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Indole derivatives inhibit hepatitis C virus replication through induction of pro-inflammatory cytokines

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Summary. – Previously, we discovered a series of indole derivatives as a new class of hepatitis C virus (HCV) replication inhibitors by using a target-free chemical genetic strategy. Through a structure-activity relationship study, the compound 12e was identified as the most potent inhibitor of this class (EC_{50} = 1.1 μmol/l) with minimal cytotoxicity (CC_{50} = 61.8 μmol/l). In order to gain insight into its detailed antiviral mechanism of action, we performed PCR array analyses and found that 12e was able to activate transcription of a number of pro-inflammatory as well as antiviral cytokine genes including CXCL-8, IL-1α, TNF-α, IL-3, IRAK-1, and DDX58. Their induction by 12e was verified by individual RT-PCR analyses. In addition, 12e was found to stimulate secretion of soluble factors with anti-HCV replication activity. Among the 12e-induced pro-inflammatory cytokines, CXCL-8 showed a strong positive correlation between its transcriptional activation and antiviral potency. Interestingly, a recombinant CXCL-8 protein also reduced HCV replication, though only moderately. In conclusion, we found a novel mode of action of indole derivatives in inhibiting HCV replication, particularly the induction of pro-inflammatory cytokines.

Keywords: hepatitis C virus; indole derivatives; replication inhibitors; CXCL-8; pro-inflammatory cytokines; transcriptional activation

Introduction

Hepatitis C virus (HCV) is a hepatotropic single-stranded RNA virus. It is responsible for chronic inflammatory liver diseases including liver cirrhosis and hepatocellular carcinoma. Around 170 million people are estimated to be active HCV-positive carriers worldwide (Shepard *et al.*, 2005). Approximately 40 ~ 45 % of all liver transplantations, which are performed in United States, are directly related to HCV infection (MukherjeeSorrell, 2008). Therefore, HCV-associated morbidity and mortality has been a severe burden on the health care system of many countries with a high incidence rate of HCV infection.

Combined treatment with pegylated interferon (IFN)-α and ribavirin has served as standard of care for most of HCV patients (Wilby *et al.*, 2012). However, undesirable side effects including flu-like symptoms, anemia, depression, and suicidal thoughts have been consistently associated with this IFN-based therapy. Thanks to recently approved direct-acting antivirals (DAAs) including NS3 protease inhibitors (Telaprevir and Boceprevir) and an NS5B polymerase inhibitor (Sofosbuvir), the current standard of care for HCV-infected patients is starting to move toward a multiple combination regimen composed of one of these DAAs plus pegylated IFN-α and ribavirin (Lee, 2013a). In addition, a number of promising clinical results were revealed recently regarding a possibility of IFN-free combination treatment with only DAAs (Everson *et al.*, 2014). However, in spite of their high efficacy and safety profiles, DAAs alone do not

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Abbreviations: DAA = direct-acting antiviral; EMCV = encephalomyocarditis virus; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; HCV = hepatitis C virus; IFN = interferon; IRES = internal ribosomal entry site

seem to play a major role in combination therapy for most HCV patients in the near future due to their relatively high price. Therefore, in order to develop a more affordable and effective regimen for treatment of HCV infection, a new class of anti-HCV therapeutics with a novel mechanism of action is still urgently needed.

For this purpose, we screened our in-house library comprising ~ 6000 compounds and identified compound 12e as the most potent inhibitor with minimal cytotoxicity (EC_{50} = 1.1 μmol/l and CC_{50} = 61.8 μmol/l) (Jin *et al.*, 2014). However, its detailed antiviral mode of action was not clear. Here, we demonstrated that the anti-HCV activity of 12e depends on the induction of pro-inflammatory as well as antiviral cytokine genes at transcriptional level. Especially, the induction of CXCL-8 gene seems to play an important role in the suppression of HCV replication by 12e.

Materials and Methods

Indole derivatives. All of the indole compounds including (*E*)-3-(1*H*-indol-3-yl)-*N*-isopropylacrylamide (2b), (*E*)-*N*-(3,4-dimethoxybenzyl)-3-(1*H*-indol-3-yl)acrylamide (2c), (*E*)-*N,N*-diethyl-3-(1*H*-indol-3-yl)acrylamide (2d), (*E*)-3-(1*H*-indol-3-yl)-2-methyl-*N*-(4-(trifluoromethyl)phenyl)acrylamide (8), (*E*)-*N*-(2-(dimethylamino)ethyl)-3-(1*H*-indol-3-yl)-2-methylacrylamide (10), (*E*)-*N*-(3-(1*H*-imidazol-1-yl)propyl)-3-(1*H*-indol-3-yl)-2-methylacrylamide (11), (*E*)-*N*-(4-*tert*-butylphenyl)-3-(1*H*-indol-3-yl)-2-methylacrylamide (12a), (*E*)-*N*-(4-*tert*-butylphenyl)-2-methyl-3-(5-methyl-1*H*-indol-3-yl)acrylamide (12c), (*E*)-*N*-(4-*tert*-butylphenyl)-3-(5-methoxy-1*H*-indol-3-yl)-2-methylacrylamide (12d), (*E*)-*N*-(4-*tert*-butylphenyl)-3-(5-cyano-1*H*-indol-3-yl)-2-methylacrylamide (12e), and (*E*)-*N*-(4-*tert*-butylphenyl)-2-methyl-3-(6-methyl-1*H*-indol-3-yl)acrylamide (12g) were synthesized as described previously (Jin *et al.*, 2014) (Table 2 and 3, Fig. 1a).

