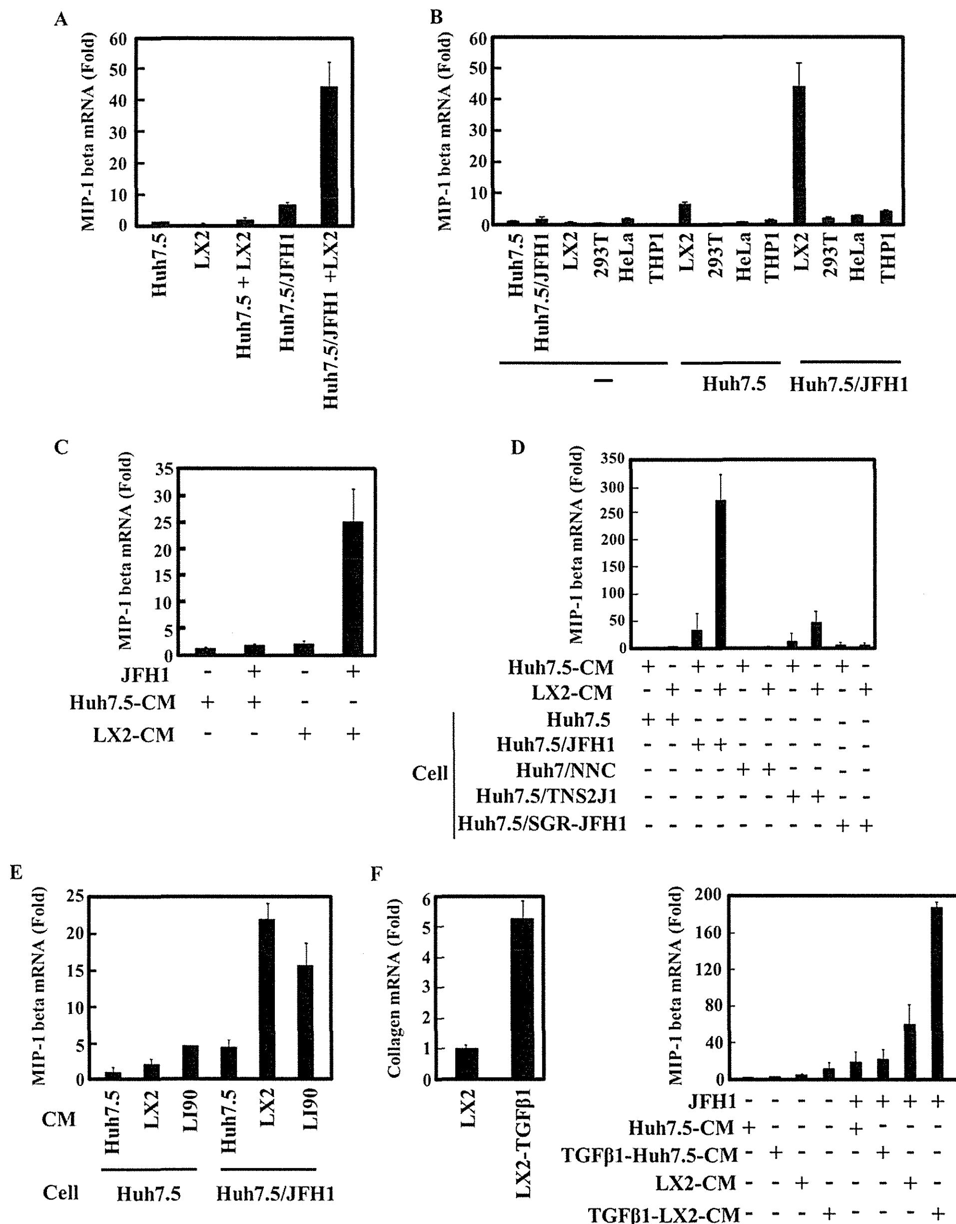

FIG 1 Conditioned medium from LX2 induces MIP-1β expression in JFH1-infected Huh7.5 cells. (A) Huh7.5 cells (1×10^5 cells) and JFH1-infected Huh7.5 (Huh7.5/JFH1) cells (1×10^5 cells) were cultured alone or in the presence of LX2 cells (1×10^5 cells) for 24 h. The level of MIP-1β was measured by qRT-PCR. Quantitative analysis of the PCR data was performed using the $2^{-\Delta\Delta CT}$ method, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) C_T values were used for normalization. The fold changes are relative to values for Huh7.5 cells. (B) Huh7.5 cells and JFH1-infected Huh7.5 cells (Huh7.5/JFH1) were cultured alone or in the presence of LX2, 293T, HeLa, or THP-1 cells for 24 h. The expression level of MIP-1β was measured by qRT-PCR as described for panel A. (C) Huh7.5 cells and JFH1-infected Huh7.5 cells were treated with conditioned medium from Huh7.5 (Huh7.5 CM) or LX2 (LX2 CM) cells for 24 h. The expression level of MIP-1β was measured by qRT-PCR as described for panel A. (D) Huh7.5, JFH1-infected Huh7.5 (Huh7.5/JFH1), Huh7/NNC, Huh7.5/TNS2J1, and Huh7.5/SGR-JFH1 cells were treated with CM from Huh7.5 or LX2 cells for 24 h. The expression level of MIP-1β was measured by qRT-PCR as described for panel A. (E) Huh7.5 and JFH1-infected Huh7.5 (Huh7.5/JFH1) cells were treated with Huh7.5 CM, LX2 CM, or LI90 CM for 24 h. The expression level of MIP-1β was measured by qRT-PCR as described for panel A. (F) LX2 cells were treated with 2.5 ng/ml TGF-β1 for 24 h, and then the level of collagen mRNA in these cells was determined by qRT-PCR (left). Huh7.5 or JFH1-infected Huh7.5 cells were treated with LX2 CM or TGF-β1-stimulated LX2 CM for 24 h. The expression level of MIP-1β was measured by qRT-PCR as described for panel A (right). The results are representative of three independent experiments, and the error bars represent the standard deviation of the means.

was significantly enhanced in JFH1-infected Huh7.5 cells after they were cocultured with LX2 cells (Fig. 1A, Huh7.5/JFH1+LX2). Importantly, MIP-1β expression was undetectable or very low in LX2 cells alone or in uninfected Huh7.5 cells cocultured with LX2 (Fig. 1A, LX2 and Huh7.5+LX2). Interestingly, increased MIP-1β expression in JFH1-infected Huh7.5 cells was specifically induced by cocultivation with LX2 cells. Cocultivation with other cell lines, such as 293T, HeLa, and THP-1, had no effect on MIP-1β expression in JFH1-infected Huh7.5 cells (Fig. 1B). These results suggest that Huh7.5 cells produce MIP-1β in re-

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sponse to HCV infection and that LX2 cells increase MIP-1β expression in HCV-infected Huh7.5 cells.

We next determined whether MIP-1β induction by LX2 cells in HCV-infected cells was mediated by a secreted soluble factor(s). Huh7.5 and JFH1-infected Huh7.5 cells were treated with conditioned medium (CM) from Huh7.5 or LX2 cells (Fig. 1C). As expected, Huh7.5 cells had no response to Huh7.5 CM or LX2 CM. However, we observed that LX2 CM, but not Huh7.5 CM, stimulated MIP-1β expression in JFH1-infected Huh7.5 cells. These results indicated that LX2 cells secrete a factor that stimulates MIP-1β expression.

To address whether MIP-1β stimulation after culturing with LX2 CM is dependent on HCV genotype or on the maintenance of the HCV replicon in cells, we used Huh7.5 cells that carry different types of the HCV genome. Huh7/NNC cells are Huh7 cells that contain the noninfectious full HCV genotype 1b, Huh7.5/SGR-JFH1 cells contain the subgenome replicon of JFH1, and Huh7.5/TNS2J1 cells have the infectious chimeric HCV genome, which consists of an HCV-1b-derived sequence in the structural-protein-coding region and a JFH-derived sequence in nonstructural-protein-coding region (Fig. 1D). In Huh7.5/TNS2J1 cells, MIP-1β expression was significantly induced by LX2 CM (47-fold), though there was a lower level of expression than what was observed in JFH1-infected cells (272-fold). By contrast, NNC and SGR-JFH1 had no effect on MIP-1β expression. These results demonstrate that LX2 CM-induced stimulation of MIP-1β expression may require a productive HCV infection (see also Fig. 6).

