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# Helioxanthin inhibits interleukin-1β-induced MIP-1β production by reduction of c-jun expression and binding of the c-jun/CREB1 complex to the AP-1/CRE site of the MIP-1β promoter in Huh7 cells

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ABSTRACT

An elevated level of macrophage inflammatory protein-1β (MIP-1β) induced by IL-1β has been correlated with chronic hepatic inflammatory disease. However, molecular mechanism of IL-1β-induced MIP-1β expression in hepatic cells is obscure. Previously, we reported the mechanism of the anti-hepatitis B virus (HBV) activity of helioxanthin (HE-145). Here, we demonstrated that HE-145 inhibited IL-1β-induced MIP-1β expression in a dose-dependent manner in Huh7 cells. To understand the mode of action of HE-145, we first examined how IL-1β induced MIP-1β expression at the molecular level. Using selective inhibitors, we found that JNK and p38 pathways participated in IL-1β-induced MIP-1β expression. HE-145 specifically suppressed IL-1β-induced c-jun mRNA and protein expression and prevented c-jun-mediated AP-1 DNA-binding activity, whereas it had no effect on IL-1β-induced activation of JNK, p38 and ATF2. Further studies indicated that HE-145 may downregulate c-jun mRNA expression directly at transcriptional level without requirement of *de novo* protein synthesis. Mutational analysis and supershift assays indicated that IL-1β stimulated c-jun and CREB1 binding to the essential AP-1/CRE site of the MIP-1β promoter. The inhibitory effect of HE-145 on IL-1β-induced MIP-1β promoter activity was completely reversed by overexpressing c-jun. Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay consistently revealed that HE-145 reduced c-jun binding to the AP-1/CRE site *in vitro* and *in vivo*. Our results established a major role for c-jun in IL-1β-induced MIP-1β expression in hepatic cells. The reduction in IL-1β-induced c-jun expression and subsequent binding of the c-jun/CREB1 complex to AP-1/CRE site mainly contributed to the inhibitory action of HE-145 on IL-1β-induced MIP-1β production.



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*Abbreviations:* MIP-1β, macrophage inflammatory protein-1β; IL-1β, interleukin-1β; HE-145, helioxanthin; MAPK, mitogen-activated protein kinase; JNK, c-jun $NH_2$-terminal kinase; AP-1, activator protein-1; ATF2, activating transcription factor 2; SP600125, anthra[1,9-*cd*]pyrazole-6 (2*H*)-one; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; CREB1, cAMP responsive element binding protein 1.

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## 1. Introduction

Interleukin-1 (IL-1) is one of the most important pro-inflammatory cytokines secreted by monocytes and tissue macrophages during microbial infection [1]. IL-1 mediates various inflammatory responses, including inflammatory bowel disease, sepsis syndrome and both chronic and acute inflammation of many organs [2,3]. IL-1 binds to cell surface IL-1 type I receptor to activate downstream signaling pathways such as IKK-NF-κB, ERK, JNK and p38; these induce expression of genes essential for immune responses [4].

An increased serum level of IL-1 has been reported to be associated with chronic liver-related inflammatory diseases, such as alcoholism, primary biliary cirrhosis and chronic hepatitis B or C viral infection [5–9]. A recent study has shown that hepatitis B viral e antigen physically interacts with cellular interleukin-1 receptor accessory protein *in vitro* [10], suggesting that human hepatitis B virus (HBV) can trigger an IL-1 response that activates downstream inflammatory responses in the host.

Chemokines are critical inflammatory-mediators activated by pro-inflammatory signals. Macrophage inflammatory protein-1β (MIP-1β), also known as chemokine CC motif ligand 4 (CCL4), was first isolated from the cultured medium of LPS-activated macrophages [11]. MIP-1β mediates inflammatory responses by stimulating the chemotaxis of monocytes, NK cells and activated eosinophils [12–15]. Numerous studies have demonstrated that MIP-1β expression can be regulated at the transcriptional level by LPS, hydrogen peroxide and IL-1β [16–19]. Meanwhile, the conserved proximal AP-1/CRE site (−104 to −97 bp relative to the transcription start site) in MIP-1β promoter, which contains the overlapping consensus sequence for both AP-1 and CRE sites, has been shown to play an important role in regulating MIP-1β gene expression. Recently, the pro-inflammatory IL-1β has been reported to induce MIP-1β expression in human hepatic cells [20], thereby emphasizing the involvement of IL-1β and MIP-1β in hepatic immune surveillance [21–24].

During a screening of the anti-inflammatory activity of natural products, we have found that helioxanthin (HE-145), a naturally occurring lignan extracted from *Taiwania cryptomerioides*, can inhibit IL-1β-induced MIP-1β production in human hepatoma Huh7 cells. HE-145 has also been shown to suppress HBV gene expression and viral replication in cultured human hepatoma HepG2 cells [25–28]. Additionally, HE-145 also reduces $PGE_2$ production in rat peritoneal macrophages [29]. In this study, we examined the molecular mechanism of HE-145's action on IL-1β-induced MIP-1β production and reported that HE-145 inhibited IL-1β-induced c-jun expression and reduced subsequent binding of the c-jun/CREB1 complex to the AP-1/CRE site of the MIP-1β promoter in Huh7 cells.

## 2. Materials and methods

### 2.1. Preparation of helioxanthin (HE-145)

The heartwood of *T. cryptomerioides* Hayata was extracted with methanol and subsequently partitioned with *n*-hexane (1:1) following a modified purification procedure from a previous report [30]. The *n*-hexane soluble material was fractionated by sequential column chromatography using silica-gel. The active component was eluted using a solution of *n*-hexane/EtOAc (95:5) and was further purified by reverse phase high performance liquid chromatography to homogeneity. Derivatives of HE-145 were organically synthesized following standard methods. The structure of HE-145 and its derivatives HE-145-3 and HE-145-24 were determined by H NMR, IR and MS spectroscopy. For the bioassays, the compound was dissolved in DMSO and filtered through a 0.25 μm fluoropore filter (Millipore, MA, USA).

### 2.2. Cell culture

Huh7 cells, a human hepatoma cell line, were maintained in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fatal calf serum (FETALCLONE III; Hyclone, Logan, UT), 100 U/ml penicillin/streptomycin, 2 mM L-glutamine and 0.1 mM non-essential amino acids (Biological Industries, Ashrat, Israel) in a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C.

### 2.3. Reagents

IL-1β was purchased from R&D systems (Abingdon, UK). SP600125, SB203580 and PD98059 were obtained from Calbiochem (San Diego, CA). Actinomycin D (Act D), cycloheximide (CHX), MG115 and MTT (Thiazolyl Blue Tetrazolium Bromide) were from Sigma–Aldrich (St. Louis, MO). Transfection reagent Lipofectamine™ 2000 was purchased from Invitrogen (Carlsbad, CA). The following antibodies were used for Western blotting and immunoprecipitation: polyclonal antibodies against p46/p54 JNK1/2 [31], p44/p42 ERK1/2 [32] (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal antibodies against p38 MAPK, c-jun, CREB1, phosphorylated forms of Ser73 c-jun, Thr180/Tyr182 p38 MAPK, Thr202/Thr204 ERK1/2 and Thr71 ATF2 (Cell Signaling Technology, Danvers, MA), monoclonal antibody against Thr183/Tyr185 phosphorylated JNK1/2 and α-tubulin antibody (Sigma–Aldrich, St. Louis, MO). HRP-conjugated anti-rabbit and anti-mouse IgG were purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Nitrocellulose membranes (Protran NC) were purchased from Schleicher & Schull (Boston, MA). RQ1 DNase I, Improm II RT reverse transcriptase, oligo dT primer and the luciferase assay kit were obtained from Promega (Madison, WI). Protease inhibitor cocktail and LightCycler Fast Start DNA Master SYBR Green I Kit were purchased from Roche Diagnostics (Mannheim, Germany).