Cell culture. Huh7.5 cell line of the human hepatoma origin was cultured in monolayers as described previously (Blight *et al.*, 2002; Sklan *et al.*, 2007), in media consisting of DMEM (Sigma) supplemented with 1% L-glutamine (Hyclone), 1% penicillin, 1% streptomycin (Hyclone), and 10 % FBS (JR Scientific).

Plasmids. Rluc-J6/JFH1 (FL-J6/JFH1-5'C19Rluc2AUbi) is a monocistronic, full-length HCV genome that expresses a renilla luciferase and was derived from the previously described infectious genotype 2a HCV genome J6/JFH1 (Lindenbach *et al.*, 2005; Tscherne *et al.*, 2006). Bart79I is a high-efficiency bicistronic subgenomic replicon of HCV derived from an HCV genotype 1b Con1 sequence that harbors the neomycin phosphotransferase gene in the first cistron and the HCV nonstructural proteins in the second cistron under the translational control of an EMCV IRES (Blight *et al.*, 2002). This plasmid also has an adaptive mutation (S2204I) in NS5A, which increases replication efficiency. FL-J6/JFH1, FL-J6/JFH1-5'C19Rluc2AUbi and Bart79I plasmids are gifts from Dr. Charles Rice at the Rockefeller University. An IL-8 promoter-luciferase reporter vector (pGL3-pIL-8) was obtained from Dr. J.-S. Chun in Gwangju Institute of Science and Technology (Korea) (Choi *et al.*, 2006).

Production of HCV RNA by in vitro transcription. In vitro transcription to produce HCV RNA genomes was performed as previously described (Lee, 2013b). Briefly, WT Bart79I, J6/JFH1, or Rluc-J6/JFH1 plasmids were linearized by digestion with *ScaI* (Bart79I) or *XbaI* (J6/JFH1 and Rluc-J6/JFH1) (NEB). The T7 promoter-driven *in vitro* transcription was performed on the digested plasmid to produce the WT HCV RNA genomes using a MEGAscript kit (Ambion).

Generation of stable HCV replicon cell lines. The establishment of Huh7.5 cells stably maintaining a Bart79I subgenomic replicon in the presence of G418 selection was described elsewhere (Cho *et al.*, 2010). Briefly, the *in vitro*-transcribed Bart79I RNA was transfected into Huh7.5 cells using the Lipofectamine 2000 transfection reagent (*Invitrogen*). The transfected cells were supplemented with G418 to the final concentration of 125 μg/ml. This selection medium was replaced every 3 days for 3 weeks. The establishment of Huh7.5 cells stably maintaining a J6/JFH1 infectious clone was performed by transfecting the *in vitro*-transcribed J6/JFH1 RNA into Huh7.5 cells using the Lipofectamine 2000 transfection reagent. The establishment of Huh7.5 cells stably maintaining an Rluc-J6/JFH1 clone was performed similarly.

Cytotoxicity and luciferase assays. 1.8 x 10^4 cells stably transfected with the HCV replicon were plated onto a white 96-well plate (Costar 3610) and supplemented with DMSO or 1 nmol/l, 10 nmol/l, 100 nmol/l, 1 μmol/l, 10 μmol/l, and 100 μmol/l, respectively, of designated compound for 3 days. Cells were incubated for 3 hr at 37°C in the presence of EZ-CYTOX (10% tetrazolium salt; Dogen) reagent to assess cytotoxicity of the compound. Simultaneously, renilla luciferase activities were measured using a luciferase reagent (1 mmol/l coelenerazine in Methanol-HCL, Goldbio).

Real-time RT-PCR. 3 X 10^5 Huh7.5-J6/JFH1 or Bart79I cells were plated onto a 6-well plate (Costar 3610) and supplemented with DMSO or designated compound with indicated concentrations. At 3 days after incubation, total cellular RNA was extracted using the RNeasy® mini kit (Qiagen) in accordance with the manufacturer's instructions. The yield of extracted RNA was assessed spectrophotometrically. The expression of HCV and cellular RNA was measured by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis as previously described (Jin *et al.*, 2014). Each sample was normalized to the endogenous reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The cDNA quantification was performed by the CFX384 qRT-PCR detection system (Bio-Rad, US). The primers used in the qRT-PCR reactions were as follows: FW-J6/JFH1: 5'-CTCCGCCATGAATCACTC-3', RV-J6/JFH1 5'-ACGACACTCATACTAACGC-3', FW-Bart79I 5'-AGAGCCATAGTGGTCT-3', RV-Bart79I 5'-CCAAATCTCCAGGCATTGAGC-3', FW-GAPDH 5'-TGGTCTCCTCTGACTTCA-3', and RV-GAPDH 5'-CGTTGTCATACCAGGAAATG-3'. 7 sets of primers used for qRT-PCR analyses of following genes - CXCL-8, IL-1α, TNF-α, IL-3, TLR-7, IRAK-1, and DDX-58 - were purchased from Qiagen.