Because LX2 cells are a human hepatic stellate cell line that was established by immortalization, we confirmed our findings by using another human hepatic stellate cell line, LI90, which was derived from a human mesenchymal liver tumor (16). When uninfected or JFH1-infected Huh7.5 cells were treated with LI90 CM, MIP-1β expression was increased only in the JFH1-infected Huh7.5 cells. These results are similar to those found after addition of LX2 CM to JFH1-infected Huh7.5 cells (Fig. 1E).

Activated hepatic stellate cells play a critical role in inflammation, yet the functional impact they have on hepatocytes has not yet been determined. Therefore, we evaluated whether the activation of LX2 cells affects the expression of MIP-1β in JFH1-infected Huh7.5 cells. LX2 cells were treated with TGF-β1, and the mRNA expression of the collagen gene, a marker of HSC activation, was measured (Fig. 1F, left). LX2 CM from activated cells significantly enhanced MIP-1β expression in JFH1-infected Huh7.5 cells but not in uninfected Huh7.5 cells, compared to the increase with nonactivated LX2 CM (Fig. 1F, right). In parallel experiments, TGF-β1-treated Huh7.5 CM did not affect MIP-1β expression in Huh7.5 or JFH1-infected Huh7.5 cells.

The supernatant from JFH1-infected Huh7.5 cells cultured with LX2 CM induces migration of NP-2-CCR5 cells. MIP-1β is a physiological ligand for the CCR5 receptor. To test whether MIP-1β produced by HCV-infected hepatocytes that have been cultured with LX2 CM has this activity, we performed a chemotactic assay using NP-2-CCR5 cells, a human glioma-derived cell line expressing CCR5 on its cell surface (Fig. 2). The treatment of NP-2-CCR5 cells with supernatant from LX2 CM-stimulated JFH1-infected Huh7.5 cells increased their migration by 2-fold compared to treatment with supernatant from uninfected Huh7.5 cells treated with LX2 CM. This increase in NP-2-CCR5 cell migration was blocked with maraviroc (a CCR5 antagonist) treatment. These results indicated that LX2 CM induces secretion of a physiologically functional MIP-1β by JFH1-infected Huh7.5 cells.

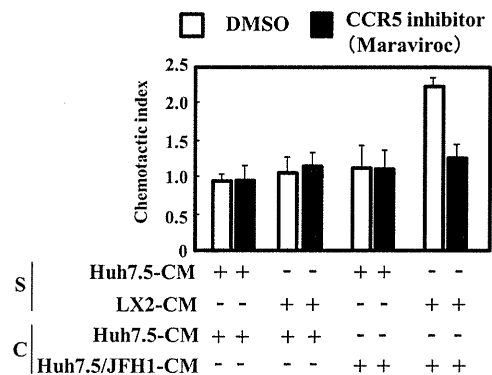

FIG 2 Conditioned medium from JFH1-infected Huh7.5 cells treated with LX2-conditioned medium induces chemotaxis of NP-2-CCR5 cells. Huh7.5 CM or LX2 CM was concentrated 60-fold using a 100,000-molecular-weight-cutoff membrane filter. Huh7.5 and Huh7.5/JFH1 cells were treated with each concentrated medium (S). After 24 h of treatment, the medium was changed to serum free DMEM for 24 h. The chemotactic activity of each conditioned medium (C) was determined by using 5 nM maraviroc (CCR5 inhibitor) and NP-2-CCR5 cells, as described in Materials and Methods. The results are representative of three independent experiments, and the error bars represent the standard deviations of the means. DMSO, dimethyl sulfoxide.

Identification of the factor in LX2 CM responsible for MIP-1β stimulation. We first fractionated culture medium of Huh7.5 or LX2 cells using a membrane filter which cut off the 100-kDa-molecular-mass marker protein and then collected the trapped and flowthrough fractions. As expected, MIP-1β expression in uninfected Huh7.5 or JFH1-infected Huh7.5 cells did not increase after treatment with the trap or the flowthrough fraction of the Huh7.5 CM (Fig. 3A). By contrast, the trap fraction of LX2 CM enhanced MIP-1β expression in JFH1-infected Huh7.5 cells, suggesting that the stimulator in the LX2 CM was enriched in the 100-kDa-molecular-mass-cutoff filter. To further analyze the trap fraction of LX2 CM, we created a cytokine antibody array (Fig. 3B). By analyzing 507 cytokines and chemokines in the array, we found four candidates (TSG-14, monocyte chemoattractant protein 2 [MCP-2], MCP-3, and IL-1α), which were more concentrated by the 100-kDa-molecular-mass-cutoff filter than by the flowthrough fraction of LX2 CM (Fig. 3B, compare 100K-Flow through to 100K-Trap). We tested the effects of these candidates on stimulation of MIP-1β expression (Fig. 4A). Although recombinant TSG-14, MCP-2, or MCP-3 did not stimulate MIP-1β expression in Huh7.5 cells or JFH1-infected cells, recombinant IL-1α induced MIP-1β expression in only JFH1-infected Huh7.5 cells. This effect was dose dependent (Fig. 4B). To evaluate whether IL-1α is required for MIP-1β stimulation in JFH1-infected Huh7.5 cells by LX2 CM, we used a neutralizing antibody against IL-1α and the IL-1 receptor antagonist. LX2 CM stimulated MIP-1β expression in JFH1-infected Huh7.5 cells, whereas anti-IL-1α and the IL-1 receptor antagonist (IL-1RA) blocked MIP-1β stimulation (Fig. 4C). Additionally, the neutralizing antibody against IL-1β had no effect (data not shown). It is not clear why IL-1α of about 30 kDa was concentrated into the trap fraction. However, it is likely that IL-1α is formed at a large mass with other proteins in the culture medium to be contained. Moreover, knockdown of IRAK1, which is essential for the downstream sig-

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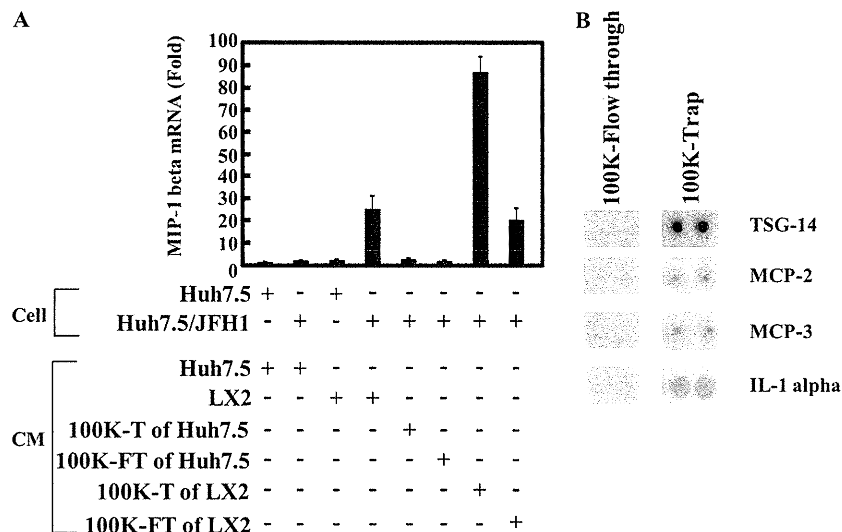

FIG 3 Identification of a factor(s) in the LX2 conditioned medium that is responsible for induction of MIP-1β expression. (A) Huh7.5 CM or LX2 CM was concentrated using a 100,000-molecular-weight-cutoff membrane filter. Huh7.5 and JFH1-infected Huh7.5 (Huh7.5/JFH1) cells were treated with each unconcentrated conditioned medium, with the flowthrough fraction (100K-FT), or with the trap fraction (100K-T). The MIP-1β expression level was analyzed by qRT-PCR as described for Fig. 1A. (B) LX2 CM was concentrated using a 100,000-molecular-weight-cutoff membrane filter. A cytokine antibody array (RayBiotech) was used for the simultaneous detection of 507 inflammatory factors. The antibody-coated membrane was incubated with the trap fraction (100K-Trap) or with the flowthrough fraction (100K-Flow through). Representative spots (TSG-14, MCP-1, MCP-3, and IL-1α) are shown.

nal of IL-1R, impaired the response to LX2 CM (Fig. 4D). These results suggest that IL-1α contributes to stimulation of MIP-1β expression by LX2 CM and that JFH1-infected Huh7.5 cells are highly sensitive to IL-1α. Furthermore, considering the molecular weight of IL-1α, it is possible that an unknown amount of IL-1α, undetectable by the cytokine antibody array, passed through the filter, which caused activation of MIP-1b by the 100,000-molecular-weight-cutoff flowthrough fraction. Alternatively, a factor(s) other than IL-1α in the 100,000-molecular-weight-cutoff flowthrough fraction might have been responsible for the activation.