### 2.4. RT-PCR analysis and quantitative RT-PCR

Primer sets for six genes were synthesized from Blossom Biotechnologies Inc. (Taipei, Taiwan) and sequences were as follows: MCP-1 (sense, 5′-GAA GAA TCA CCA GCA GCA AG-3′; antisense, 5′-GGT TGT GGA GTG AGT GTT C-3′), MIP-1α (sense, 5′-TCT CCA CTG CTG CCC TTG-3′; antisense, 5′-GCT GCT CGT CTC AAA GTA G-3′), MIP-1β (sense, 5′-CTC TGC GTG ACT GTG CTG-3′; antisense, 5′-AGG CTG CTG GTC TCA TAG T-3′), RANTES (sense, 5′-CGAAAGAACCGCCAAGTGT-3′; antisense, 5′-GGG TAG GAT AGT GAG GGG A-3′), c-jun (sense, 5′-GAC TCC

GAG GAA CCG CTG-3′; antisense, 5′-TGG CTG TCG TCC CCG CTG-3′) and porphobilinogen deaminase (PBGD) (sense, 5′-TCT GGT AAC GGC AAT GCG G-3′; antisense, 5′-GAA TCT TGT CCC CTG TGG TG-3′). Total RNA from cultured Huh7 cells was extracted using REzol C & T reagent (PROtech Technologies Inc., Taipei, Taiwan), a new improved isolation method based on the acid guanidinium–thiocyanate–phenol–chloroform extract procedure of Chromczynski and Sacchi. After DNase I digestion of the total extracted RNA, 3 μg of RNA was used to synthesize single-strand cDNA using oligo dT primer and Impron II reverse transcriptase. PCR was performed with 26 cycles for the MIP-1β gene and 30 cycles for the porphobilinogen deaminase gene; finally the PCR products were checked by loading onto 1.2% agarose gels. Furthermore, real-time quantitative RT-PCR was performed with the double-stranded DNA-binding dye SYBR Green I using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany). The mRNA expression level of MIP-1β and c-jun was normalized against the average expression of a housekeeping gene (PBGD).

### 2.5. Enzyme-linked immunosorbent assay

An instant enzyme-linked immunosorbent assay (ELISA) kit for human MIP-1β detection was purchased from Bender MedSystems (Vienna, Austria) and the assay was performed according to the provided protocol. The microwell plate was coated with lyophilized polyclonal antibody to MIP-1β, biotin-conjugated anti-MIP-1β and streptavidin–HRP. Next 50 μl of cultured Huh7 cell supernatant was added into the microwells and the plates were then incubated for 3 h at room temperature. The plate was washed with the provided buffer solution, which was followed by pipetting 100 μl TMB substrate solution to each well without light exposure. After 20 min, the reaction was stopped by the addition of 100 μl of stop solution. The absorbance was measured on a plate reader at 450 nm against 620 nm. Simultaneously, cell numbers were monitored by an MTT assay. The amount of MIP-1β production was normalized against the cell number of that sample.

### 2.6. Western blotting

Briefly, 100 μg of total protein lysates were separated by 8.5% SDS–PAGE and blotted onto nitrocellulose membranes (Protran NC). The membranes were blocked with 4% non-fat dry milk in Tris-buffered saline (TBS) for 1 h and then probed with the appropriate primary antibody at 4 °C overnight. After washing five times with TBS, the NC membrane was incubated with secondary HRP-conjugated antibodies for 1 h. The HRP signal was detected by a Western Lightning Chemiluminescence Kit (PerkinElemer Biosciences, Boston, MA). Subsequent quantification was performed using a personal densitometer (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

### 2.7. JNK activity assay

Cell lysates were prepared from Huh7 cells grown in 100-mm plates and then immunoprecipitated with polyclonal JNK1/2 antibody overnight at 4 °C. Protein A-conjugated agarose beads (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) were then added and incubated for additional 2 h at 4 °C. The complex was washed with buffer I (20 mM Tris/HCl pH 7.4; 0.5 M NaCl) and buffer II (20 mM Tris/HCl pH 7.4; 0.5 mM DTT), each buffer twice. Kinase assays were performed by incubating immune complexes in 30 μl of reaction buffer (20 mM Tris/HCl pH 7.4, 0.5 mM DTT, 5 μM ATP, 10 mM $MgCl_2$) with 10 μCi of [$\gamma$-$^{32}$P] ATP (PerkinElemer Biosciences, Boston, MA) and 10 μg GST-c-jun (Cell Signaling Technology, Danvers, MA) at 37 °C for 15 min. The reaction mix was then denatured and analyzed by 12.5% SDS–PAGE and autoradiography.

### 2.8. Plasmid, transient transfection and luciferase assay

The human MIP-1β promoter-luciferase reporter plasmid (MIP-1β-Luc) was constructed by subcloning the upstream promoter region of the human MIP-1β gene (−1281/+12) or (−157/+12) into pGL3-basic Luc (Promega, Madison, WI). The wild type AP-1/CRE (TGACGTCA) of MIP-1β-Luc was mutated into a mutant AP-1/CRE (GGATGTCG) version of MIP-1β-Luc by Quick Change XL Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA). The c-jun expression plasmid pCMV-jun was kindly provided by Dr. Bon-Chu Chung (Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei, Taiwan) [33]. The CREB1 expression plasmid pCMV-CREB1 was provided by VYMGC (http://genome.ym.edu.tw/). The wild or mutant versions of the MIP-1β-Luc together with a CMV-driven β-galactosidase construct (pCMV-β-gal) were transiently transfected at 9:1 ratio into 80% subconfluent Huh7 cells using Lipofectamine™ 2000 reagent according to the manufacturer's recommendations. After 24 h, the transfected cells were changed to serum-free DMEM medium with various treatments for 4 h. Cell extracts were prepared and luciferase activity was measured using the Promega Luciferase Assay System and standardized against the β-galactosidase activity. The values shown are the means (±S.D.) of three replicates and at least three independent trials.

### 2.9. Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Preparation of the nuclear extracts was carried out using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, USA). The sequences of the double-stranded oligonucleotides used to detect the DNA-binding activities of AP-1 and AP-1/CRE sites were 5′-CGC TTG ATG ACT CAG CCG GAA-3′ and 5′-AGG AGC ATG ACG TCA TCT CT-3′, respectively. Each reaction contained 5 μg nuclear extract, 2.5 μg dI dC (polydeoxinosinic-deoxycytidylic acid) (Roche Diagnostics, Mannheim, Germany) and $1 \times 10^5$ cpm of $^{32}$P-labeled oligonucleotide. Reactions were incubated for 15 min on ice, another 20 min at 30 °C, then resolved on a 5% polyacrylamide gel with 0.5× TBE for 3 h. Gels were dried and subjected to autoradiography. For cold oligonucleotide competition, the binding reactions were set up as described above except that 50-fold unlabeled oligonucleotide was added prior to the reaction. Mutated AP-1/CRE oligonucleotide was used in a cold excess competition experiment and the sequence was as follows: 5′-AGG AGC AgG AtG TCg TCT CT-3′. For the

supershift assays, the binding reactions were set up as described above except for pre-incubation with rabbit polyclonal antibody against c-jun, CREB1 or phospho-ATF2 antibody (1 μg) for 20 min at room temperature.

### 2.10. Chromatin immunoprecipitation (ChIP) assay

Huh7 cells were grown in 100-mm plates and treated with HE-145 or SP600125 before stimulation of IL-1β. After 1 h, the protein–DNA complexes were cross-linked by formaldehyde (1% final concentration) for 10 min and then Glycine (0.125 M final concentration) was added to terminate the reaction. Cells were scraped and resuspended in SDS lysis buffer (50 mM Tris pH 8.1, 0.5% SDS, 5 mM EDTA and complete protease inhibitor mixture). The collected nuclei was resuspended in IP buffer (100 mM Tris pH 8.6, 0.3% SDS, 5 mM EDTA, 1.7% Triton X-100 and complete protease inhibitor mixture) and subjected to three cycles of sonication on ice with 20-s pulses. The sonicated samples were centrifuged to spin down cell debris and the soluble chromatin solution was immunoprecipitated using polyclonal antibody against c-jun and pulled down by protein A-conjugated agarose beads. One part of the chromatin without immunoprecipitation was subjected to DNA purification and served as the input DNA. The protein-bound immunoprecipitated DNA was washed with Buffer 500, LiCl wash buffer and TE buffer, then immune complexes were eluted by adding elution buffer followed by incubation for at least 6 h at 65 °C to reverse cross-links and incubation for 1 h at 37 °C with RNase A (50 μg/ml). Finally, the DNA was purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Primer sets for AP-1/CRE site of the human MIP-1β promoter and a random region of the human β-actin were designed. PCR and quantitative-PCR were then performed and the amount of immunoprecipitated chromatin associated with AP-1/CRE site was normalized against the corresponding input DNA which was referenced by β-actin.

### 2.11. Statistical analysis

All experimental results and measurements are triplicates and expressed as the means ± standard deviation (S.D.). To confirm reproducibility, all experiments were repeated at least three times. Statistical analysis was performed using Student's paired *t* test.

## 3. Results

### 3.1. HE-145 suppresses IL-1β-induced MIP-1β mRNA and protein expression

The effects of IL-1β on expression of four members of CC-chemokines including MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5) were examined in human hepatoma Huh7 cells. Using quantitative RT-PCR analysis, we found that IL-1β stimulated expression of all 4 CC-chemokines in Huh7 cells (Fig. 2A). MIP-1β was the most IL-1β-responsive gene (>1000-fold induction) among different chemokines we examined. Therefore, we focused on IL-1β-induced MIP-1β gene expression for further investigations.