Table 1. Result of PCR array analyses of (A) cytokine and (B) antiviral genes induced by treatment with either 12e or IFN-α (Huh7.5-J6/JFH1 cells were treated with DMSO, 10 μmol/l of 12e, or 10 pg/ml of IFN-α for 3 days. PCR array analyses were performed on isolated RNA using antiviral and cytokine PCR array kits)

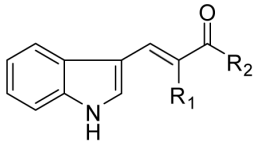
A

Gene	Fold induction by 12e	Fold induction by IFN-α	Gene	Fold induction by 12e	Fold induction by IFN-α
CXCL-8	6.67	1.47	CCL-3	1.96	1.22
IL-1α	5.98	3.22	CCL-8	1.96	1.22
TNF-α	5.69	1.19	CSF-2	1.96	1.22
IL-3	4.64	2.10	CXCL-13	1.96	1.22
IL-15	4.49	2.13	FASLG	1.96	1.22
CSF-3	4.21	2.23	IFN-α2	1.96	1.22
CXCL-12	4.18	0.70	IFN-γ	1.96	1.22
IL-5	3.91	1.06	IL-12β	1.96	1.22
IL-7	3.88	1.92	IL-17α	1.96	1.22
MSTN	3.85	1.50	IL-1β	1.96	1.22
SPP-1	3.81	1.80	IL-2	1.96	1.22
CCL-20	3.55	1.32	IL-4	1.96	1.66
IL-9	3.44	1.14	IL-6	1.96	1.22
CSF-1	3.38	1.47	OSM	1.96	1.22
CXCL-2	3.38	1.53	PPBP	1.96	1.22
TGF-β2	3.35	1.90	CXCL-16	1.9	1.28
IL-24	3.21	1.45	CCL-7	1.88	1.22
CXCL-5	3.09	1.37	IL-27	1.87	1.43
CCL-17	2.76	1.23	TNFSF-11	1.83	1.03
CXCL-1	2.71	1.43	IL-13	1.71	1.35
CCL-19	2.68	1.91	XCL-1	1.65	1.50
CNTF	2.61	1.47	MIF	1.63	1.26
BMP-6	2.47	1.22	BMP-2	1.6	1.27
CCL-5	2.46	3.37	BMP-4	1.59	1.17
CXCL-10	2.41	1.37	CX3CL-1	1.58	1.34
LTB	2.34	0.96	LTA	1.58	1.20
IL-18	2.08	1.23	LIF	1.55	1.14
IL-12α	2.07	1.13	IL-10	1.52	0.95
IL-22	2.02	1.71	VEGF-α	1.48	1.28
IL23α	2.02	1.69	RPLP0	1.29	0.95
IL17F	2.00	2.54	CD40Lγ	1.26	0.74
TNFSF13β	2.00	1.22	IL-16	1.18	0.68
CXCL9	1.99	1.41	CCL-13	1.16	1.37
TNFRSF11β	1.99	1.40	CXCL-11	1.16	1.73
ADIPOQ	1.96	1.22	GPI	1.14	1.21
BMP-7	1.96	1.22	ACTB	1.13	0.95
CCL-11	1.96	1.22	B2M	1.13	1.22
CCL-18	1.96	1.22	NODAL	1.08	0.90
CCL-2	1.96	1.22	IL-11	1.07	0.67
CCL-21	1.96	1.22	C5	0.98	1.16
CCL-22	1.96	1.22	CCL-1	0.97	1.11
CCL-24	1.96	1.22	IL-21	0.94	0.89

B

Gene	Fold induction by 12e	Fold induction by IFN-α	Gene	Fold induction by 12e	Fold induction by IFN-α
TLR-7	7.75	10.14	AZI-2	1.15	1.88
IRAK-1	6.03	2.40	CD-80	1.15	0.93
DDX-58	4.24	20.57	TBK-1	1.14	1.39
CXCL-8	3.68	4.32	TICAM-1	1.12	1.78
CASP-1	2.99	3.15	ATG-5	1.08	1.54
SPP-1	2.84	3.22	TRAF-6	1.07	1.40
CTSS	2.82	4.09	MX-1	1.06	484.86
TNF-α	2.55	1.11	MAP2K-1	1.03	0.6
JUN	2.47	2.10	ACTB	1.02	0.98
PIN-1	2.40	0.76	PSTPIP-1	0.98	1.54
CXCL-10	2.36	3.54	STAT-1	0.94	7.36
MAP3K-1	2.35	1.70	CXCL-9	0.93	1.78
IKBKB	2.27	1.94	IRF-3	0.93	0.74
IFIH-1	2.25	70.95	ISG-15	0.91	64.38
FOS	2.22	1.82	SUGT-1	0.88	1.28
DAK	2.06	1.63	RPLP-0	0.75	0.67
MAVS	1.77	1.72	MAP2K-3	0.73	1.01
IL-18	1.75	2.68	HSP90AA-1	0.69	1.04
NF-kBIA	1.71	1.44	HPRT-1	0.64	0.68
MAPK-3	1.66	1.18	CXCL-11	0.56	7.46
MAPK-14	1.59	1.45	APOBEC3G	0.55	0.93
GAPDH	1.56	1.17	CARD-9	0.55	0.93
CASP-10	1.55	1.61	CCL-3	0.55	0.93
IRF-5	1.54	0.93	CCL-5	0.55	3.24
IL-12α	1.52	1.81	CD-40	0.55	0.93
MYD-88	1.51	1.93	CD-86	0.55	0.93
CASP-8	1.49	2.20	CD-40	0.55	0.93
TRAF-3	1.49	1.53	CD-86	0.55	0.93
NF-kB1	1.47	1.5	DHX-58	0.55	0.93
IL-15	1.45	3.19	IFN- α1	0.55	0.93
CHUK	1.43	1.28	IFN- α2	0.55	0.93
FADD	1.42	1.61	IFN- β1	0.55	0.93
MAP3K7	1.41	1.12	IL-12β	0.55	0.93
IFN-aR1	1.39	1.21	IL-1β	0.55	0.93
CTSL-1	1.38	1.63	IL-6	0.55	1.07
DDX3X	1.36	1.50	IRF-7	0.55	0.93
TRIM-25	1.35	5.27	NLRP-3	0.55	0.93
MAPK-8	1.30	1.33	NOD-2	0.55	0.93
B2M	1.30	1.90	OAS-2	0.55	224.36
PYCARD	1.27	0.93	PYDC-1	0.55	0.93
MAPK-1	1.26	1.34	TLR-8	0.55	0.93
RIPK-1	1.17	1.11	TLR-9	0.55	0.93
CTSB	1.16	1.51	TRADD	0.53	1.4
TLR-3	1.16	5.65			

