which indicates that bicyclol inhibits the activation of p38 and ERK1/2 in CpG-ODN-stimulated L02 hepatocytes. As p38 and ERK1/2 are important transcription factors in the MAPK signaling pathway, which plays a pivotal role in regulating inflammatory factor production, this finding suggests that the inhibitory effects of bicyclol on cytokine and chemokine secretions in CpG-ODN-stimulated L02 hepatocytes might be associated with a suppression of the activation of the MAPK signaling pathway.

2.4. Bicyclol inhibits NF- κ B signaling pathway activation in CpG-ODN-stimulated L02 hepatocytes

As shown in Fig. 4, NF- κ B-p65 is slightly phosphorylated in the control L02 cells, and CpG-ODN stimulation dramatically promotes the phosphorylation of NF- κ B-p65 (p-p65). Bicyclol alone hardly influences phospho-p65 levels in the L02 cells in the absence of a CpG-ODN treatment but obviously inhibits phospho-p65 levels in CpG-ODN-stimulated L02

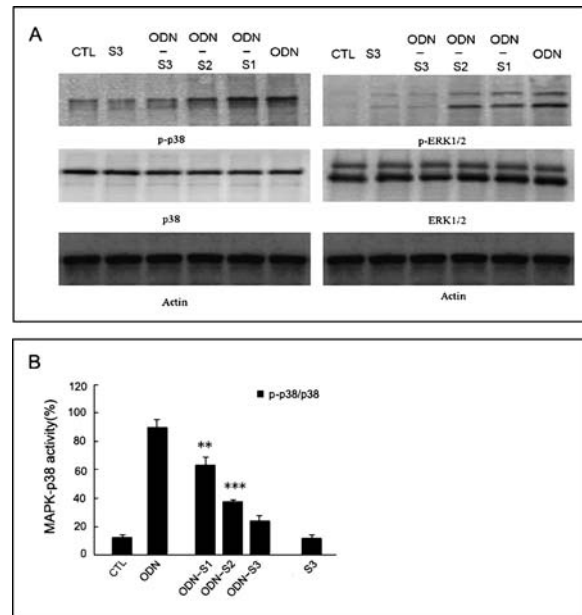


Fig. 3: Bicyclol inhibits CpG-ODN-induced activation of the MAPK signaling pathway in L02 hepatocytes.

L02 hepatocytes were pre-treated with a series of concentrations of bicyclol (0.01 mM, 0.1 mM and 0.5 mM) for 2 h, and co-incubated with 10 μ g/ml CpG-ODN (2006) for another 18 h. CTL: Blank control group; S3: Bicyclol 0.5 mM; ODN: CpG-ODN (10 μ g/ml); ODN-S1: CpG-ODN (10 μ g/ml) + bicyclol (0.01 mM); ODN-S2: CpG-ODN (10 μ g/ml) + bicyclol (0.1 mM); ODN-S3: CpG-ODN (10 μ g/ml) + bicyclol (0.5 mM).

(A) After the treatments, the phosphorylation levels of MAPK-p38 and Erk1/2 were analyzed by western blot;

(B) The activities of MAPK-p38 (p-p38/p38) were determined with the Fast Activated Cell-based ELISA (FACE) Kit. Bicyclol pretreatment inhibited MAPK-p38 activation in a dose-dependent manner ($*p < 0.05$, $**p < 0.01$)

cells (Fig. 4A). The inhibitory effect of bicyclol increases with increasing dose.

FACE analysis also indicates that upon CpG-ODN stimulation, the ratio of phosphorylated (activated) p65 (phospho-p65/p65 %) is increased to $54.58 \pm 2.13\%$, which is 4.856 and 4.264 times that in the control CTL and S3 groups, respectively. These differences are statistically significant ($p < 0.01$). In addition, all three doses of bicyclol inhibit the phospho-p65/p65 ratio in CpG-ODN-stimulated L02 hepatocytes. Compared to the ODN group, median and high doses of bicyclol inhibit the phospho-p65/p65 ratio to $27.48 \pm 1.26\%$ and $15.27 \pm 4.19\%$, respectively ($p < 0.05$) (Fig. 4B). This result is consistent with that of the western-blot analysis, which indicates that bicyclol attenuates the activation of the nuclear transcription factor p65 in CpG-ODN-stimulated L02 hepatocytes. As p65 plays an important role in regulating the production of inflammatory factors, this finding suggests that the inhibitory effects of bicyclol on cytokine and chemokine secretions in CpG-ODN-stimulated L02 hepatocytes might be associated with a suppression of p65 activation.