A time- and dose-dependent analysis of IL-1β-induced MIP-1β mRNA was performed by both RT-PCR and real-time RT-PCR. The MIP-1β mRNA was barely detectable in the control Huh7 cells but was rapidly induced after IL-1β treatment in a time- and dose-dependent manner (Fig. 2B and C).

The effect of HE-145 on IL-1β-induced MIP-1β expression in Huh7 cells was then examined. As shown in Fig. 2D and E, HE-145 specifically inhibited IL-1β-induced MIP-1β mRNA with an $IC_{50}$ of about 1.5 μM, whereas two structurally related analogs, HE-145-3 and HE-145-24 (Fig. 1), had no effect up to 3 μM. The suppressive effect of HE-145 on IL-1β-induced MIP-1β gene expression can also be observed at the protein level. Using ELISA to measure secreted MIP-1β protein in the culture medium, IL-1β alone increased MIP-1β protein production by 6-fold in Huh7 cells, but HE-145 suppressed IL-1β-induced MIP-1β protein production with a similar $IC_{50}$ of about 1.5 μM (Fig. 2F).

### 3.2. HE-145 suppresses IL-1β-induced activation of JNK/c-jun but not p38 MAPK pathways

To determine which specific IL-1β signaling pathway(s) were inhibited by HE-145, we first examined which MAPK pathways were involved in IL-1β-induced MIP-1β regulation in Huh7 cells. As shown in Fig. 3A, the JNK inhibitor SP600125 severely inhibited IL-1β-induced MIP-1β expression (91%), whereas the p38 MAPK inhibitor SB203580 only exerted a moderate inhibitory effect (50%). In contrast to the JNK or p38 pathways, the MEK 1 inhibitor PD98059 had no effect at all on IL-1β-induced MIP-1β expression. This result indicated that JNK and

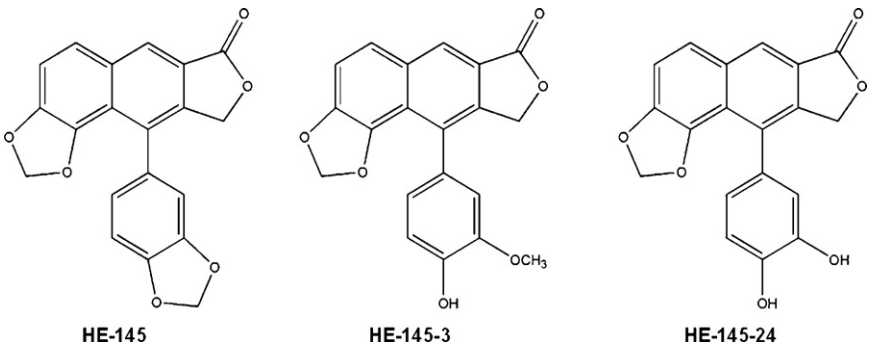


**Fig. 1 – Chemical structures of HE-145 and its derivatives.**

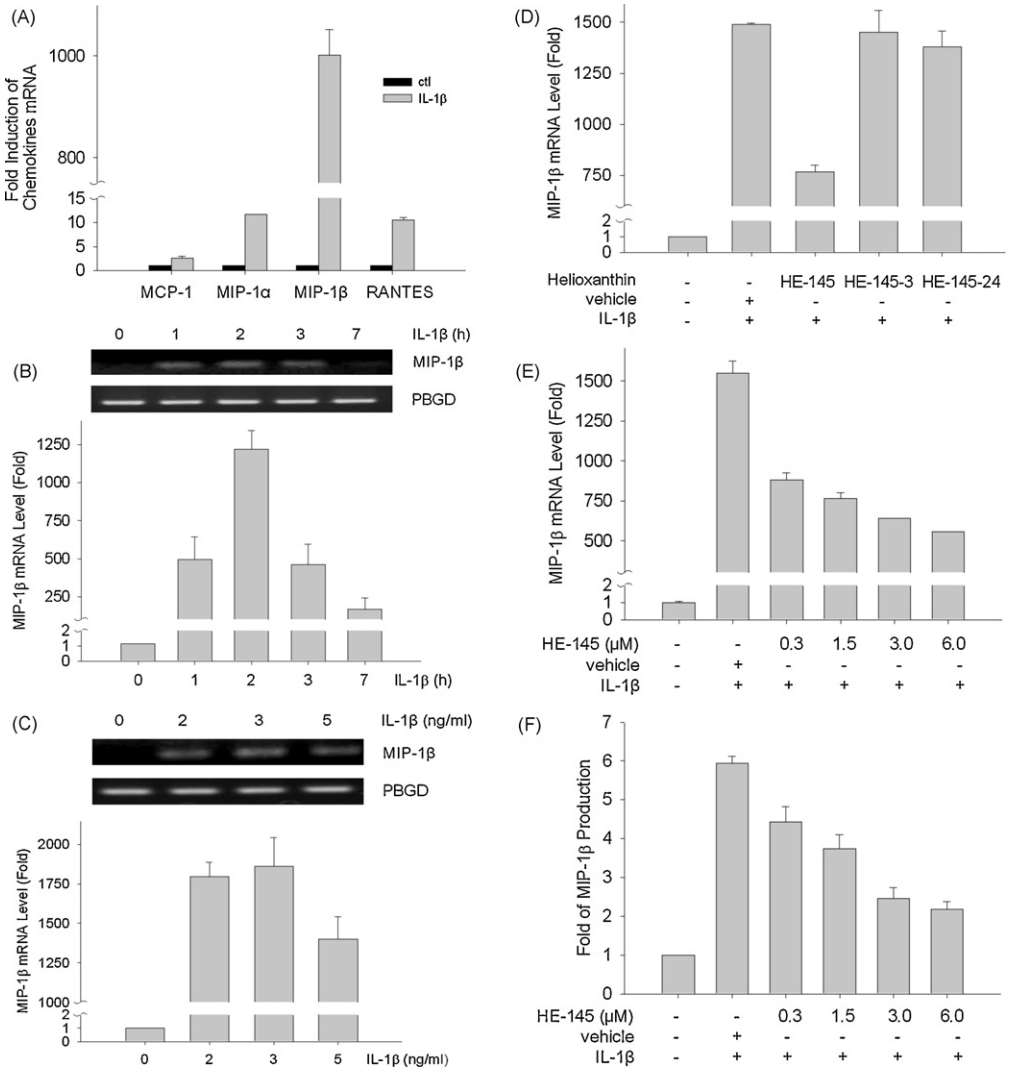


**Fig. 2 – Effect of IL-1β and HE-145 on MIP-1β expression in Huh7 cells. (A) Huh7 cells were treated with IL-1β (2 ng/ml) for 2 h. Cellular RNA was extracted and subjected to real-time RT-PCR analysis to measure the mRNA level of MCP-1, MIP-1α, MIP-1β and RANTES. After normalizing against the corresponding PBGD mRNA level, the fold induction of MCP-1, MIP-1α, MIP-1β and RANTES mRNA by IL-1β was shown as means ± S.D. (B) Huh7 cells were incubated with IL-1β (2 ng/ml) for up to 7 h. Cellular RNA was extracted and subjected to RT-PCR and real-time RT-PCR to analyze the MIP-1β and PBGD mRNA expression levels. The MIP-1β mRNA level was normalized against the PBGD mRNA level and the fold induction of MIP-1β mRNA expression is shown as means ± S.D. (C) Huh7 cells were incubated with various concentrations of IL-1β for 2 h. The fold of IL-1β-induced MIP-1β mRNA level was analyzed by RT-PCR and real-time RT-PCR. Values are expressed as means ± S.D. (D) Huh7 cells were exposed to vehicle and 3.0 μM of HE-145, HE-145-3 or HE-145-24 for 24 h and this was followed by stimulation with IL-1β (2 ng/ml) for another 2 h. The MIP-1β mRNA level was quantified by real-time RT-PCR and plotted as means ± S.D. (E) Huh7 cells were exposed to vehicle and HE-145 (0–6.0 μM) for 24 h, followed by stimulation with IL-1β (2 ng/ml) for another 2 h. The MIP-1β mRNA level was quantified by real-time RT-PCR and plotted as means ± S.D. (F) Huh7 cells were treated with vehicle and HE-145 (0–6.0 μM) for 24 h in the presence of IL-1β (2 ng/ml). The amount of MIP-1β in the medium was detected and normalized against cell number, which was assayed simultaneously by MTT assay. The fold of MIP-1β production is expressed as means ± S.D.**

p38 but not MEK 1/2 MAPK play important roles in IL-1β-induced MIP-1β expression.