Table 2. Chemical structures of *trans*-3-indole acrylate derivatives including 2b, 8, 10, 11, and 12a



Code No.	Structure	
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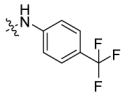
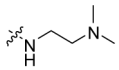
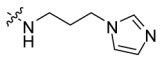
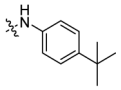
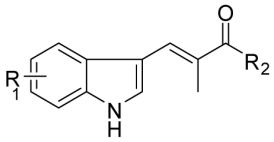
8	Me	
10	Me	
11	Me	
12a	Me	

Me = *Methyl* (CH_3).

Table 3. Chemical structures of indole 2-methylacrylamide derivatives including 12c, 12d, 12e, and 12g



Code No	Structure	
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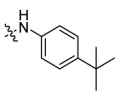
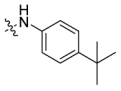
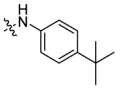
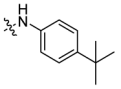
12c	5-Me	
12d	5-OMe	
12e	5-CN	
12g	6-Me	

Me = *Methyl* (CH_3).

Western blot analysis. 1.5 x 10^5 Huh7.5-J6/JFH1 or Bart79I cells were plated onto a 6-well plate (Costar 3610) and supplemented with DMSO or compound 12e with indicated concentrations. At 5 days after incubation, whole-cell extracts were prepared in RIPA buffer (150 mmol/l NaCl, 1 % Triton X-100, 1 % deoxycholic acid sodium salt, 0.1 % SDS, 50 mmol/l Tris-HCl, pH 7.5, 2 mmol/l EDTA; genDEPOT) containing a cocktail of protease inhibitors (Complete, Roche Diagnostic at final concentration of 1 tablet per 50 ml RIPA buffer) and quantitated by the Bradford assay (Bio-Rad). Equal amounts of protein were electrophoresed on an SDS–PAGE, subsequently transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford), and probed with a mouse anti-NS3 monoclonal antibody (1:1000, MAb 1847 for NS3, Virostat). Proteins were visualized via enhanced chemiluminescence (Amersham Pharmacia).

PCR array analyses. Total RNA was isolated from Huh7.5-J6/JFH1 cells treated with either DMSO or 12e for 3 days. The quality of isolated RNA was confirmed by spectrophotometric analysis of 260/280nm UV absorbance ratio. PCR array analyses were performed on isolated RNA using cytokine and anti-viral PCR array kits (PHAS-122ZE and PHAS-150ZE, Qiagen) according to the manufacturer's instructions. All individual experimental data were analyzed using the recommended software.

Effect of 12e-treated media on HCV replication. 3 × 10^6 Huh-7.5 cells were seeded in a 10 cm plate and supplemented with either DMSO or 10 μmol/l of compound 12e. After 2 days of incubation, cells were incubated with 12e-free media for additional 2 days to collect media containing secreted cytokines. After 3 × 10^3 Huh7.5-Rluc-J6/JFH1 cells were plated onto a white 96-well plate, these media were then transferred to Huh7.5-Rluc-J6/JFH1 cells to examine their effect on HCV replication by performing renilla luciferase and cytotoxicity assays after 3 days of incubation.

FACS analysis. FACS analysis was conducted as described previously (Min *et al.*, 2012). Briefly, 3 ×10^5 Huh7.5, Huh7.5-J6/JFH1, or Huh7.5-Bart79I cells were plated onto a 6-well plate (Costar 3516) and supplemented with either DMSO or 10 μmol/l of 12e. After 3 days of incubation, cells were fixed at room temperature for 20 min in 4 % paraformaldehyde, permeabilized in 0.1% Triton-X in PBS for 5 min, rinsed three times in PBS, and blocked with PBS with 2% FBS. A mouse anti-CXCL-8 antibody (M801, Thermo Scientific) was diluted in blocking buffer, and the mixture was incubated for overnight at 4°C. After three washes in PBS, cells were incubated with an Alexa 488-conjugated anti-mouse IgG secondary antibody (Invitrogen, Carlsbad, CA) for 1 hr. Following three washes with PBS, cells were resuspended in PBS. FACS analysis was performed by the FACSAria 3 system (BD, USA).

ELISA of CXCL-8 protein. 1.5 × 10^5 Huh7.5, Huh7.5-J6/JFH1 or Bart79I cells were plated onto a 6-well plate (Costar 3610) and supplemented with DMSO or compound 12e with indicated concentrations for 3 days. The concentration of CXCL-8 protein in media was measured using human IL-8 ELISA kit (Thermo Scientific). The analysis was performed according to the manufacturer's protocol.