The transcription factor C/EBPβ mediates LX2 CM-stimulated MIP-1β production. IL-1 is one of the most important proinflammatory cytokines and binds to the cell surface IL-1 type I receptor to activate downstream signaling pathways such as IKK–NF-κB, extracellular signal-regulated kinase (ERK), Jun N-terminal protein kinase (JNK), p38, and C/EBPβ. Moreover, the MIP-1β promoter contains a C/EBPβ motif located between bp −222 and −100, and the C/EBPβ promoter is required for a functional response to IL-1β in human chondrocytes (17). To evaluate whether LX2 CM-stimulated MIP-1β expression involves C/EBPβ stimulation, the level of C/EBPβ mRNA was measured in uninfected and HCV-infected cells. C/EBPβ expression was higher in Huh7.5/JFH1 and Huh7.5/TNS2J1 cells than in Huh7.5, Huh7/NNC, and Huh7.5/SGR-JFH1 cells (Fig. 5A). This result correlates with MIP-1β stimulation shown in Fig. 1D. Additionally, LX2 CM induced low levels of C/EBPβ expression in Huh7.5/JFH1 and Huh7.5/TNS2J1 cells; induction was likely caused by IL-1α (Fig. 5A). To further confirm that the enhancement of MIP-1β expression is mediated by C/EBPβ, we performed experiments where uninfected or JFH1-infected Huh7.5 cells were transduced with either a control or a C/EBPβ-specific siRNA and then treated with Huh7.5 CM or LX2 CM. Quantitative RT-PCR analysis demonstrated that the siRNAs targeting C/EBPβ significantly suppressed endogenous C/EBPβ expression (Fig. 5B). Depletion of C/EBPβ significantly reduced the MIP-1β expression stimulated by LX2 CM in JFH1-infected Huh7.5 cells. Residual stimulation of MIP-1β seems to be attributable to imperfect knockdown of C/EBPβ (70 to 80%). However, currently we cannot rule out the possibility of the involvement of other transcription factor(s) in the activation of MIP-1β expression. Furthermore, cytokines (IL-6, IL-8, CXCL2, CXCL1, MIP-1β, and MIP-1α) that were enhanced by C/EBPβ expression were upregulated by LX2 CM only in JFH1-infected Huh7.5 cells (Fig. 5D). There was no induction of C/EBPβ-independent cytokines (IL-7, CXCL6, CXCL5, IL-1α, and IL-1R) after the addition of LX2 CM. None of these cytokines were induced by LX2 CM in uninfected Huh7.5 cells. These data indicate that HCV stimulates C/EBPβ expression, which confers upon HCV-infected Huh7.5 cells the ability to produce proinflammatory cytokines in response to LX2 CM.

Early steps of the HCV life cycle trigger MIP-1β stimulation by LX2 CM. We observed that Huh7.5/TNS2J1 and Huh7.5/JFH1 cells, but not Huh7/NNC or Huh7.5/SGR-JFH1 cells, could respond to LX2 CM (Fig. 1D), suggesting that the production of infectious HCV is required for MIP-1β induction. However, it remains to be determined whether infectious HCV particles actually induce MIP-1β expression in the presence of LX2 CM. To address this question, we used JFH1-CL3B, which is a virus that is defective in the production of virus particles because of mutations in domain III of NS5A; however, the self-replication ability of its genome is normal (18). As shown in Fig. 6A, JFH1-CL3B genome-bearing Huh7.5 cells responded to neither Huh7.5 CM nor LX2 CM. These data suggest that productive infection of HCV is an essential event for MIP-1β induction. To characterize the mechanism that mediates LX2 CM-stimulated MIP-1β induction in HCV-infected cells, we studied the temporal kinetics of MIP-1β expression. In JFH1-infected Huh7.5 cells, MIP-1β expression did not increase in the first 2 h after infection but it started to increase after 4 h in the presence of LX2 CM (Fig. 6B). Furthermore,

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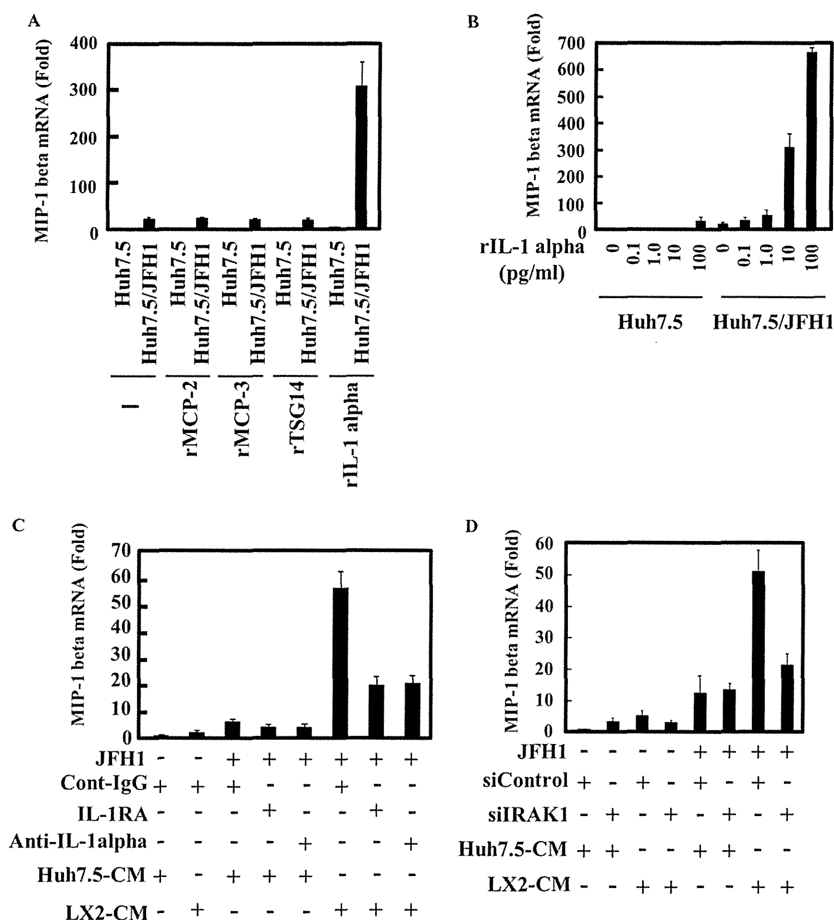

FIG 4 IL-1α induces MIP-1β expression in JFH1-infected Huh7.5 cells. (A) Huh7.5 and JFH1-infected Huh7.5 (Huh7.5/JFH1) cells were treated with recombinant MCP-2 (10 ng/ml), recombinant MCP-3 (rMCB-3; 10 ng/ml), recombinant TSG14 (10 ng/ml), or recombinant IL-1α (10 pg/ml) for 24 h. The level of MIP-1β expression was analyzed by qRT-PCR as described for Fig. 1A. (B) Huh7.5 and JFH1-infected Huh7.5 (Huh7.5/JFH1) cells were treated with various amounts of recombinant IL-1α (0, 0.1, 1.0, 10, or 100 pg/ml) for 24 h. The level of MIP-1β expression was analyzed by qRT-PCR as described for Fig. 1A. (C) JFH1-infected Huh7.5 cells were treated with Huh7.5 CM or LX2 CM along with an isotype control (0.2 μg/ml), anti-IL-1α (0.2 μg/ml), or IL-1RA (100 μg/ml) for 24 h. The level of MIP-1β expression was analyzed by qRT-PCR as described for Fig. 1A. (D) Huh7.5 and Huh7.5/JFH1 cells were transfected with 50 nM control siRNA (siControl) or 50 nM IRAK1 siRNA (siIRAK1). At 48 h after transfection, cells were treated with Huh7.5 CM or LX2 CM. The level of MIP-1β expression was analyzed by qRT-PCR as described for Fig. 1A. The results are representative of three independent experiments, and the error bars represent the standard deviations of the means.