As shown in Fig. 3B, IL-1β rapidly induced phosphorylation of JNK, c-jun and p38, which peaked at around 15–30 min in Huh7 cells. However, the activation of ERK1/2 was not significantly affected by IL-1β. Treatment with HE-145 or the JNK inhibitor SP600125 suppressed both the IL-1β-induced c-jun protein and the phosphorylation level of c-jun, whereas the phosphorylation level of JNK was not decreased by HE-145 (Fig. 3C). On the other hand, IL-1β-induced phosphorylation of p38 MAPK and ATF2, a signaling downstream transcription factor of JNK or p38 MAPK, were not inhibited by HE-145 (Fig. 3D).

### 3.3. HE-145 suppresses IL-1β-induced c-jun gene expression but not JNK activity directly in vitro

Next, Huh7 cells were pre-treated with HE-145 for different time periods, which were followed by IL-1β induction. As shown in Fig. 4A, pre-treatment with HE-145 for 6 h was sufficient to inhibit not only the basal c-jun protein, but also IL-1β-induced protein and the phosphorylation of c-jun. We then wondered whether HE-145 also reduced IL-1β-induced c-jun mRNA level. IL-1β induced a transient induction of c-jun mRNA with the maximum expression at around 1 h (Fig. 4B). However, HE-145 reduced both basal c-jun ($IC_{50} \sim 3.0$ μM) and IL-1β-induced c-jun ($IC_{50} < 1.5$ μM) mRNA expression (Fig. 4C).

Furthermore, the effect of HE-145 on c-jun-mediated AP-1-binding activity was examined by electrophoretic mobility shift assay. The c-jun-mediated AP-1-binding activity in cell extract was induced by IL-1β treatment (Fig. 4D, compare lanes 2 and 4). The AP-1-binding activity was completely competed by the specific AP-1 oligonucleotide but not by the random non-specific oligonucleotide (Fig. 4D, lanes 8 and 9). The

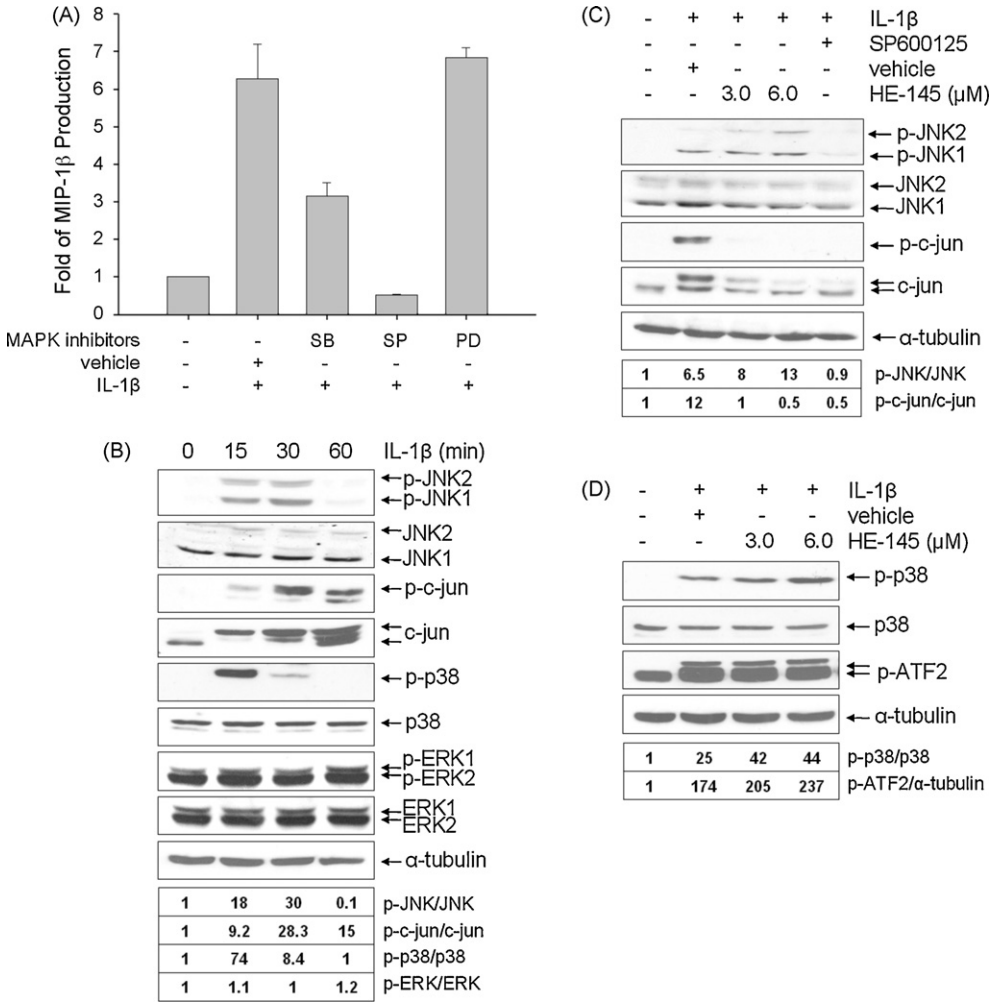


**Fig. 3 – Effect of HE-145 on IL-1β-induced MAPK activations. (A) Huh7 cells were incubated with vehicle or 10 μM of the p38 MAPK inhibitor SB203580 (SB), JNK inhibitor SP600125 (SP), MEK1 inhibitor PD98059 (PD) for 2 h and followed by stimulation with IL-1β (2 ng/ml) for another 24 h. The production of MIP-1β in culture medium was examined by ELISA. The ratio of MIP-1β production was expressed as means ± S.D. (B) Huh7 cells were treated with IL-1β (2 ng/ml) for indicated times and then phosphorylation levels and the corresponding protein levels of JNK1/2, c-jun, p38 and ERK1/2 were detected by Western blotting. The ratio of densities of phospho-JNK1/2, phospho-c-jun, phospho-p38 and phospho-ERK1/2 relative to their corresponding proteins was shown as measured by densiotometer. (C) The effect of HE-145 on IL-1β-induced JNK/c-jun signaling was examined. Huh7 cells were incubated with HE-145 (3.0, 6.0 μM) for 24 h or SP600125 (10 μM) for 2 h, followed by stimulation with IL-1β (2 ng/ml) for another 30 min. The phospho-JNK and phospho-c-jun levels relative to their corresponding protein levels were analyzed using α-tubulin to give equal loading of the cell lysates. The ratio of relative densities of phospho-JNK/JNK and phospho-c-jun/c-jun was shown. (D) The effect of HE-145 on IL-1β-induced p38 MAPK signaling was examined. Huh7 cells were incubated with vehicle and HE-145 (3.0 μM) for 24 h, which was followed by stimulation with IL-1β (2 ng/ml) for another 30 min. The phosphorylation levels of p38 and ATF2 were analyzed; the corresponding protein levels and the amount of α-tubulin present were also probed as internal controls. The ratio of relative densities of phospho-p38/p38 and phospho-ATF2/α-tubulin was shown.**

specificity of the AP-1-binding activity was also verified by a supershift assay using specific anti-c-jun Ab (Fig. 4D, lanes 10 and 11). Thus, HE-145 severely reduced IL-1β-induced AP-1-binding activity which was mediated by c-jun in Huh7 cells (Fig. 4D, lanes 5 to 6).

Since SP600125, a specific JNK inhibitor, has also been shown to inhibit IL-1β-induced c-jun protein, c-jun mRNA, c-jun phosphorylation and AP-1 DNA-binding activity, it is possible that HE-145 just acts in an exactly similar manner to SP600125 by directly inhibiting JNK. To test this hypothesis, we assayed JNK activity *in vitro* using a GST-c-jun fusion protein as the substrate. The result clearly showed that HE-145 did not affect the kinase activity of JNK *in vitro* at concentrations of HE-145 up to 10 μM (Fig. 4E).