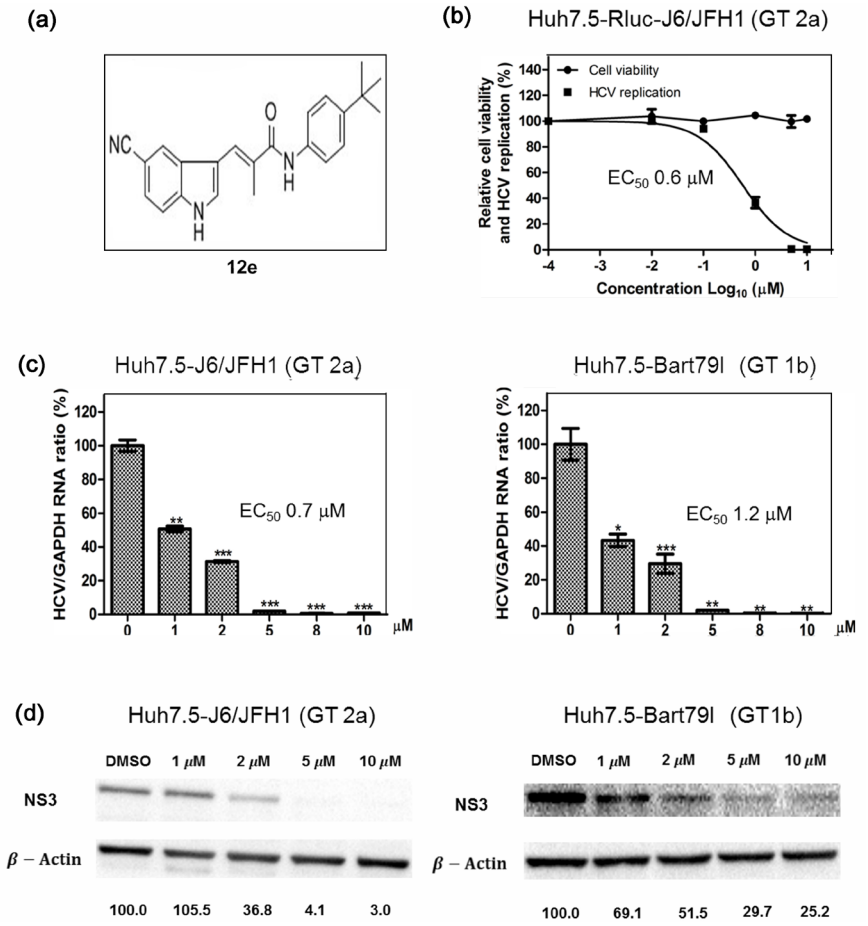


Fig. 1
Inhibition of HCV replication by 12e

(a) The chemical structure of compound 12e (b) Dose-response curve was determined by assaying relative cell viability and luciferase activity in Rluc-J6/JFH1 RNA-transfected Huh7.5 cells treated with increasing concentrations of the compound 12e for 72 hr. (c) Dose-response graphs were determined by assaying relative HCV as well as GAPDH RNA levels via qRT-PCR analysis of either J6/JFH1 or Bart79I RNA-transfected Huh7.5 cells treated with increasing concentrations of the compound 12e for 72 hr. The statistical significance of differences relative to controls (DMSO) is indicated as *p <0.1, **p <0.01 or ***p <0.001 by Student's t test. (d) Dose-response was determined by assaying relative levels of HCV NS3 as well as host β-actin proteins via Western blot analyses of either J6/JFH1 or Bart79I RNA-transfected Huh7.5 cells treated with increasing concentrations of the compound 12e for 120 hr. Error bars represent SD values. GT = genotype.

Effect of a recombinant CXCL-8 protein on HCV replication. 1.8×10^4 Huh7.5-Rluc-J6/JFH cells were plated onto a white 96-well plate (Costar 3610) and supplemented with DMSO or a recombinant CXCL-8 protein at a concentration starting in the range from 2 pg/ml to 20 ng/ml (GTX48422-PRO, GeneTex). After 3 days of incubation, cells were incubated for 3 hr at 37°C in the presence of EZ-CYTOX (10 % tetrazolium salt; Dogen) reagent to assess the cytotoxicity. Renilla luciferase activities were measured using a luciferase reagent (1 mmol/l coelenerazine in Methanol-HCL, Goldbio).

CXCL-8 promoter reporter assay. An IL-8 promoter-luciferase reporter vector (pGL3-pIL-8) was transfected into Huh7.5-Rluc-J6/JFH1 cells. The transfected cells were then treated with 10 μmol/l of indole compound for 48 hr. A dual luciferase assay was conducted using Glomax dual luciferase assay kit (Promega) to simultaneously measure the firefly luciferase for CXCL-8 promoter activity and the renilla luciferase for HCV replication.

Results

12e inhibits HCV replication

Before we started the study of the mechanism of action for compound 12e (its chemical structure shown in Fig. 1a), we decided to reconfirm its anti-HCV replication activ-

ity. For this purpose, Huh7.5 hepatocarcinoma cells were transfected with renilla luciferase-linked J6/JFH1 RNAs (Huh7.5-Rluc-J6/JFH1 (genotype (GT) 2a)) (Tscherne *et al.*, 2006) and the luciferase activity was measured to assess the HCV RNA replication levels. As shown in Fig. 1b, treatment with 12e at the concentration of 0.6 μmol/l (EC_{50}) was able to suppress HCV replication by half without any significant cytotoxicity until 10 μmol/l. A reporter-free infectious clone system (Huh7.5-J6/JFH1 (GT 2a)) as well as a subgenomic replicon system (Huh7.5-Bart79I (GT 1b)) reached a similar range of EC_{50} values (0.7 μmol/l and 1.2 μmol/l, respectively) by qRT-PCR analyses (Fig. 1c). The dose-dependent down-regulation of expression of a viral protein, NS3, upon the treatment with an increasing dose of 12e was further confirmed by two sets of Western blot analyses using Huh7.5-J6/JFH1 as well as Huh7.5-Bart79I cells (Fig. 1d). These data demonstrated a potent inhibitory activity of 12e against HCV replication.