MIP-1β induction in Huh7.5/JFH1 cells that were cultured in the presence of LX2 CM was suppressed by pretreatment of the cells with a neutralizing antibody against anti-CD81. To further address whether MIP-1β induction by LX2 CM in Huh7.5/JFH1 cells is due to the binding of HCV E1/E2 to the receptor, JFH1-infected cells were UV irradiated and used to infect Huh7.5 cells. Huh7.5 cells infected with UV-irradiated JFH1 did not show MIP-1β induction stimulated by LX2 CM (Fig. 6D). In addition, ectopic expression of HCV proteins in Huh7.5 cells did not respond to the LX2 CM (Fig. 6E). These results suggest that MIP-1β expression is required for HCV infection at postentry.

DISCUSSION

HCV-specific CTLs are concentrated within the liver during chronic infection, and they may control viral replication and contribute to progressive liver disease (19). Recruitment of T cells to the liver is required for the expression of CCR5 on activated T cells (20). Intrahepatic expression of the ligands for CCR5 (RANTES, MIP-1β, and MIP-1α) is elevated in HCV patients, and these chemokines have been linked to a high degree of liver inflammation (21). However, the mechanism mediating the stimulation of these cytokines is unclear. In this study, we demonstrated that cross talk between HCV-infected hepatocytes and human stellate cells (HSCs) induced inflammatory cytokines and chemokines. Importantly, uninfected hepatocytes are tolerant of HSC stimulation. Of note, HCV has no direct effect on LX2 cells with regard to MIP-1β stimulation because MIP-1β expression in Huh7.5/JFH1 cells was induced by treatment with CM from JFH1-treated LX2 cells as well as LX2 CM.

We identified IL-1α as an inducer of cytokines and chemokines in HCV-infected hepatocytes. These cells are highly sensitive to IL-1α (Fig. 4). Previous reports have indicated that the HCV core and NS3 proteins, which induced IL-1 receptor-associated kinase (IRAK) activity, triggered inflammatory cell activation through TLR-2 (22). IRAK activation by HCV infection may contribute to the induction of cytokines and chemokines by HSCs. We addressed this point and found that knockdown of IRAK1 in HCV-

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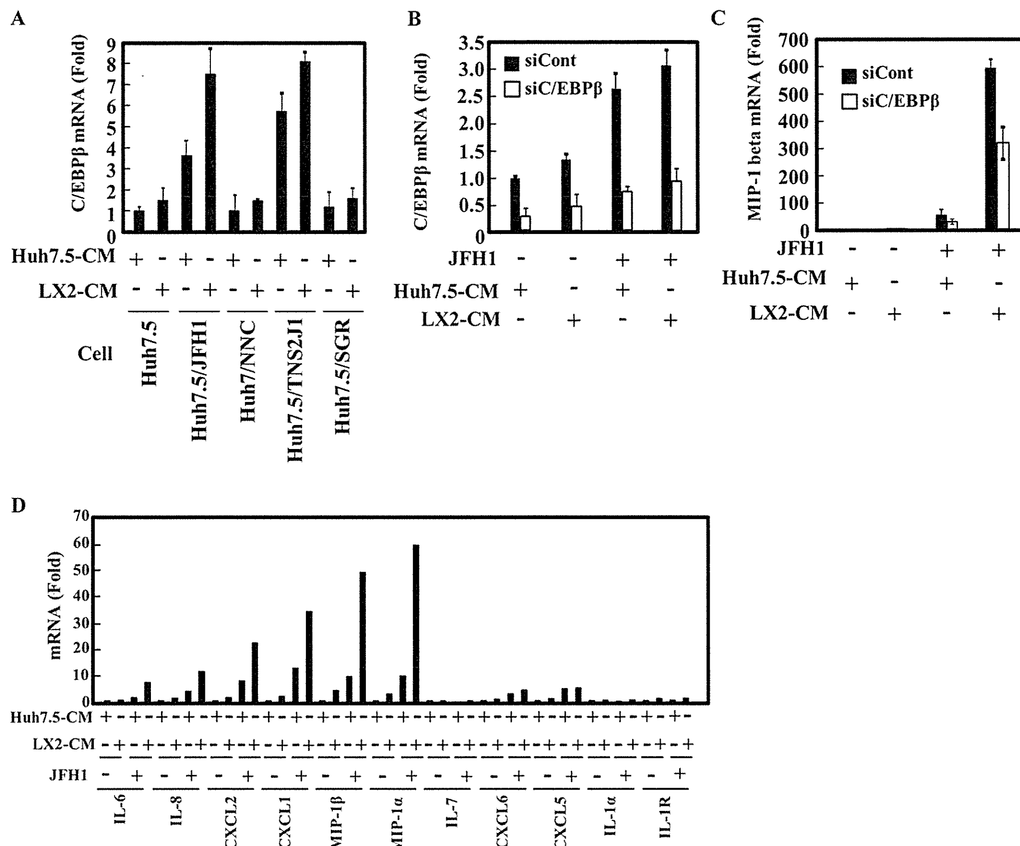

FIG 5 Conditioned medium from LX2 induces C/EBPβ-mediated proinflammatory gene expression in JFH1-infected Huh7.5 cells. (A) The indicated cells were treated with Huh7.5 CM or LX2 CM for 24 h. The level of C/EBPβ expression was analyzed by qRT-PCR as described for Fig. 1A. (B and C) Huh7.5 cells and JFH1-infected Huh7.5 cells were transfected with 50 nM control siRNA or siC/EBPβ. After 24 h of transfection, the cells were treated with Huh7.5 CM or LX2 CM for 24 h. The levels of C/EBPβ (B) and MIP-1β (C) expression were analyzed by qRT-PCR as described for Fig. 1A. (D) Huh7.5 cells and JFH1-infected Huh7.5 cells were treated with Huh7.5 CM or LX2 CM for 24 h. The levels of the indicated cytokines and chemokines were measured by qRT-PCR, as described for Fig. 1A. The results are representative of three independent experiments.

infected hepatocytes indicated that the response to HSC CM was impaired (Fig. 4D). By contrast, another group reported that HCV NS5A inhibits recruitment of IRAK to MyD88, resulting in suppression of the TLR signaling pathways in macrophage cell lines that stably expressed HCV NS5A (23). Currently, there is no evidence to suggest that HCV infects macrophages or that macrophages uptake functional NS5A to modulate TLR signaling. These conflicting results may be explained by the differences in cell types.

Our results suggest that IL-1α produced from hepatic stellate cells contributes to the inflammation by HCV infection *in vivo*. Another report shows augmented expression of IL-1α, tumor necrosis factor alpha (TNF-α), and IL-2 in HCV-infected patients compared to controls (24). Further, IL-1α plays roles in the liver to induce the acute-phase response of inflammation and autoactivation of Kupffer cells (25). Brain tissue from HCV patients, which is positive for HCV RNA and virus proteins, showed a significantly higher level of IL-1α than HCV-negative controls. Higher levels of proinflammatory cytokines such as IL-1α, IL-1β, TNF-α, IL-12, and IL-18 correlate with neurocognitive dysfunction among HCV patients. These results suggest that IL-1α is an important factor of HCV-related inflammation (26).