3. Discussion

The Toll-like receptor (TLR) family consists of phylogenetically-conserved transmembrane pattern recognition receptors (PRRs) that function as mediators of innate immunity to recognize pathogen-derived ligands (pathogen associated molecular patterns, PAMPs) and to activate downstream effectors via the Toll/IL-1R signaling pathway (Aderem and Ulevitch 2000; Akira et al. 2001; Hemmi et al. 2000). To date, TLR9 (one of 13 TLRs) is found to recognize the CpG motif of bacterial and viral DNA and to activate the NF- κ B and MAPK signaling pathways through binding to the adaptor protein myeloid differentiation protein 88 (MyD88). Activation of the NF- κ B and

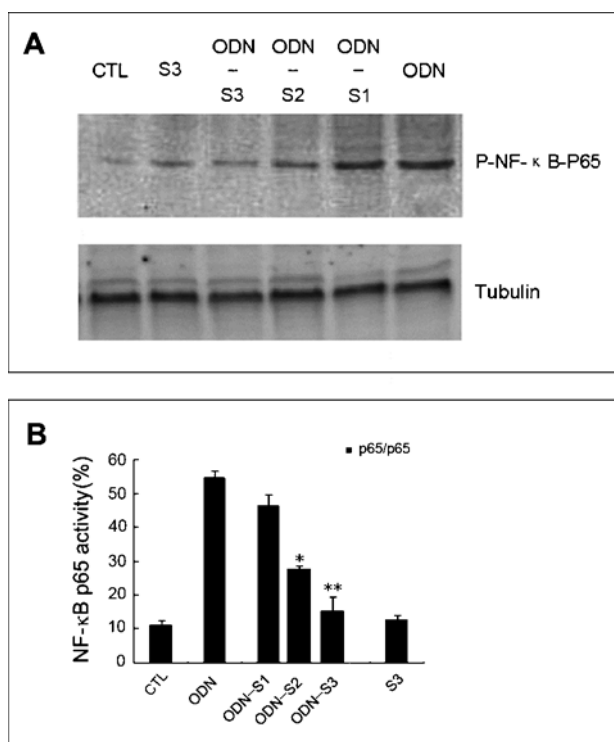


Fig. 4: Bicyclol inhibits CpG-ODN-induced activation of the NF- κ B signaling pathway in L02 hepatocytes.

L02 hepatocytes were pre-treated with a series of concentrations of bicyclol (0.01 mM, 0.1 mM and 0.5 mM) for 2 h and co-incubated with 10 μ g/ml CpG-ODN (2006) for another 18 h. CTL: Blank control group; S3: Bicyclol 0.5 mM; ODN: CpG-ODN (10 μ g/ml); ODN-S1: CpG-ODN (10 μ g/ml) + bicyclol (0.01 mM); ODN-S2: CpG-ODN (10 μ g/ml) + bicyclol (0.1 mM); ODN-S3: CpG-ODN (10 μ g/ml) + bicyclol (0.5 mM).

(A) After the treatments, the phosphorylation levels of NF- κ B-p65 were analyzed by western blot;

(B) The activities of NF- κ B-p65 (p-p65/p65) were determined with the Fast Activated Cell-based ELISA (FACE) Kit. Bicyclol pretreatment inhibited NF- κ B activation in a dose-dependent manner (* $p < 0.05$, ** $p < 0.01$)

MAPK signaling pathways then leads to the transactivation of many cytokines, such as IFN- γ , IL-2, IL-12 and IL-18, which elicits Th1-type cell-mediated immunity (Akira 2003; Hacker et al. 2000; Klinman et al. 1996).

CpG-ODN is a synthetic oligodeoxyribonucleotide sequence that can mimic the immunostimulatory effects of the non-methylated CpG motifs in viral and bacterial DNA (Bauer et al. 2001; Krieg et al. 1995). It is specifically recognized by TLR9, which can initiate several intracellular signaling pathways to produce and secrete cytokines and other immune molecules that elicit innate and adaptive immunity (Akira 2003; Hacker et al. 2000; Klinman et al. 1996; Roman et al. 1997). Although the effects of TLR9 on hepatitis B pathogenesis are not fully revealed (Xu et al. 2008), a large number of studies have indicated that the activation of the TLR9 signaling pathway can induce the production and secretion of pro-inflammatory cytokines and chemokines, which elicit a Th1 immunity and lead to a local inflammatory response. Small doses of CpG-ODN can induce Th1 cytokine release by monocytes and macrophages and subsequent B cell proliferation and T cell activation (Bjersing et al. 2005; Li et al. 2005; Marshall et al. 2006; Yoshinaga et al. 2007). Thus, they can be used in immunotherapies to treat infections, allergies, immunodeficiency diseases and tumors and be used as immunoadjuvants (Dalpke and Heeg 2004; Ishii et al. 2004; Kline 2007; Zimmermann et al. 2008). However, high doses of CpG-ODN can cause systemic inflammatory response syndrome (SIRS) and pyemia (Li et al. 2008; Wang et al. 2007). ODNs are also reported to induce hepatitis and