### 3.4. *HE-145 does not affect the stability of c-jun mRNA or protein, nor requires de novo protein synthesis to suppress IL-1β-induced c-jun gene expression*

To elucidate the molecular mechanism of HE-145 on IL-1β-induced c-jun gene expression, the effect of HE-145 on c-jun mRNA or on protein stability was examined. Using actinomycin

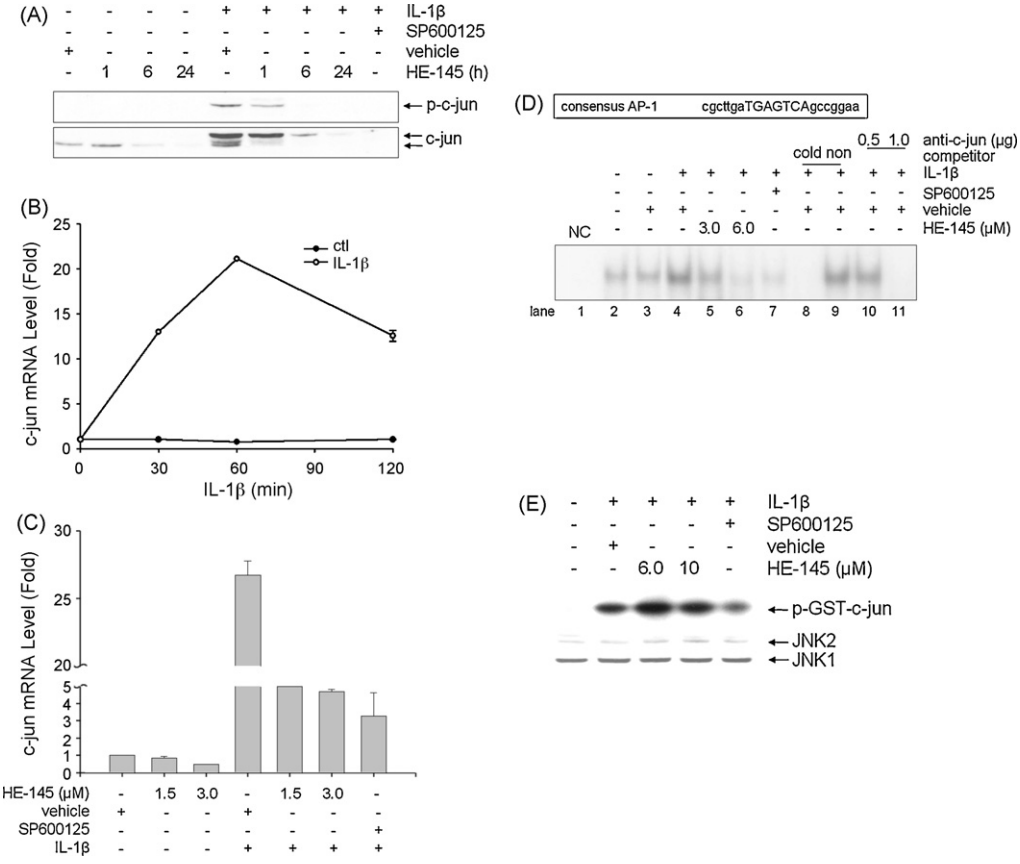


**Fig. 4 – Effect of HE-145 on IL-1β-induced c-jun phosphorylation, expression, AP-1 DNA-binding activity and JNK activity. (A) Huh7 cells were pre-treated with HE-145 (6.0 μM) for indicated times or SP600125 (10 μM) for 2 h and this was followed by stimulation with IL-1β (2 ng/ml) for another 30 min. The level of phospho-c-jun relative to its protein level is shown. (B) A time-course analysis of IL-1β-induced c-jun mRNA expression. Huh7 cells were incubated with IL-1β (2 ng/ml) for indicated times and then total RNA was extracted. The c-jun mRNA level was normalized against the PBGD mRNA level and the fold induction of c-jun mRNA expression was expressed as means ± S.D. (C) The effect of HE-145 on c-jun mRNA expression was analyzed. Huh7 cells were incubated with the indicated concentrations of HE-145 for 6 h or SP600125 (10 μM) for 2 h and this was followed by stimulation with IL-1β (2 ng/ml) for another 30 min. The relative fold of c-jun mRNA level was analyzed as described above and expressed as means ± S.D. (D) The effect of HE-145 on c-jun-mediated DNA-binding activity of the AP-1 complex was examined. Huh7 cells were incubated with vehicle, HE-145 (3.0 and 6.0 μM) for 24 h or SP600125 (10 μM) for 2 h and this was followed by stimulation with IL-1β (2 ng/ml) for another 1 h. Subsequently, nuclear extracts were prepared and AP-1 DNA-binding activity was assayed by EMSA (in lanes 2–7) as described in Section 2. "NC" (lane 1) represented the negative control without nuclear extract. Cold competition assays were performed by co-incubation with a 50-fold excess of cold specific AP-1 oligonucleotide (lane 8) or non-specific oligonucleotide (lane 9). Supershift assays were carried out by pre-incubation with anti-c-jun Ab (0.5 and 1.0 μg, lane 10–11). (E) The effect of HE-145 on JNK1/2 activity was determined. Huh7 cells were incubated with HE-145 (6.0 and 10 μM) for 24 h or SP600125 (10 μM) for 2 h and this was followed by stimulation of IL-1β (2 ng/ml) for 30 min. Cell lysates were extracted and immunoprecipitated with specific anti-JNK 1/2 Ab and the JNK1/2 immunocomplex was reacted with GST-c-jun and [γ-$^{32}$P]-ATP. The presence of equal amount of JNK1/2 protein in total cell lysates is shown by Western blotting analysis.**

D to block newly synthesized transcripts, we revealed that the half-life of IL-1β-induced c-jun mRNA was about 0.5 h and that this was not altered by HE-145 treatment (Fig. 5A). When *de novo* protein synthesis was blocked by cycloheximide (CHX), both the basal and IL-1β-induced c-jun mRNA levels were reduced by HE-145 (Fig. 5A and B). Moreover, in the presence of the proteosomal inhibitor MG115, which blocks proteosome-mediated protein degradation, the c-jun protein level increased dramatically but was still subjected to HE-145's inhibition (Fig. 5C). Similarly, HE-145 had no effect on the degradation rate of preexisting c-jun protein when protein synthesis was inhibited by CHX (Fig. 5C). Based on these results, we suggested that HE-145 may directly inhibit c-jun gene expression at the transcription level but that this is not mediated through alterations in the stability of c-jun mRNA or protein; neither does it depend on the synthesis of any regulatory proteins.

### 3.5. *Binding of c-jun and CREB1 to an essential AP-1/CRE cis-element of MIP-1β promoter*

To understand the role of c-jun in regulating human MIP-1β promoter activity, we next analyzed the human MIP-1β gene promoter sequence using the AliBaba2 program [34]. Four putative AP-1 sites and one conserved AP-1/CRE site, consisting of an overlapped AP-1 and CRE site, were identified in the promoter region of the human MIP-1β gene (−1281/+12) (Fig. 6A). We found one base mismatch in the AP-1/CRE sequence (TGACGTCA, −104 to −97 bp relative to the transcription start site of MIP-1β promoter between our construct and that reported in the literature (see detailed description in Section 4). To identify the key regulatory cis-element important to the transcriptional regulation of IL-1β-induced MIP-1β expression, both wild type (−1281/+12) and specifically mutated MIP-1β promoter driven luciferase constructs were examined. As shown in Fig. 6B, IL-1β significantly stimulated promoter activity of the wild type MIP-1β promoter construct (−1281/+12) (2.3-fold; $^{**}P < 0.01$). When the upstream three AP-1 sites (AP-1-2, AP-1-3 and AP-1-4) were deleted, the activity of the del-MIP-1β promoter (−157/+12) was similar to that of the wild type MIP-1β promoter and still can be activated by IL-1β. However, both basal and IL-1β-induced MIP-1β promoter activity was completely abolished in the mutated promoter, where the AP-1/CRE site 5′-TGACGTCA-3′ was mutated to 5′-gGAtGTCg-3′. These results indicated that the proximal AP-1/CRE site, but not the upstream three AP-1 sites of the MIP-1β promoter, is essential for both basal and IL-1β-induced MIP-1β promoter activation in human hepatic cells.