12e activates pro-inflammatory and antiviral cytokine genes

Induction of host antiviral cytokine genes by viral infection plays a major role in combating viral replication in host cells. Especially, several well-characterized anti-HCV therapeutics including IFN-α and ribavirin were shown to exhibit their antiviral activities through induction of specific host antiviral cytokine genes at the transcriptional level (Tokumoto *et al.*, 2012). After reconfirming suppression of HCV replication by 12e, we hypothesized that 12e might be able to alter transcriptional profiles of key host cytokines as well as antiviral genes in order to facilitate its antiviral activity. To verify this hypothesis, we performed two sets of PCR array analyses, which enabled us to examine effects of 12e on transcriptional levels of 84 cytokines (Table 1A) and 87 antiviral (Table 1B) genes from Huh7.5-J6/JFH1 cells. As shown in Table 1B, well-known IFN-α-responsive genes including MX1 (484.85-fold), OAS2 (224.36-fold), IFIH1 (70.95-fold), ISG15 (64.38-fold), DDX58 (20.57-fold), and TLR7 (10.14 fold) were highly induced by treatment with 10 pg/ml of IFN-α, validating the specificity of these PCR array analyses. Interestingly, 12e was able to activate transcription of a subset of pro-inflammatory genes including CXCL-8 (6.67-fold), IL-1α (5.98-fold), TNF-α (5.69-fold), and IL-3 (4.64-fold), and antiviral genes including TLR-7 (7.75-fold), IRAK-1 (6.03-fold), DDX-58 (4.24-fold), and CXCL-8 (3.68-fold). Especially, CXCL-8 gene showed the first and fifth highest fold-induction values among cytokine and antiviral genes induced by treatment of 12e, respectively. In order to verify the transcriptional activation of these seven individual genes by 12e, we also conducted a series of individual qRT-PCR analyses. As shown in Fig. 2, the dose-dependent increase in the mRNA levels of CXCL-8, TNF-α, IL-3, TLR-7, and IRAK-1 genes after the treatment with 12e was further confirmed except for IL-1α. These data suggest that 12e is able to activate transcription of a subset of key pro-inflammatory and antiviral cytokine genes.

12e stimulates extracellular secretion of soluble factors leading to the suppression of HCV replication

After confirming transcriptional up-regulation of a unique subset of cytokine genes, we hypothesized that 12e might be able to stimulate extracellular secretion of specific cytokine proteins, which might be responsible for indirect inhibition of HCV replication by 12e via a paracrine manner. To test this hypothesis, Huh7.5 cells were treated with either DMSO or 12e for 48 hr, and then incubated with drug-free fresh media for another 48 hr. These conditioned media were then transferred to Huh7.5-Rluc-J6/JFH1 cells to examine their effect on HCV replication. Unlike DMSO-pretreated media (Fig. 3a), 12e-treated media showed a strong inhibitory activity against HCV replication in a dose-dependent manner without any major effect on cell viability (Fig. 3b). The dose-dependent 12e-specific antiviral activity was more evident when relative cell viability and HCV replication values were plotted as a ratio (Fig. 3c). These data suggest that 12e is able to stimulate secretion of soluble factors with anti-HCV replication activities.

Transcriptional activation of CXCL-8 gene by indole derivatives correlates with their antiviral potentials

According to our previous PCR array analyses, CXCL-8 was the only gene whose transcriptional activation was validated in both pro-inflammatory and antiviral PCR array analyses. Therefore, we decided to focus on a potential role of CXCL-8 gene in the suppression of HCV replication by 12e. In order to study a direct relationship between transcriptional activation of CXCL-8 and down-regulation of HCV replication by 12e in a more detailed manner, we treated Huh7.5-J6/JFH1 and Huh7.5-Bart79I cells with an increasing dose of 12e and examined levels of CXCL-8 mRNA and HCV RNA simultaneously. As expected, both Huh7.5-J6/JFH1 and Huh7.5-Bart79I cells exhibited around 8- and 3-fold increase, respectively, in the amount of CXCL-8 mRNAs upon the treatment with 10 μmol/l of 12e (Fig. 4a and 4b). In addition, we were also able to observe a sharp decrease in HCV RNA levels in both Huh7.5-J6/JFH1 and Huh7.5-Bart79I cells treated with 12e. Even Huh7.5 cells without any HCV genome showed a robust increase in CXCL-8 mRNA level upon treatment of 12e in a dose-dependent manner (Fig. 4c). Of note, BMS-790052, an NS5A inhibitor did not affect a transcriptional level of CXCL-8 gene despite its superior inhibitory potency against HCV replication (Fig. 4a,b,c). These data imply that transcriptional activation of CXCL-8 gene by 12e is not due to a mere indirect result of the loss of HCV replication.

In order to further validate this negative relationship between transcriptional activation of CXCL-8 gene and HCV replication, we examined the effects of other previously identified indole derivatives on levels of CXCL-8 mRNA. For this purpose, we used a qRT-PCR analysis as well as a CXCL-8 promoter-linked luciferase reporter assay. As shown in Fig. 5a, two indole compounds such as 12a (chemical structure shown in Table 2) and 12e, which exhibited strongest antiviral potencies, also showed highest level of a transcriptional activation of CXCL-8 gene. On the other hand, three other indole compounds – 2b, 8, and 10 (chemical structures shown in Table 2), which exhibited weaker antiviral potencies - also showed lower levels of a transcriptional activation of CXCL-8 gene. This trend was also true for other compounds including 12c, 12d, 12e, and 12g (structures shown in Table 3). Their high CXCL-8 promoter-stimulating activities always resulted in potent antiviral capabilities in Huh7.5-Rluc-J6/JFH1 cells (Fig. 5b). These data strongly suggest that the induction CXCL-8 gene is closely linked with the inhibition of HCV replication by indole derivatives.