even cause liver damage (Abe et al. 2005; Sacher et al. 2002; Yi et al. 2006). Collectively, these results indicate that CpG-ODN can mimic the immunostimulatory effects of bacterial DNA to elicit Th1 type immune responses in the mammalian immune system (Dalpke et al. 2002).

In the present study, we investigated the inhibitory effects of bicyclol on the inflammatory response of L02 hepatocytes and the related molecular mechanisms and downstream signaling pathways that follow from this inhibitory effect. Firstly, CpG-ODN stimulation potentially up-regulates TLR-9 expression, activates the MAPK and NF- κ B signaling pathways and induces the production of IL-18, TNF- α , MCP-1, MIP-1 α and Rantes in L02 hepatocytes. This result suggests that viral DNA can stimulate hepatocytes through up-regulation of TLR-9 signaling pathway-mediated cytokine and chemokine secretion. Secondly, bicyclol pretreatment significantly inhibits the secretion of pro-inflammatory factors. Finally, the anti-inflammatory effects of bicyclol are partly mediated through the TLR-9-MAPK/NF- κ B signaling pathways.

It has been proven that pro-inflammatory cytokines and chemokines play important roles in the pathogenesis of viral hepatitis. CC chemokines, especially CCL3 (MIP-1 α), CCL4 (MIP-1 β) and CCL5 (RANTES), can recruit monocytes, eosinophils granulocytes, basophile granulocytes and T cells to sites of infection and injury. These factors are indispensable in the recruitment, differentiation, localization and activation of Th1 cytokine-secreting helper T cells and cytotoxic T lymphocytes (Gutierrez-Reyes et al. 2007; Loomba and Liang 2007). However, the aberrant expression of chemokines can induce excessive inflammatory responses. Recent studies found that intrahepatic chemokine signaling plays a key role in regulating significant pathological events during chronic hepatitis C (Apolinario et al. 2002; Promrat et al. 2003). In addition, liver chemokine expression levels are closely correlated with the degree of inflammatory activity in chronic hepatitis B (CHB) (Cheong et al. 2007; Duan et al. 2005; Masihi 2006). Therefore, the ability to alleviate the inflammatory damages caused by excessive cytokine and chemokine activation will be helpful to prevent the aggravation of clinical hepatitis. We found that 10 μ g/ml CpG-ODN stimulation greatly increased the secretion of cytokines (IL-18 and TNF- α) and chemokines (MCP-1, MIP-1 α and Rantes) by the L02 hepatocytes into the culture media. Nevertheless, pre-treating L02 hepatocytes with 0.01, 0.1 or 0.5 mM Bicyclol significantly inhibits the stimulatory effects of CpG-ODN on cytokine and chemokine secretions in a dose-dependent manner. These results provide evidence that bicyclol has a potent inhibitory effect on cytokine and chemokine secretions in CpG-ODN-stimulated L02 hepatocytes.

Furthermore, the aberrant expression of cytokines and the over-activation of the TNF- α -mediated inflammatory response are primary causes of severe hepatitis B (Tilg et al. 2006; Zou et al. 2009). It was also reported that IL-18 and TNF- α are key factors that induce hepatocyte toxicity (Falasca et al. 2006). These factors are closely related to hepatocyte necrosis and hepatitis. Their expression levels are positively correlated with the severity of liver damage and could thus reflect the degree of liver damage (Dinarello 2000; Lebel-Binay et al. 2000; Tsutsui et al. 2000). It was also reported that bicyclol could inhibit ConA- and LPS-induced hepatitis through down-regulating serum and intrahepatic TNF- α levels and repressing lymphocyte factor interferon- γ (IFN γ) expression (Li et al. 2004; Wang et al. 2006). Consistent with previous research, we found that L02 hepatocytes respond directly to CpG-ODN stimulation and produce more cytokines (IL-18 and TNF- α) and chemokines (MCP-1, MIP-1 α and Rantes) as a result. In addition, pre-treatment with bicyclol attenuates this stimulatory effect of CpG-ODN. This finding suggests that the protective effect of bicyclol

Table: Grouping and Treatments

Group	Descriptions	Treatment (final concentration)
CTL	Blank control group	-
S3	High dose Bicyclol control group	Bicyclol 0.5 mM
ODN	CpG-ODN stimulation group	CpG-ODN (10 µg/ml)
ODN-S1	ODN + Bicyclol group	CpG-ODN (10 µg/ml) + Bicyclol (0.01 mM)
ODN-S2	ODN + Bicyclol group	CpG-ODN (10 µg/ml) + Bicyclol (0.1 mM)
ODN-S3	ODN + Bicyclol group	CpG-ODN (10 µg/ml) + Bicyclol (0.5 mM)

on the liver might be partially mediated through attenuating the excessive secretions of pro-inflammatory factors by hepatocytes.