The specific up-shift band of the AP-1/CRE complex in an EMSA assay was completely competed by oligonucleotides containing either a specific CRE or a specific AP-1 sequence, which suggests that both CREB and c-jun protein possibly bind to this AP-1/CRE site *in vitro* (Fig. 6C, lanes 2–6). This possibility was further supported by the observation that only specific anti-CREB1 and anti-c-jun antibodies could induce supershift of the AP-1/CRE complex (Fig. 6C, lanes 7–9). Nuclear extracts prepared from c-jun overexpressing cells showed great enhancement in the intensity of the specific up-shift band of the AP-1/CRE sequence, whereas nuclear extracts prepared from CREB1 overexpressing cells only showed a small enhancement (Fig. 6C, lanes 10–12). These results suggested that both c-jun and CREB1 are able to bind to the essential AP-

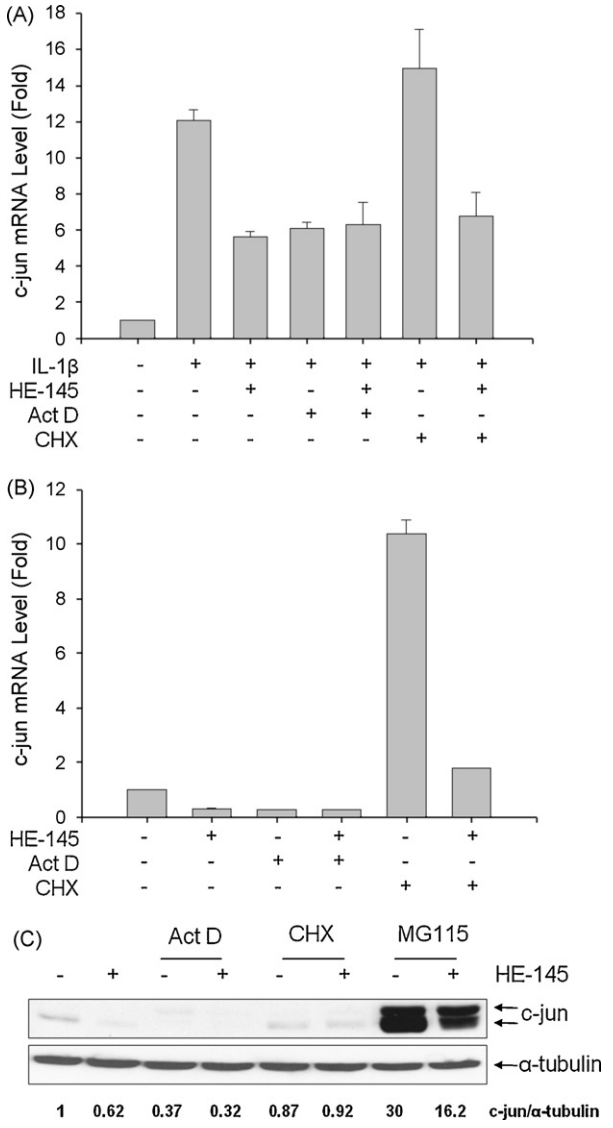


**Fig. 5 – Effect of HE-145 on the transcriptional level, mRNA and protein stabilities of c-jun. (A) The effect of HE-145 on the stability of IL-1β-induced c-jun mRNA transcript in the presence of actinomycin D (Act D) or cycloheximide (CHX). c-jun mRNA was induced by IL-1β (2 ng/ml) for 30 min and this was followed by treatment with HE-145 together with either Act D (2 μg/ml) or CHX (20 μM) for another 30 min. The relative fold of c-jun mRNA was analyzed and expressed as means ± S.D. (B) The effect of HE-145 on the stability of basal c-jun mRNA transcript in the presence of Act D or CHX. Huh7 cells were treated with HE-145 (6.0 μM) together with either Act D (2 μg/ml) or CHX (20 μM) for 2 h. Thereafter, the relative fold of c-jun mRNA level was analyzed and expressed as means ± S.D. (C) The effect of HE-145 on c-jun protein stability. Huh7 cells were treated with HE-145 (6.0 μM) together with either Act D (2 μg/ml), CHX (20 μM) or MG115 (10 μM) for 6 h and then the cell lysates were subjected to Western blotting analysis. The c-jun protein levels were detected using α-tubulin as a loading control. The density of c-jun protein relative to α-tubulin is shown as a fold change as measured by densiotometer.**

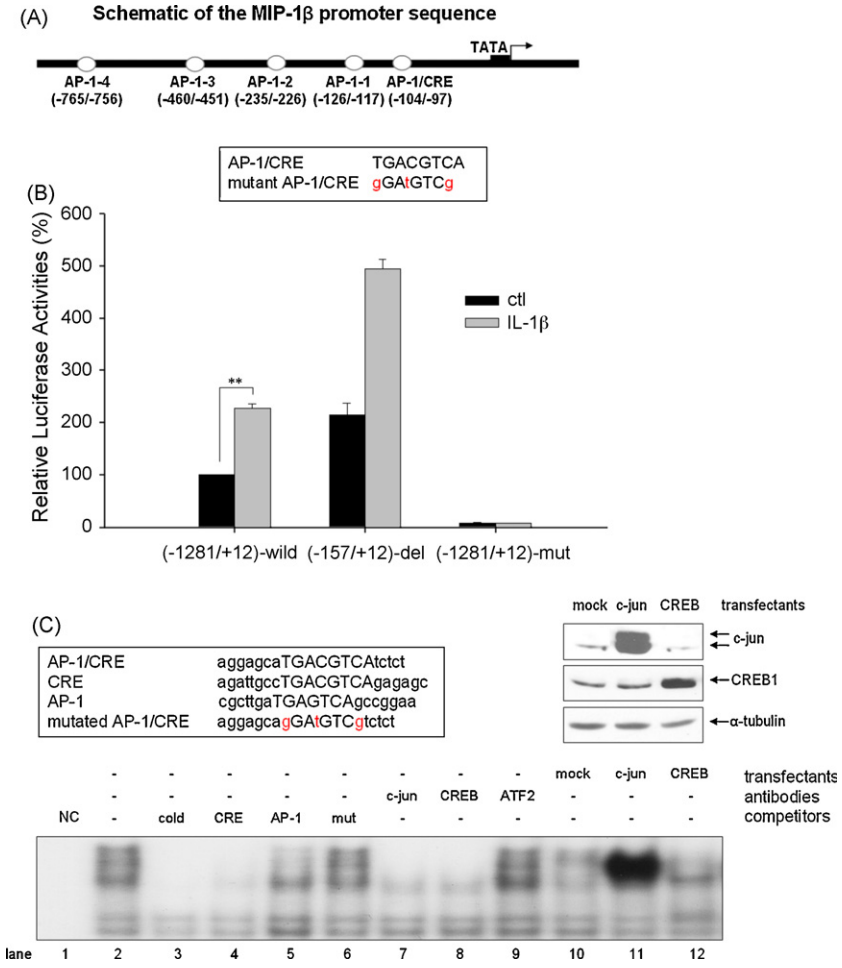


**Fig. 6 – Analysis of the cis-element AP-1/CRE found in the MIP-1β promoter region. (A) A schematic of the human MIP-1β promoter sequence. The numbers indicate nucleotide positions in relation to the transcription initiation site. The one putative AP-1/CRE and four AP-1 binding sites are indicated. (B) The MIP-1β reporter plasmids (−1281/+12)-wild, (−157/+12)-del and (−1281/+12)-mut (AP-1/CRE 5′-TGACGTCA-3′ mutated to 5′-gGAtGTCg-3′) were transiently co-transfected with a CMV-driven β-galactosidase (pCMV-β-gal) expression construct into Huh7 cells. After 24 h, the transfected cells were stimulated with IL-1β (2 ng/ml) for 4 h. Luciferase activity was measured and normalized against the β-galactosidase activity. The relative wild type MIP-1β luciferase activity of the untreated control was set to 100%. The fold of MIP-1β luciferase activity was expressed as means ± S.D. $^{**}P < 0.01$. (C) The components of the nucleoprotein complex bound to AP-1/CRE site were analyzed. "NC" (lane 1) represented the negative control without nuclear extract. The specificity and affinities of the complex between AP-1, CRE and AP-1/CRE site were analyzed by co-incubation with 50-fold cold oligonucleotides of AP-1/CRE (lane 3), CRE (lane 4), AP-1 (lane 5) and mutated AP-1/CRE (lane 6), which competed with the specific AP-1/CRE binding band (lane 2). Supershift assays were also performed by pre-incubating nuclear extracts with anti-c-jun (lane 7), anti-CREB1 (lane 8) and anti-phospho-ATF2 Abs (lane 9). Furthermore, Huh7 cells were transiently transfected with mock (lane 10), pCMV-c-jun (lane 11) and pCMV-CREB1 (lane 12) plasmids and subjected to analysis of AP-1/CRE DNA-binding activity by EMSA. In addition, the protein levels of overexpressing c-jun and CREB1 were demonstrated by Western blotting; again, α-tubulin was used as a loading control.**

1/CRE site of the MIP-1β promoter and c-jun may have stronger binding affinity than CREB1 to this site.