The relative importance of C/EBPβ in the stimulation of IL-1-inducible genes, such as the MIP-1α, MIP-1β, and IL-6 genes, is still not fully understood. Our results indicate that HCV-infected cells, which exhibited a 3- to 5-fold enhancement of C/EBPβ mRNA expression compared to uninfected cells (Fig. 5A), responded to IL-1α for the induction of MIP-1β mRNA synthesis. MIP-1β expression required the presence of C/EBPβ because knockdown of C/EBPβ significantly decreased LX2 CM-induced MIP-1β expression (Fig. 5C). To further address the relationship between C/EBPβ and MIP-1β, we analyzed the levels of C/EBPβ and MIP-1β mRNAs in uninfected Huh7.5 and Huh7.5/JFH1 cells in the presence of IL-1α (1 to 100 pg/ml) (data not shown). Compared with Huh7.5 cells, C/EBPβ expression was modestly induced in Huh7.5/JFH1 cells (about 4-fold) in the presence of 100 pg/ml IL-1α. By contrast, MIP-1β expression in Huh7.5/JFH1 cells at this dose of IL-1α was strongly induced (300-fold). These results indicate that IL-1α-induced MIP-1β mRNA induction in Huh7.5/JFH1 cells cannot be fully explained by the slight increase of C/EBPβ mRNA expression. It is known that C/EBPβ is modified posttranslationally (27, 28). C/EBPβ is phosphorylated by the Ras/mitogen-activated protein kinase (Ras/MAPK) pathway (29, 30), and a Ca^{2+}/calmodulin-dependent protein kinase (31) or ERK1/ERK2 stimulates the transactivation potential of C/EBPβ (32). C/EBPβ in HCV-infected cells may also be activated by a

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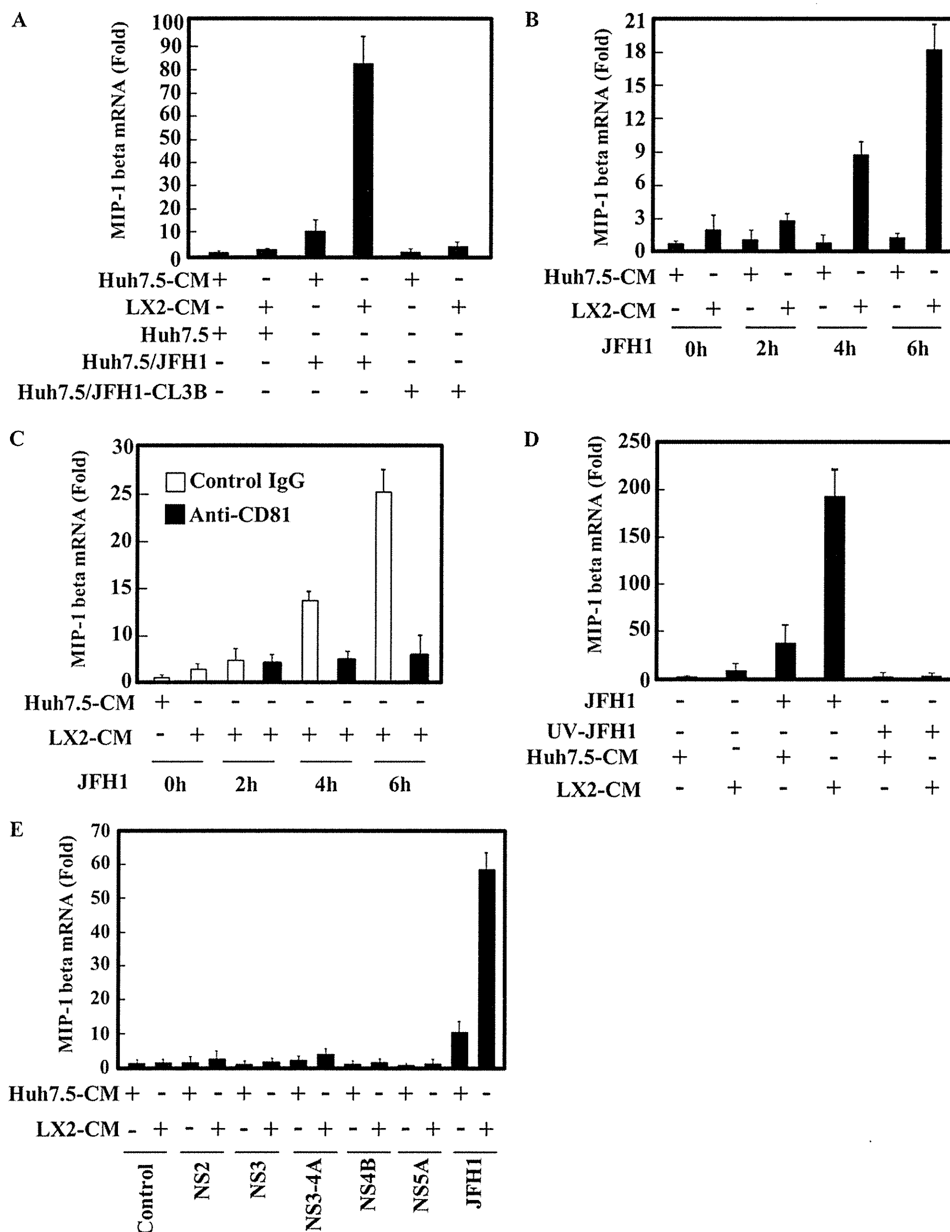

FIG 6 HCV entry is required for induction of MIP-1β expression by LX2 CM. (A) Huh7.5, JFH1-infected Huh7.5 (Huh7.5/JFH1), and JFH1-CL3B-infected Huh7.5 (Huh7.5/JFH1-CL3B) cells were treated with Huh7.5 CM or LX2 CM for 24 h. The level of MIP-1β expression was analyzed by qRT-PCR as described for Fig. 1A. (B) Huh7.5 cells were treated with Huh7.5 CM or LX2 CM. After 24 h of treatment, cells were infected with JFH1 for 2, 4, or 6 h. The level of MIP-1β expression was analyzed by qRT-PCR as described for Fig. 1A. (C) Huh7.5 cells were treated with Huh7.5 CM or LX2 CM in the presence of an isotype control IgG or anti-CD81 antibody (1 μg/ml). After 24 h of treatment, the cells were infected with JFH1 in the presence of an isotype control IgG or anti-CD81 antibody (1 μg/ml) for 2, 4, or 6 h. The level of MIP-1β expression was analyzed by qRT-PCR as described for Fig. 1A. (D) HCV JFH1 was UV irradiated and used to infect Huh7.5 cells. At 24 h after infection, cells were treated with Huh7.5 CM or LX2 CM. The level of MIP-1β expression was analyzed by qRT-PCR as described for Fig. 1A. (E) Huh7.5 cells expressing the indicated HCV proteins were treated with Huh7.5 CM or LX2 CM for 24 h. The levels of MIP-1β expression were analyzed by qRT-PCR as described for Fig. 1A. The results are representative of three independent experiments, and the error bars represent the standard deviations of the means.

posttranslational modification in response to IL-1 treatment. It is possible that activation of the MAPK (33) and ERK pathways (34) by HCV infection induces phosphorylation of C/EBPβ, leading to a highly sensitive IL-1 response. Further studies will be required to clarify the details of how HCV modulates increased MIP1-β production through both the C/EBPβ and IL-1 receptor signaling pathways.

This study revealed that induction of inflammatory cytokines and chemokines by cross talk between HSCs and HCV-infected hepatocytes primarily involves the activation of C/EBPβ-dependent gene transcription, such as transcription of the IL-6, IL-8, CXCL-1, and MIP-1β genes (Fig. 5). A recent study demonstrated that the HCV NS5A protein induces C/EBPβ expression (35). However, treatment of Huh7.5 cells that expressed NS5A with HCS

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CM did not induce MIP-1β expression significantly (Fig. 6E). We also observed only a marginal increase in MIP-1β mRNA level by solitary expression of each HCV protein in Huh7.5 cells by addition of LX2 CM (Fig. 6E).

Moreover, previous reports have suggested that ER stress, which is induced by HCV infection, leads to the generation of mature sterol regulatory element-binding protein 1 (SREBP-1) (36, 37) and that the mature SREBP-1c can bind to the C/EBPβ promoter (36, 38), leading to induction of C/EBPβ. Additionally, SREBP-1c promoter activity was upregulated in core-transgenic mice in a PA28γ-dependent manner (39). Indeed, we observed that endoplasmic reticulum (ER) stress-related genes and SREBP-1c expression were enhanced by HCV infection. However, induction of SREBP-1c is not involved in this process because HCV-infected cells that had SREBP-1c knocked down could still respond to HSC CM (data not shown).