Transcriptional activation of CXCR-8 gene by 12e leads to the induction of CXCR-8 protein

Based on previous results, we hypothesized that the transcriptional activation of CXCL-8 gene by 12e should lead

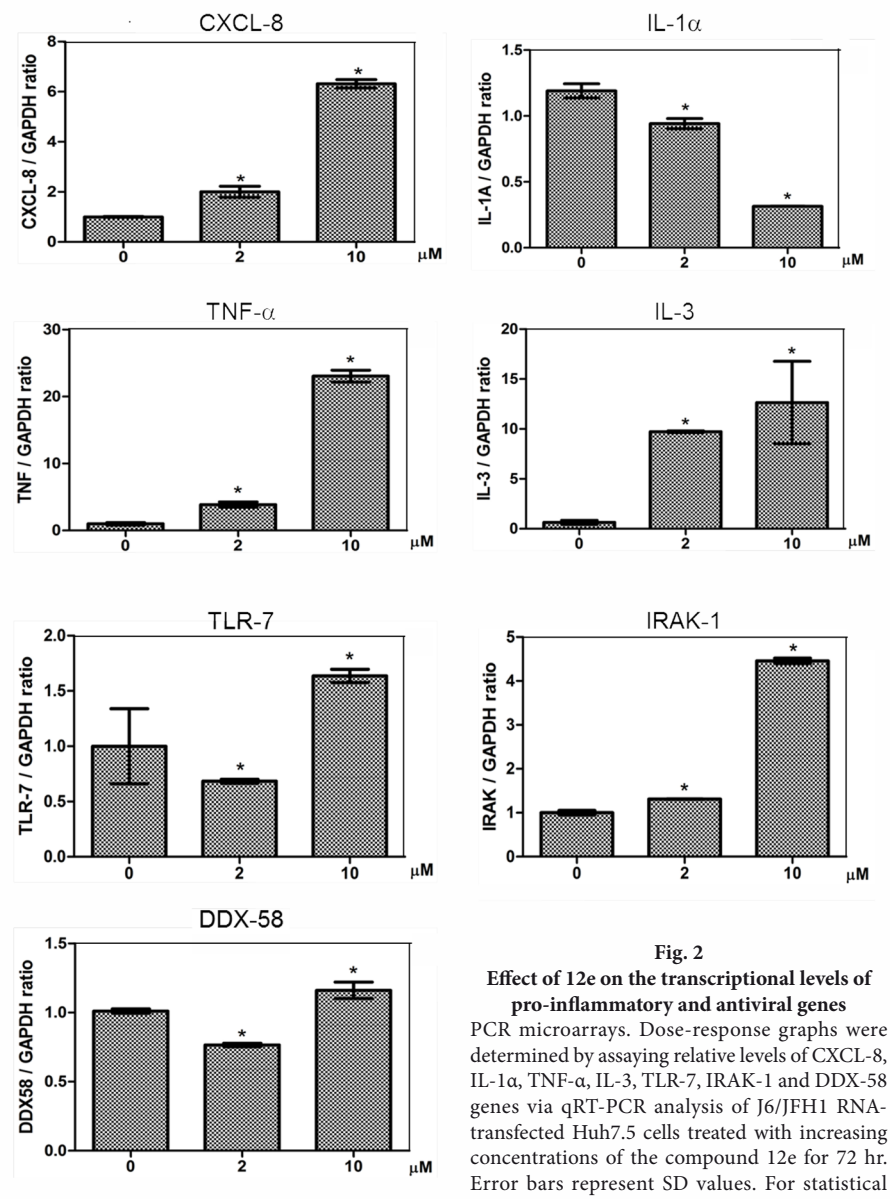


Fig. 2
Effect of 12e on the transcriptional levels of pro-inflammatory and antiviral genes
PCR microarrays. Dose-response graphs were determined by assaying relative levels of CXCL-8, IL-1α, TNF-α, IL-3, TLR-7, IRAK-1 and DDX-58 genes via qRT-PCR analysis of J6/JFH1 RNA-transfected Huh7.5 cells treated with increasing concentrations of the compound 12e for 72 hr. Error bars represent SD values. For statistical significance and error bars see above.

to an increased extracellular secretion of CXCL-8 protein. In order to test this hypothesis, we decided to stain Huh7.5, Huh7.5-J6/JFH1, and Huh7.5-Bart79I cells with an anti-CXCL-8 antibody after treatment with 12e for 3 days. An Alexa 488-conjugated polyclonal anti-mouse antibody was used as a secondary antibody for FACS analysis in order to quantitate the percentage of CXCR-8-positive cells. As shown in Fig. 6a, upon treatment with 12e, the number of CXCR-8-positive cells increased by 67, 121, and 20 % in Huh7.5, Huh7.5-J6/FHJ1, and Huh7.5-Bart79I cells, respectively, when compared with DMSO-treated cells. A significant increase in the extracellular level of CXCR-8 protein upon treatment of 12e was further verified by ELISA analysis of extracellular levels of secreted CXCL-8 protein in the media from Huh7.5, Huh7.5-J6/FHJ1, and Huh7.5-Bart79I cells (Fig. 6b). Of note, BMS-790052 again failed to induce any CXCL-8 protein, suggesting suppression of HCV replication itself is not sufficient for induction of extracellular level of CXCL-8 protein. These data suggest that the transcriptional activation of CXCR-8 gene by 12e leads to the induction and secretion of CXCR-8 protein.