### 3.6. *HE-145 suppresses IL-1β-induced MIP-1β promoter activity mainly through reducing IL-1β-induced c-jun expression and binding of c-jun/CREB1 complex to the AP-1/CRE site of MIP-1β promoter*

To investigate how HE-145 suppressed IL-1β-induced MIP-1β gene expression, we next examined the effect of HE-145 on the promoter activity of MIP-1β gene. As shown in Fig. 7A, HE-145 suppressed both basal and IL-1β-induced MIP-1β promoter activity in a dose-dependent manner. The JNK specific inhibitor SP600125 also suppressed IL-1β-induced MIP-1β promoter activity, suggesting that c-jun is required for MIP-1β promoter activity. Since HE-145 has been shown to reduce IL-1β-induced c-jun expression, one possibility is that HE-145 may suppress IL-1β-induced MIP-1β promoter activity mainly through inhibition of IL-1β-induced c-jun expression. If this is the case, it should be possible to reverse the suppressive effect

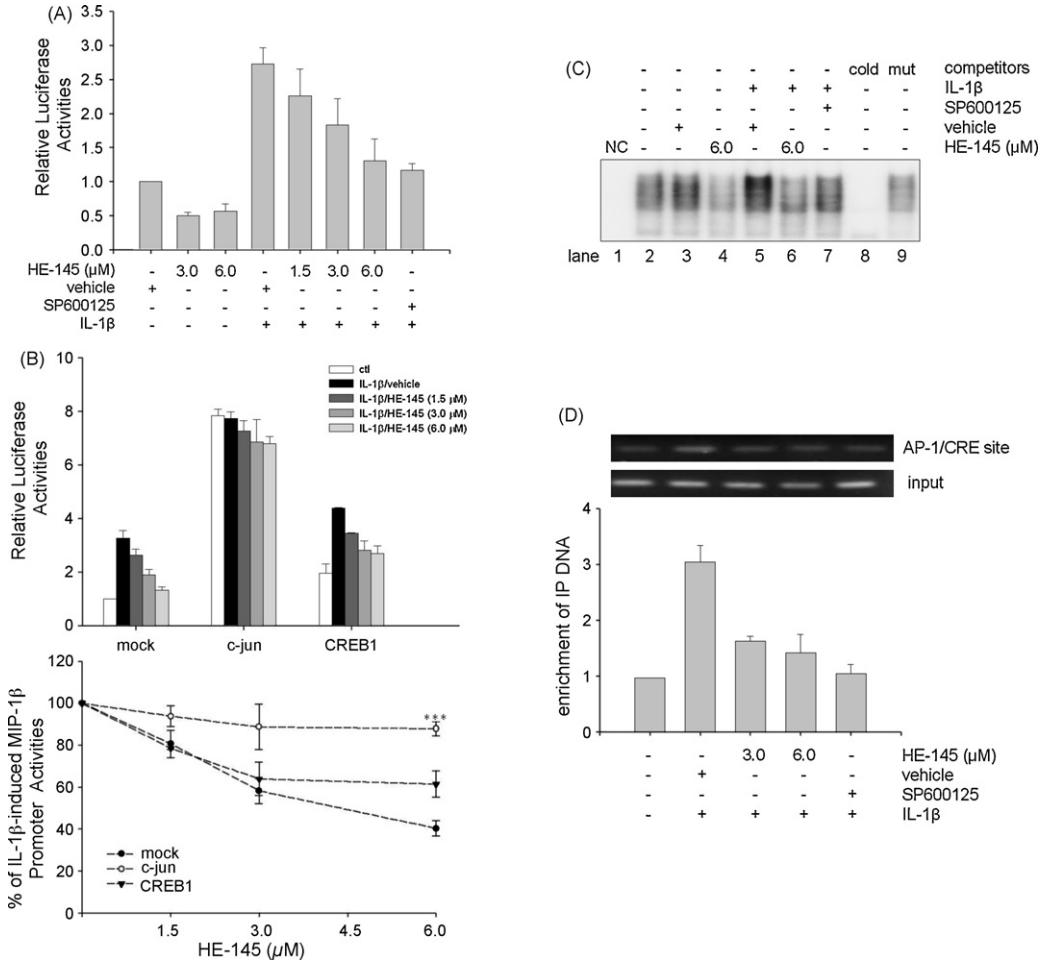


**Fig. 7 – Suppressive effect of HE-145 on IL-1β-induced MIP-1β promoter activity and c-jun-meditated DNA-binding activity of the AP-1/CRE site. (A) Huh7 cells were transiently co-transfected with MIP-1β-Luc and pCMV-β-gal. After 24 h, the cells were changed into serum-free medium for 16 h, then treated with vehicle or the indicated concentrations of HE-145 and SP600125 (10 μM) in the presence of IL-1β (2 ng/ml) for 4 h. The relative MIP-1β luciferase activity was expressed as means ± S.D. (B) Mock, pCMV-c-jun or pCMV-CREB1 plasmid was co-transfected with (−1281/+12)-MIP-1β-Luc and pCMV-β-gal in Huh7 cells. After 24 h, the cells were changed into serum-free medium for 16 h, then treated with vehicle or indicated concentrations of HE-145 in the presence of IL-1β (2 ng/ml) for 4 h. The relative IL-1β-induced MIP-1β luciferase activities in the mock, c-jun or CREB1 overexpressing cells were set to 100% individually for comparison. $^{***}P < 0.001$, comparing the suppressive effect of HE-145 (6.0 μM) between mock and c-jun overexpressing cells. (C) The DNA-binding activity of the proximal AP-1/CRE site on the MIP-1β promoter was examined. Huh7 cells were treated with vehicle, HE-145 (6.0 μM) for 24 h or SP600125 (10 μM) for 2 h and this was followed by stimulation with IL-1β (2 ng/ml) for another 1 h. AP-1/CRE DNA-binding activity was analyzed by EMSA and binding specificity was checked by competing with 50-fold excess of cold wild type (lane 8) or mutated AP-1/CRE (lane 9) oligonucleotide. (D) Huh7 cells were pre-incubated with vehicle, HE-145 (3.0, 6.0 μM) for 24 h or SP600125 (10 μM) for 2 h, then stimulated with IL-1β (2 ng/ml) for 1 h. The degree of c-jun recruitment to the proximal AP-1/CRE site on the MIP-1β promoter was determined by ChIP assay. The associated DNA fragments were monitored by PCR and further quantified by real-time PCR using a primer pair specific for the AP-1/CRE site. Values are normalized against input DNA. The fold induction of the associated DNA is expressed as the mean ± S.D.**

of HE-145 by overexpression of c-jun. As shown in Fig. 7B, MIP-1β promoter activity was fully activated (8-fold) and no longer further stimulated by IL-1β in c-jun overexpressing cells. Under this circumstance, the suppressive effect of HE-145 was completely abolished. On the other hand, overexpression of CREB1 only increased MIP-1β promoter activity by 2-fold while HE-145 still significantly inhibited IL-1β-induced MIP-1β promoter activity (Fig. 7B). Western blotting analysis was carried out to measure the c-jun and CREB1 protein levels present in the c-jun and CREB1 overexpressing cells (supplementary Fig. S1).

The conclusion that HE-145-suppressed IL-1β-induced c-jun expression was responsible for its suppressive effect on IL-1β-induced MIP-1β promoter activation was further substantiated by an *in vitro* EMSA analysis and an *in vivo* chromatin immunoprecipitation (ChIP) assay. As shown in Fig. 7C, both

HE-145 (6.0 μM) and the specific JNK inhibitor SP600125 (10 μM) suppressed the basal and IL-1β-induced DNA-binding activity of the AP-1/CRE site of MIP-1β promoter *in vitro*. Similarly, IL-1β induced a 3-fold increase in c-jun recruitment to the MIP-1β promoter *in vivo*, which was totally abolished by either HE-145 or SP600125 treatment (Fig. 7D).

## 4. Discussion

A new class of lignans, helioxanthin (HE-145), isolated from *T. cryptomerioides*, has been shown to be a potent inhibitor of HBV gene expression and replication in cultured human hepatoma cells [27,28]. In this study, we unambiguously demonstrate that HE-145 has another important pharmacological activity of suppressing IL-1β-induced MIP-1β expression at the transcriptional level in Huh7 cells. The inhibitory activity of HE-145 on IL-1β-stimulated MIP-1β expression is structurally specific, since two other structurally related analogs did not show any activity at all (Figs. 1 and 2D). The specific structure activity relationship of HE-145 suggests that HE-145 may act on a specific cellular target to suppress IL-1β-induced MIP-1β expression.