It is well known that the immunostimulatory activities of CpG-ODN are mediated by its specific TLR9 receptor that can activate a series of intracellular signaling pathways and induce an immune response. The NF- κ B and MAPK signaling pathways are two primary intracellular signaling pathways involved in CpG-ODN-stimulated immunity. In the NF- κ B signaling pathway, the activation of the NF- κ B transcription factor is based on the phosphorylation of p65 (Kawai and Akira 2007). In the MAPK signaling pathway, signals culminate in the phosphorylation and activation of three transcription factors, the extracellular signal regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (Alvarez et al. 2006). In this study, we find that the level of TLR9 expression and the levels of phospho-p65, phospho-ERK and phospho-p38 are significantly up-regulated in CpG-ODN-stimulated L02 hepatocytes, indicating that both the NF- κ B and MAPK signaling pathways are activated. This result is consistent with previous reports (Lim et al. 2006; Liu et al. 2002; Sato et al. 2007). With this model, we found that bicyclol significantly inhibits the CpG-ODN-dependent up-regulation of TLR9 expression and the CpG-ODN-dependent activation of the p65, p38 and ERK1/2 transcription factors. A recent study reported by Liu, et al. (2005) also found that bicyclol could protect the liver from ConA-induced damage, which might be associated with an inhibition of NF- κ B signaling. Together, these results suggest that the anti-inflammatory effects of bicyclol are mediated through regulation of intracellular signaling pathways (Li et al. 2004). These findings suggest that bicyclol might function to interfere with CpG-ODN immunostimulatory signals through modulating receptor expression and transcription factor activation. In this way, bicyclol can prevent CpG-ODN stimulation of hepatocytes and inhibit the over-secretion of cytokines and chemokines by hepatocytes to protect hepatocytes from inflammatory damage. In addition to modulating their expression, bicyclol can also directly inhibit the secretion of cytokines and chemokines. However, whether the inhibitory effects of bicyclol on pro-inflammatory cytokine and chemokines secretion is mediated through inhibition of MAPK and NF- κ B signaling warrants further study.

Taken together, our findings demonstrate for the first time that bicyclol can attenuate the production of pro-inflammatory cytokines and chemokines in CpG-ODN-stimulated L02 hepatocytes by inhibiting TLR9-mediated intracellular signaling pathways, including the NF- κ B and MAPK pathways. However, as inflammation is cross-regulated by multiple signaling pathways, the CpG-ODN/TLR9 signaling pathway only accounts for one important part of inflammatory regulation. Whether bicyclol modulates other inflammatory signaling pathways might need further investigation. Regardless, this study provides evidence for the use of combined medications with Bicyclol and other drugs that regulate additional signaling pathways to achieve a synergistic control of hepatitis.

4. Experimental

4.1. Materials (CPG-ODN synthesis and bicyclol solution preparation)

Phosphorothioate-modified CPG-ODN 2006 sequence (5'-TCG TCG TTT TGT CGT TTT GTC GTT-3') was synthesized by Shanghai Sangon Biological Technology & Services CO., LTD. Bicyclol (MW: 390.34) was kindly provided by Prof. Gengtao Liu. It is a white crystalline solid with 99.9% purity and high lipid solubility (Beijing Union Pharmaceutical Factory). Bicyclol is dissolved in DMSO at a concentration of 39 mg/ml (0.1 mol/L), preserved in a 4 °C refrigerator, and diluted in culture media prior to use.

4.2. Cell culture and treatments

L02 human hepatocytes were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Cells were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a humidified CO₂ incubator (5% CO₂, 95% air). Cells at a confluence of 90% were digested with 2.5 g/L Trypsin (Gibco) for cell passage and subcultivation.

Aliquots of 1×10^6 exponential-phase L02 cells were seeded in each well of a 6-well plate (Falcon). After cell attachment, the original cell culture media was discarded, and the cells were pretreated with a series of concentrations of bicyclol (0~0.5 mM) in fresh media for 2 h. Next, the cells were treated with CpG-ODN at a final concentration of 10 µg/ml and incubated for another 18 h. After treatment, the cells and media were collected for further analysis. The grouping method is shown in Fig. 1. Three parallel wells were used for each treatment.