Chronic inflammation is triggered by many events that also can increase the risk of developing cancer. For example, *Helicobacter pylori* is associated with gastric cancer, and inflammatory bowel disease is associated with colon cancer. In particular, IL-6 is one of the clinical targets for cancer-related inflammation (2). The level of IL-6 expression correlates with a rapid progression from hepatitis to hepatocellular carcinomas (40). The Ras and Jak/Stat pathway, which is downstream of the IL-6 receptor, is activated in hepatocellular carcinomas that have a poor prognosis (41). Additionally, IL-8 is a proinflammatory cytokine that specifically attracts and activates human neutrophils. In HCV patients, pegylated IFN-α-2a (PEG-IFN-α-2a) and ribavirin therapy decreased the neutrophil count in virologic responders compared to nonresponders (42). Enhancement of expression of these cytokines as well as MIP-1β, described here, suggests that the cross talk between HCV-infected hepatocytes and HSCs polarizes the cytokine profile toward a Th2-type immune response.

In conclusion, we have demonstrated that the cross talk between HSCs and HCV-infected hepatocytes results in the induction of inflammatory cytokines and chemokines, which promote the migration of CCR5-expressing cells in *in vitro* experiments. These results suggest that the induction of inflammatory cytokines and chemokines by HCV infection may recruit inflammatory cells such as cytotoxic T lymphocytes (CTL) and neutrophils to the liver, which induces liver cell injury leading to chronic hepatitis.

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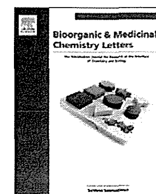

2′-Fluoro-6′-methylene-carbocyclic adenosine phosphoramidate (FMCAP) prodrug: In vitro anti-HBV activity against the lamivudine–entecavir resistant triple mutant and its mechanism of action

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ABSTRACT

Novel 2′-fluoro-6′-methylene-carbocyclic adenosine (FMCA) monophosphate prodrug (FMCAP) was synthesized and evaluated for its in vitro anti-HBV potency against a lamivudine–entecavir resistant clone (L180M + M204V + S202G). FMCA demonstrated significant antiviral activity against wild-type as well as lamivudine–entecavir resistant triple mutant (L180M + M204V + S202G). The monophosphate prodrug (FMCAP) demonstrated greater than 12-fold (12×) increase in anti-HBV activity without increased cellular toxicity. Mitochondrial and cellular toxicity studies of FMCA indicated that there is no significant toxicity up to 100 μM. Mode of action studies by molecular modeling indicate that the 2′-fluoro moiety by hydrogen bond as well as the Van der Waals interaction of the carbocyclic ring with the phenylalanine moiety of the polymerase promote the positive binding, even in the drug-resistant mutants.

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The chronic HBV infection is strongly associated with liver diseases like chronic hepatic insufficiency, cirrhosis and hepatocellular carcinoma (HCC).[1] According to the World Health Organization (WHO), currently about 2 billion people world-wide have been infected with HBV and more than 350 million live with chronic infection. Acute or chronic outcomes of HBV infection are estimated to cause the deaths of 600,000 people worldwide every year.[2]

Currently, there are several nucleos(t)ide analogues available to treat chronic hepatitis B virus infection.[3–6] The major target of these drugs is to inhibit the viral reverse transcriptase (RT)/DNA polymerase, which is responsible for the synthesis of the minus-strand DNA. Although the currently used agents are well tolerated and effective in suppressing the viral replication for extended periods, the significant rate of virological relapse caused by drug resistance remains a critical issue.

Lamivudine (LVD) was first introduced as the orally active anti-HBV agent in 1998. Lamivudine profoundly suppresses HBV replication in patients with chronic hepatitis B infection; however, lamivudine-resistant HBV (LVDr) was isolated from a significant numbers of patients during the treatment with lamivudine. Currently, there are several antiviral options exist for these patients viz., to use adefovir or high dose (1.0 mg/day) of entecavir, or more recently tenofovir. However, this resulted in also the development of resistance mutants during the long term therapy. At present, entecavir is the most prescribed drug, and is recommended for patients with the wild-type as well as for those harboring adefovir and lamivudine-resistant strains. However, recent clinical studies by Tanaka and his co-workers suggested that the entecavir mutant in the lamivudine-resistant patients (L180M + M204V + S202G) causes a viral breakthrough: 4.9% of patients at baseline increases to 14.6%, 24% and 44.8% at weeks 48, 96 and 144, respectively.[7] Therefore, it is of great interest to discover novel anti-HBV agent, which is effective against lamivudine- and entecavir-resistant triple mutants (L180M + M204V + S202G).

The potency of a nucleos(t)ide analogue is determined by its ability to serve as a competitive inhibitor of the HBV polymerase relative to that of the natural substrate, the nucleotide triphosphate.[8] However, host cellular kinases limit the pharmacological potency of nucleoside analogues by phosphorylation to their corresponding triphosphates. Particularly, the initial kinase action on the nucleoside to the monophosphate is the rate-limiting step. However, many synthetic nucleosides are not phosphorylated or the rate of phosphorylation is very slow due to the structural requirement of the kinases, resulting in only generating a low quantity of the triphosphate. To overcome this phosphorylation issue, nucleoside phosphoramidate prodrugs have been introduced,[8,9] which

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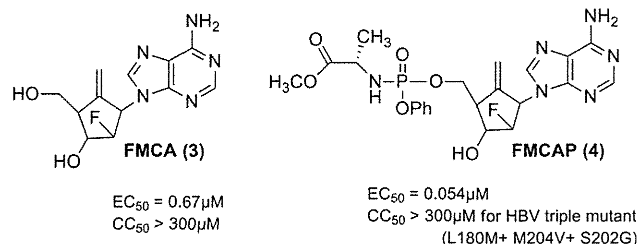


Figure 1. Structures of 2′-fluoro-6′-methylene-carbocyclic adenosine (FMCA; **3**) and its prodrug (FMCAP; **4**).

can bypass the rate-limiting first step of monophosphorylation. Phosphoramidate prodrugs have demonstrated to enhance the nucleoside potency in cell culture as well as in patients.[10,11] This methodology greatly increases the lipophilicity of the nucleotide to increase the cell penetration as well as to target the liver cells in vivo.

In this communication, we present that a FMCA phosphoramidate prodrug is such an agent, which can potentially be used for the treatment of patients who experience viral breakthrough due to the triple mutants caused by the use of lamivudine and entecavir.

In our previous report, we have demonstrated that the novel carbocyclic adenosine analog **3** (FMCA Fig. 1) exhibits significant anti-HBV activity against wild type as well as adefovir/lamivudine resistant strains.[12] The present study describes the synthesis and antiviral evaluation of a phosphoramidate of FMCA (FMCAP), which demonstrated the significantly improved in vitro potency. Additionally, we studied its mechanism of action how FMCA-TP can effectively bind to the HBV polymerase by molecular modeling and still exerts the antiviral activity against the lamivudine–entecavir triple mutant (L180M + M204V + S202G).

FMCAP (**4**, Scheme 1)[13] was synthesized using a known method in the literature,[14,15] in which the phosphorylation of phenol with phosphorus oxychloride generates phenyl dichlorophosphate **1**, which was coupled with L-alanine methyl ester in the presence of *tri*-ethyl amine in dichloromethane to give chlorophosphoramidate reagent **2**, which, in turn, was coupled with FMCA **3** in the presence of 1-methyl imidazole in THF to furnish the phosphoramidate **4** in good yield.