IL-1β-stimulated MIP-1β expression has been reported in human hepatoma cells [20]. However, the molecular mechanism of IL-1β-induced MIP-1β expression in hepatic cells has not been fully explored. Using chemical inhibitors to block specific signaling pathways, we first showed that IL-1β can simultaneously activate both JNK and p38 MAPK, but not ERK1/2 (Fig. 3B). However, only SP600125, a JNK specific inhibitor, completely blocked the IL-1β-induced MIP-1β production (Fig. 3A). We further demonstrated that SP600125 not only blocked IL-1β-induced c-jun phosphorylation, but also IL-1β-induced c-jun mRNA and protein production (Figs. 3C and 4C). The dominant role of c-jun in MIP-1β gene expression was supported by results showing that SP600125 inhibited IL-1β-induced MIP-1β promoter activation (Fig. 7A). However, direct elimination of c-jun protein expression by siRNA knockdown will further substantiate the essential role of c-jun on IL-1β-mediated MIP-1β regulation.

Bioinformatics analysis revealed that human MIP-1β gene (−1281/+12) contains four putative AP-1 sites and one conserved overlapped AP-1 and CRE binding sequence (AP-1/CRE site) in the MIP-1β promoter region. Deletion and mutational analysis suggest that the putative AP-1 sites may be dispensable, but the conserved AP-1/CRE site is essential for the human MIP-1β promoter activity (Fig. 6B). This conclusion was supported by the result of the ChIP assay, which indicated that c-jun protein associated only with the AP-1/CRE site (Fig. 7D) but not with the putative AP-1 sites *in vivo* (data not shown). Interestingly, we have noticed that the AP-1/CRE sequence in human MIP-1β promoter (TGACGTCA, −104 to −97 bp relative to the transcription start site) of our construct has one base mismatch to the reported AP-1/CRE sequence (TGACATCA, −104 to −97 bp) in human MIP-1β promoter isolated from human T cells [18] and the conserved mouse ATF/CRE sequence in mouse MIP-1β promoter (TGACATCA, −104 to −97 bp) [16,19]. Detailed sequence analysis revealed that the MIP-1β promoter previously cloned from human T cells actually is not the promoter of human MIP-1β gene, but the promoter of a paralogue MIP-1β gene called CCL4L1, MIP-1β2 or LAG-1 (geneID9560) (data not shown) [35,36].

We propose that IL-1β activates JNK to phosphorylate c-jun protein and to induce c-jun gene expression. Elevated c-jun protein is then subsequently recruited to the AP-1/CRE site and activates MIP-1β promoter activity. Several lines of experimental evidences support this hypothesis. First of all, specific mutation of the conserved AP-1/CRE site completely abolished both basal and IL-1β-induced MIP-1β promoter activity (Fig. 6B). Secondly, overexpression of c-jun dramatically increased binding activity of cell extract to a DNA fragment containing the AP-1/CRE site. The supershift and ChIP assays further demonstrated that there is an association of c-jun on the essential AP-1/CRE site of the MIP-1β promoter *in vitro* and *in vivo* (Figs. 6C and 7D). Thirdly, overexpression of c-jun fully activated MIP-1β promoter and made the MIP-1β promoter no longer able to respond to IL-1β (Fig. 7B).

We found that HE-145 selectively suppressed IL-1β-induced c-jun phosphorylation and c-jun protein expression without altering the phosphorylation level of p38 MAPK and ATF2 (Fig. 3C and D). We wondered whether HE-145 may act like a specific kinase inhibitor to inhibit JNK or an upstream kinase involved in JNK signaling such as MKK4/MKK7. Two lines of evidences conclude that this is not true. Firstly, HE-145 does not affect IL-1β-induced phosphorylation of JNK1/2. Therefore, an upstream kinase of JNK such as MKK4/MKK7 or JNK itself cannot be the target of HE-145 (Fig. 3C). Secondly, when we used purified GST-fused c-jun protein as the substrate to assay the activity of JNK *in vitro*, we were unable to detect any inhibitory activity of HE-145 on JNK up to a concentration of 10 μM (Fig. 4E). In addition, HE-145 did not reduce IL-1β-induced phosphorylation of overexpressed c-jun protein while HE-145 could not suppress CMV promoter-driven ectopic c-jun protein expression (data not shown). Therefore, we conclude that HE-145 does not actually suppress IL-1β-induced c-jun phosphorylation and this suppressive effect may just reflect the fact that IL-1β-induced c-jun protein level is reduced by HE-145 treatment.

Exactly how HE-145 suppresses IL-1β-induced MIP-1β expression is not completely understood. We propose that the major effect of HE-145 on IL-1β-induced MIP-1β expression is by a suppression of IL-1β-induced c-jun expression. This hypothesis is supported by the observation that HE-145 reduces IL-1β-induced c-jun mRNA (Fig. 4C), AP-1 DNA-binding activity (Fig. 4D), MIP-1β promoter activity (Fig. 7A) and the binding of c-jun protein to AP-1/CRE site of MIP-1β promoter *in vivo* (Fig. 7D). If IL-1β-induced c-jun expression is the major target of HE-145 during the suppression of IL-1β-induced MIP-1β expression, ectopic expression of c-jun should override the inhibitory activity of HE-145. This is indeed the case. Only c-jun and not CREB1 is able to fully activate MIP-1β promoter activity and completely override the inhibitory activity of HE-145 (Fig. 7B).

The molecular mechanism of HE-145 suppression of IL-1β-induced c-jun expression is another question that still needs to be answered. Kinetic analysis has revealed that 6 h pre-treatment with HE-145 is sufficient to inhibit transiently induced c-jun protein and mRNA on treatment with IL-1β for 30 min (Fig. 4A and C). This result suggests that HE-145 may either directly inhibit c-jun expression at the transcription

level or HE-145 may enhance degradation of c-jun mRNA or protein. However, inhibition of basal and transient induced c-jun mRNA by HE-145 was not affected by cycloheximide treatment, which is a strong evidence against the possibility that HE-145 may alter the stability of c-jun mRNA or needs *de novo* protein synthesis to suppress basal c-jun and IL-1β-induced c-jun mRNA expression (Fig. 5A and B). When protein stability is considered, HE-145 was not able to accelerate degradation of preexisted c-jun protein when newly protein synthesis was inhibited by cycloheximide (Fig. 5C). Furthermore, when proteosomal-mediated protein degradation was blocked by MG115, the accumulated c-jun protein was still subjected to inhibition by HE-145. These observations are consistent with the notion that the major effect of HE-145 on c-jun expression is to inhibit the transcription of the c-jun gene. In addition, the protein synthesis inhibitor cycloheximide was able to superinduce c-jun mRNA (Fig. 5B). Superinduction of immediate early genes by cycloheximide has been believed to due to delayed transcriptional shut-off, a prolong mRNA half-life or the eliciting of nuclear signals to induce transcription of immediate early genes [37]. Since we have shown that HE-145 did not alter the stability of c-jun mRNA, the inhibition of c-jun gene transcription by HE-145 should be sufficient to explain why HE-145 can potently block superinduction of c-jun mRNA (Fig. 5B). However, the mode of action of HE-145 where it suppresses IL-1β-induced c-jun gene transcription is still unknown. Recently, we have reported that HE-145 selectively suppresses promoter activity of the hepatitis B viral core antigen gene in human hepatic cells [28]. We hypothesize that HE-145 may either interfere with formation of the specific transcriptional machinery on HBV core promoter *in vivo* or HE-145 may reduce the affinity of the transcriptional machinery for the corresponding cis-elements. Whether HE-145 employs a similar mechanism to suppress IL-1β-induced activation of the c-jun promoter needs further investigation.

In addition to c-jun, CREB1 was also shown to bind the AP-1/CRE site of MIP-1β promoter using a supershift assay (Fig. 6C). However, overexpression of CREB1 only slightly enhanced (2-fold) basal MIP-1β promoter activity and this activity can be further stimulated by IL-1β and is still subject to HE-145's suppression (Fig. 7B). These results suggest that the role of CREB1 in IL-1β-induced MIP-1β expression may only be modest. However, the protein level of exogenous overexpressing CREB1 was much less than exogenous overexpressing c-jun (Fig. 6C). We cannot completely rule out the possible involvement of CREB1 in IL-1β-induced MIP-1β gene activation and this area deserves further investigations.

In conclusion, we have identified a natural product HE-145 with novel anti-inflammatory activity. We have demonstrated that HE-145 suppresses IL-1β-induced MIP-1β expression mainly through inhibiting IL-1β-induced c-jun expression and reducing subsequent binding of c-jun/CREB1 complex to the essential AP-1/CRE site of MIP-1β promoter in human hepatic cells. The dual biological activities of HE-145, namely the inhibition of HBV replication and the inhibition of IL-1β-induced MIP-1β expression, not only opens up a new research avenue involving the study of the interactions between HE-145 and various specific transcriptional machinery *in vivo* but also suggests the possibility that HE-145 can be developed into a therapeutic agent for HBV-related hepatic inflammatory diseases.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.08.023.

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