4.3. Enzyme-linked immunosorbent assay (ELISA)

The presence of proinflammatory cytokines (TNF- α and IL-18) and chemokines (Rantes, MCP-1 and MIP-1 α) in the cell culture media was detected with an ELISA kit (American R&D systems Company) according to the manual. The results were measured at 450 nm on an automatic microplate reader (Bio-Tek Elx800, America). The cytokine and chemokine levels in the sample media were calculated against the standard reaction curve.

4.4. Immunofluorescent staining and flow cytometry analysis of TLR9 expression

Cells were trypsinized, fixed with 4% paraformaldehyde (PFA) at 4 °C for 15 min, blocked with 2% BSA at room temperature (RT) for 30 min, incubated with mouse anti-human TLR9-FITC antibody (IMGEX, America) at RT for 1 h and analyzed with flow cytometry. After each step, the cells were centrifuged at 2000 rpm for 5 min and washed twice with $1 \times$ phosphate-buffered saline (PBS).

4.5. Protein extraction and Western-blot analysis

Cells were collected, lysed with $1 \times$ lysis buffer (20 mM Tris-HCl (pH7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM leupeptin and 1 mM PMSF) on ice for 30 min and centrifuged at 10000 rpm for 3 min. The supernatants were subjected to protein quantification using a Bradford Protein Assay Kit (Pierce), and the results were determined with a Beckman DU-640 Spectrophotometer. After quantification, 40 µg of proteins from each sample was mixed with $1 \times$ loading buffer and boiled for protein denaturation. For western-blot analyses, protein samples were separated by electrophoresis in 10% SDS-PAGE, transferred to a PVDF membrane (0.45 µm, Immobilon-P, Millipore) and blocked in a blocking buffer (5% defatted milk, 2% BSA and 0.05% Tween-20 in $1 \times$ PBS) at RT for 2 h. For detection of different protein products, the PVDF membrane was blotted with anti-phospho-p38 MAPK (p-p38), anti-p-38, anti-phospho-Erk1/2 (p-Erk1/2), anti-Erk1/2 (1:1000, Cell Signaling Technology) or anti-TLR-9 (1:500, IMGEX) antibody. Antibody incubations were performed in a

Hybrid at RT for 2 h. After incubation, the membrane was washed 5 min \times 3, incubated with HRP labeled secondary antibodies (1:5000, Santa Cruz) at RT for 1 h, extensively washed and developed with ECL reagents (Super-Signal West Dura Kit, Pierce). The images were captured by the automatic VersaDoc™ Model 5000 imaging system (Bio Rad). Actin was used as a loading control.

4.6. Nuclear protein extraction and Western-blot analysis

To determine the activity of the nuclear transcription factor NF- κ B, nuclear proteins were extracted with a Nuclear Extract Kit (Active Motif, America) and subjected to western-blot analysis with an anti-phospho-NF- κ B-p65 antibody (p-p65, 1:1000, Cell Signaling Technology). β -Tubulin was used as a loading control.

4.7. Analysis of the NF- κ B and p38-MAPK signaling pathways

The activities of NF- κ B-p65 and p38-MAPK were detected with the Fast Activated Cell-based ELISA (FACE) Kit (Active Motif, America) according to the manual. Based on the ELISA method, this kit provides the latest method for detecting endogenous protein phosphorylation. Briefly, cells were counted, seeded in a 96-well plate (8000 cells/per well) and treated as indicated in "Grouping and Cell Treatments". After an 18-h cultivation, cells were fixed with 4% PFA for 20 min, blocked with the blocking buffer for 1 h, washed and incubated with the diluted anti-phospho-NF- κ B p65 or anti-phospho-p38 antibody (40 μ l/per well) at 4 °C overnight. The next day, the cells were washed, incubated with the diluted HRP labeled secondary antibody (1:1000, 100 μ l/per well) at RT for 1 h, washed and developed with TMB substrate solution in the dark for 5–10 min. Next, 0.5 M H₂SO₄ was added to terminate the reaction. Finally, the OD value at A450 nm of each well was determined with an automatic microplate reader (Biotek-Elx800, America). Each OD value represents the activity of NF- κ B or p38 in the cells of each well.

4.8. Statistical analysis

Inter-group comparisons were analyzed by ANOVA variance analysis. Differences with a p value lower than 0.05 are considered statistically significant. A correlation coefficient is calculated with the linear regression method.

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