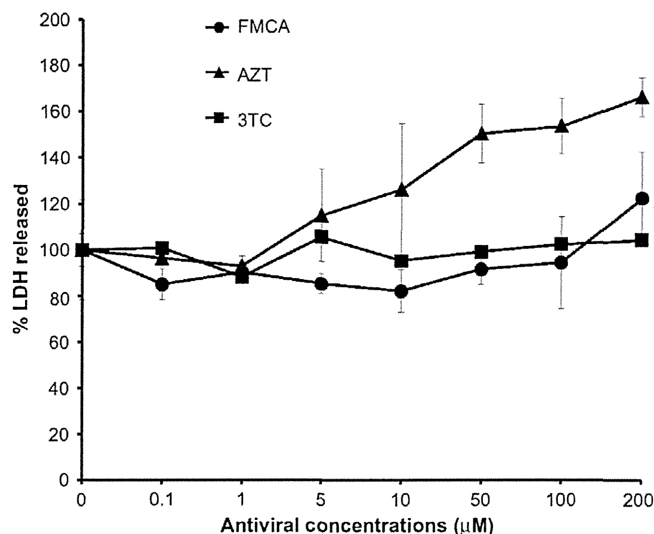
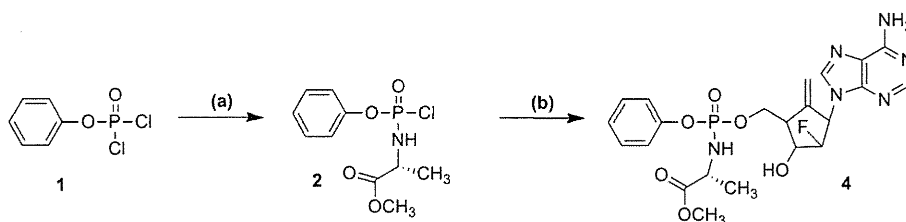


Figure 2. Mitochondrial toxicity of FMCA **3**, AZT and 3TC through lactate dehydrogenase release (LDH) assay.

FMCA **3** and FMCAP **4** were evaluated in vitro against the wild-type as well as the lamivudine–entecavir resistant clone (L180M + S202I + M202V). The FMCA **3** and FMCAP **4** demonstrated significant anti-HBV activity (EC_{50} 0.548 ± 0.056 & 0.062 ± 0.011 μM, respectively) against the wild-type virus, while lamivudine and entecavir also demonstrated potent anti-HBV activity (EC_{50} 0.056 ± 0.003 & 0.008 μM, respectively) (Table 1). It is noteworthy to mention that the anti-HBV potency of FMCAP (**4**) was increased to eight-fold (8×) in comparison to that of FMCA **3**, which indicates the importance of the initial phosphorylation of the nucleoside.

FMCA **3** and FMCAP **4** were further evaluated for their in-vitro antiviral potency against a lamivudine–entecavir resistant clone (L180M + M204V + S202G). It was observed that the anti-HBV potency of both FMCA **3** and FMCAP **4** (EC_{50} 0.67 & 0.054 μM, respectively) were maintained against the resistant clone, and furthermore, the anti-HBV activity of FMCAP **4** was enhanced a 12-fold (12×) with respect to that of FMCA without significant enhancement of cellular toxicity. It was also noteworthy to mention that the anti-HBV potency of entecavir against the mutant



Scheme 1. Reagent and conditions: (a) L-alanine methyl ester hydrochloride, Et_3N, CH_2Cl_2; (b) FMCA (3), NMI, THF, rt overnight.

Table 1
In vitro anti-HBV activity of FMCA **3**, FMCAP **4**, lamivudine and entecavir against wild-type and entecavir drug-resistant mutant (L180M + M204V + S202G) in Huh7 cells

Compounds	HBV Strains			
	Wild-type			L180M + M204V + S202G
	EC_{50} (μM)	EC_{90} (μM)	CC_{50} (μM)	EC_{50} (μM)
FMCA **3**	0.548 ± 0.056	6.0 ± 0.400	>300	0.67
FMCAP **4**	0.062 ± 0.011	0.46 ± 0.060	>300	0.054
Lamivudine	0.056 ± 0.003	0.142 ± 0.008	>300	>500[17]
Entecavir	0.008	0.033	28	1.20[16]

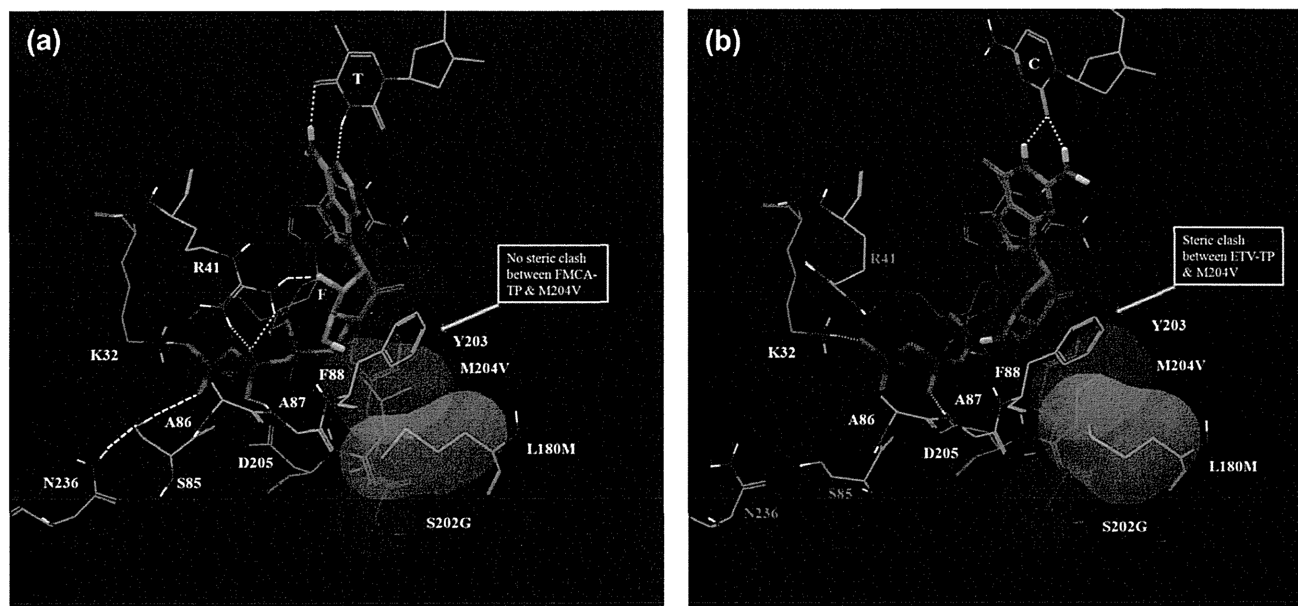


Figure 3. (a) FMCA-TP binding mode in ETVr (L180M + M204V + S202G); and (b) ETV-TP binding mode in ETVr (L180M + M204V + S202G) and there is a steric hindrance. Yellow dotted lines are hydrogen bonding interactions (<2.5 Å). The Van der Waals surface of L180M is colored yellow. The Van der Waals surface of M204V is shown in spring green. The Van der Waals surface of S202G is colored orange. The exocyclic double bond is shown blue color.

Table 2
MBAE (multi-ligand bimolecular association with energetics) calculation of FMCA-TP and ETV-TP after Glide XP docking[21] and energy minimization[22]

Strains	Compounds	Energy difference results (ΔE, kcal/mol)		
		Total energy	VdW[a]	Electrostatic
Wild-type	FMCA-TP	−588.05	375.78	−6341.08
	ETV-TP	−597.25	350.35	−6009.65
ETVr	FMCA-TP	−591.54	359.91	−6245.68
(L180M + M204V + S202G)	ETV-TP	−320.28	248.82	−4831.12

[a] Van der Waals interaction.

was reduced by 150-fold (EC_{50} 1.2 μM) in comparison to wild type.[16]

In the preliminary mitochondrial toxicity studies in HepG2 cells by measuring the lactic dehydrogenase release,[18] FMCA **3** did not exhibit any significant toxicity up to 100 μM like lamivudine (3TC), while azidothymidine (AZT) shows the increase of toxicity (Fig. 2).

In our previous report, we described molecular modeling studies for favorable anti-HBV activity of FMCA-TP in wild-type as well as in N236T adefovir resistant (ADVr) mutant.[12] In the current studies, it was of interest to know how the FMCA and its prodrug maintain the anti-HBV activity against ETVr triple mutant (L180M + M204V + S202G) in comparison to entecavir. Therefore, molecular modeling studies were conducted to obtain the insight of the molecular mechanism of FMCA-TP by using the Schrodinger Suite modules.[19] A previously described homology model was used to further explore the impact of the ETVr to the HBV-RT.[12] The homology model of HBV-RT was constructed based on the published X-ray crystal structure of HIV reverse transcriptase (PDB code: 1RTD).[20]

The binding mode of FMCA-TP and ETV-TP in ETVr (L180M + M204V + S202G) HBV-RT are depicted in Figure 3a and b, respectively. Their MBAE (multi-ligand biomolecular association with energetics)[22] calculations of FMCA-TP (total energy, wt −588.05 & ETVr −591.54 kcal/mol) and ETV-TP (total energy, wt −597.25 & ETVr −320.28 kcal/mol) after glide XP (extra precision) docking[21] and energy minimization in ETVr HBV-RT are shown in Table 2. The triphosphate of FMCA-TP forms all the network of hydrogen bonds with the active site residues (Fig. 3a), K32, R41, S85 & A87 in the similar manner as in wild-type,[12] whereas ETV-TP lose the hydrogen bonding with R41 & S85. The γ-phosphate of FMCA-TP maintains a critical H-bonding with the OH of S85 with connection of hydrogen bonds between S85 and N236 in ETVr HBV-RT also. However, γ-phosphate ETV-TP does not maintain this critical H-bonding with S85 and N236 (Fig. 3b).

The carbocyclic ring with an exocyclic double bond of FMCA-TP and ETV-TP makes the favorable Van der Waals interaction with F88 in ETVr HBV-RT (Fig. 3a and b). There is no steric clash in between exocyclic double bond of FMCA-TP and M204V residue, whereas ETV-TP exocyclic double bond has steric clash with M204V residue in ETVr HBV-RT. The 2′-fluorine substituent in the carbocyclic ring of FMCA-TP appears to promote an additional binding with the NH of R41 guanidino group as shown in Figure 3a, which is in agreement with the antiviral activity of FMCA-TP shown in Table 1. Overall, the modeling studies can qualitatively explain the favorable anti-HBV activity of FMCA-TP against ETVr mutant (L180M + M204V + S202G) in comparison to entecavir as shown in Table 1.

In conclusion, 2′-fluoro-6′-methylene-carbocyclic adenosine phosphoramidate prodrug (FMCAP) was synthesized, which demonstrated the significantly increased anti-HBV potency relative to the parent compound, FMCA in vitro. Molecular modeling studies delineated the mechanism of FMCA-TP and how it can effectively bind to the lamivudine–entecavir resistant triple mutant resulting

in maintaining the anti-HBV activity against the mutant. Furthermore, FMCA has been studied for the release of lactic dehydrogenase for potential mitochondrial toxicity and found that no significant increase of toxicity of FMCA compared with other commonly used anti-HIV nucleoside drugs. Very recently, a preliminary in vivo study in chimeric mice harboring the triple mutant, FMCAP was found to reduce HBV viral load while entecavir did not (data not shown). In view of these promising anti-HBV activities and non-toxicity of FMCAP as well as the interesting mechanism of antiviral activity, the chiral synthesis of FMCAP and its mitochondrial toxicity studies for preclinical investigation are warranted.

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13. *Compound **4***: ^{1}H NMR (500 Mz, *CDCl$_3$*) δ 8.35 (s, 1H), 7.86 (d, J = 3.0 Hz, 1H), 7.34–7.15 (m, 5H), 5.95 (m, 3H), 5.26 (d, J = 8.0 Hz, 1H), 5.01–4.90 (m, 1H), 4.83 (s, 1H), 4.50–4.41 (m, 2H), 4.25–4.04 (m, 3H), 3.71 (s, 3H), 3.07 (s, 1H), 1.40 (d, J = 6.5 Hz, 3 H); ^{19}F NMR (500 MHz, CDCl$_3$) δ −192.86 (m, 1F); ^{13}C NMR (125 MHz, *CDCl$_3$*) δ 171, 159.0, 156.5, 152.5, 150.4, 142.9, 130.1, 121.2, 120.3, 106.7, 102.4, 72.2, 71.1, 62.3, 51.9, 46.3, 43.9, 19.1; ^{31}P NMR (202 MHz, *CDCl$_3$*): δ 2.67, 2.99. Anal. Calcd For $C_{22}H_{26}FN_6O_6P.0.5H_2O$: C, 49.91; H, 5.14; N, 15.87; Found C, 49.84; H, 5.06; N, 15.22.
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Risk Factors for Long-term Persistence of Serum Hepatitis B Surface Antigen Following Acute Hepatitis B Virus Infection in Japanese Adults

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Abbreviations:

HBV, hepatitis B virus; AHB, acute hepatitis B; HIV, human immunodeficiency virus; HBeAg, hepatitis B e-antigen; NAs, nucleotide analogues; anti-HBc, antibody to hepatitis B core antigen; IgM, immunoglobulin M; HBsAg, hepatitis B surface antigen; anti-HBs, antibody to HBsAg; anti-HBe, antibody to HBeAg; CLIA, chemiluminescent enzyme immunoassay; EIA, enzyme immunoassay; ALT, alanine aminotransferase; RPHA, reverse passive hemagglutination.

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ABSTRACT

The proportion of patients who progress to chronicity following acute hepatitis B (AHB) widely varies worldwide. Moreover, the association between viral persistence after AHB and hepatitis B virus (HBV) genotypes in adults remains unclear. A nationwide multicenter study was conducted throughout Japan to evaluate the influence of clinical and virological factors on chronic outcomes in patients with AHB. For comparing factors between AHB patients with viral persistence and those with self-limited infection, 212 AHB patients without co-infection of HIV were observed in 38 liver centers until serum hepatitis B surface antigen (HBsAg) disappeared or a minimum of 6 months in cases where HBsAg persisted. The time to disappearance of HBsAg was significantly longer for genotype A patients than that of patients infected with non-A genotypes. When chronicity was defined as the persistence of HBsAg positivity for more than 6 or 12 months, rate of progression to chronicity was higher in patients with genotype A, although many cases caused by genotype A were prolonged cases of AHB, rather than chronic infection. Multivariate logistic regression analysis revealed only genotype A was independently associated with viral persistence following AHB. A higher peak level of HBV DNA and a lower peak of alanine aminotransferase (ALT) levels were characteristics of AHB caused by genotype A. Treatment with nucleotide analogues (NAs) did not prevent progression to chronic infection following AHB overall. Subanalysis suggested early NA initiation may enhance the viral clearance. ***Conclusions*****:** Genotype A was an independent risk factor for progression to chronic infection following AHB. Our data will be useful in elucidating the association between viral persistence after AHB, host genetic factors, and treatment with NAs in future studies.

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Hepatitis B virus (HBV) infection is one of the most common persistent viral infections in humans. Approximately 2 billion people worldwide have been exposed to HBV and 350 million of them remain persistently infected (1). The incidence of HBV infection and the patterns of transmission vary greatly worldwide among different population subgroups (2). In Western countries, chronic HBV infection is relatively rare and acquired primarily in adulthood via sexual transmission or the use of injectable drugs. Meanwhile, in Asia and most of Africa, the majority of infections are the result of transmission from an infected mother to her newborn. However, very few studies of acute hepatitis B (AHB) in adults have been reported.

HBV genomic sequences vary worldwide and have been classified into at least 8 genotypes (A through H) based on an intergroup divergence of 8% or more over the complete nucleotide sequence (3, 4). These genotypes have distinct geographic distributions (5-7). In particular, genotype A is predominant in North Western Europe, the United States, Central Africa, and India (8, 9). The Japanese have been infected with genotypes B and C since prehistoric times (10). Recently, many lines of evidence have revealed among the Japanese an increase in acute infection with HBV genotype A following sexual transmission (11, 12). As a result of this increasing transmission of genotype A, the distribution of HBV genotypes in Japan clearly differs among patients with acute and chronic infections (13). Moreover, recent studies suggest that acute infection with HBV genotype A may be associated with an increased risk of progression to persistent infection (15). Indeed, the prevalence of HBV genotype A in chronic hepatitis B patients doubled in Japan between 2000–2001 and 2005–2006 (1.7% versus 3.5%) (11).