Recombinant CXCL-8 protein partially reduces HCV replication

After confirming an indirect antiviral activity of extracellular proteins whose secretion was stimulated by 12e, we tested whether CXCL-8 protein itself possesses a direct antiviral activity. For this purpose, we purchased a commercially available recombinant CXCL-8 protein. Specificity of this recombinant CXCL-8 protein was first confirmed by successful detection of 50 pg of CXCL-8 protein by Western blot analysis using an anti-CXCL-8 antibody (Fig. 7a), which was employed previously in our FACS analysis (Fig. 6a). Then, Huh7.5-Rluc-J6/JFH1 cells were treated with an increasing concentration of this recombinant CXCL-8 protein to see if it has any direct effect on HCV replication. As shown in Fig. 7b, we observed around 30 % reduction in the level of HCV replication upon the treatment with the recombinant CXCL-8 protein at 200 pg/ml. This moderate inhibitory activity of CXCL-8 against HCV replication was maintained up to 20 ng/ml. These data suggest that the treatment with recombinant CXCL-8 proteins is able to induce a partial reduction in HCV replication.

Discussion

In this paper, we described that one of the most potent anti-HCV replication inhibitor with an indole moiety, 12e, was able to induce transcriptional activation of a number of pro-inflammatory as well as antiviral cytokine genes including CXCL-8, IL-1α, TNF-α, IL-3, IRAK-1, DDX58, and TLR-7. In addition, we also found that secretion of soluble factors stimulated by 12e plays a significant role in suppression of HCV replication in HCV-positive neigh-

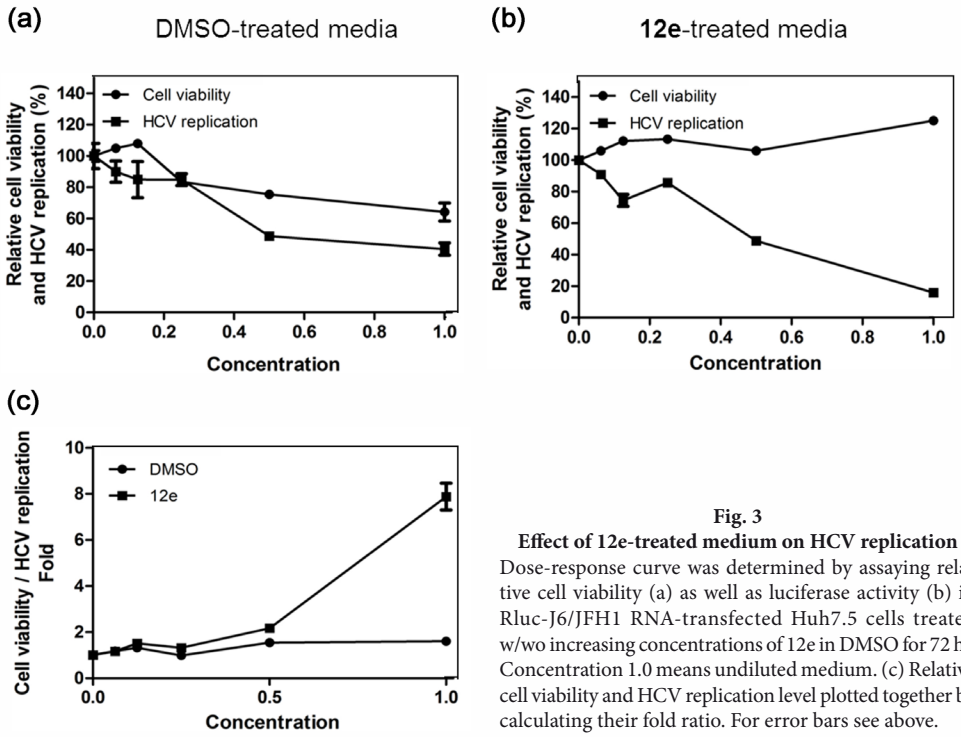


Fig. 3
Effect of 12e-treated medium on HCV replication
Dose-response curve was determined by assaying relative cell viability (a) as well as luciferase activity (b) in Rluc-J6/JFH1 RNA-transfected Huh7.5 cells treated w/wo increasing concentrations of 12e in DMSO for 72 hr. Concentration 1.0 means undiluted medium. (c) Relative cell viability and HCV replication level plotted together by calculating their fold ratio. For error bars see above.

boring cells. Existence of a strong positive correlation between transcriptional activation of CXCL-8 and antiviral potency by other members of this class of compounds was further validated by a qRT-PCR analysis and a CXCL-8 promoter-linked luciferase reporter assay.Finally, direct treatment with a recombinant CXCL-8 protein also resulted in a moderate reduction in HCV replication. All these data strongly suggest that indole derivatives tested inhibit HCV replication through induction of a number of pro-inflammatory genes.

Ribavirin is a nucleotide analog with a broad range of antiviral capability against a number of RNA and DNA viruses (Sidwell *et al.*, 1972). As explained previously, ribavirin has played a critical role in suppression of HCV infection as an essential component of the first line of HCV therapeutics together with pegylated IFN-α, since ribavirin is able to eliminate HCV only when combined with IFN. However, its detailed mode of antiviral action has been elusive so far. A recent study demonstrated that ribavirin is able to regulate HCV replication through enhancing IFN-stimulated genes

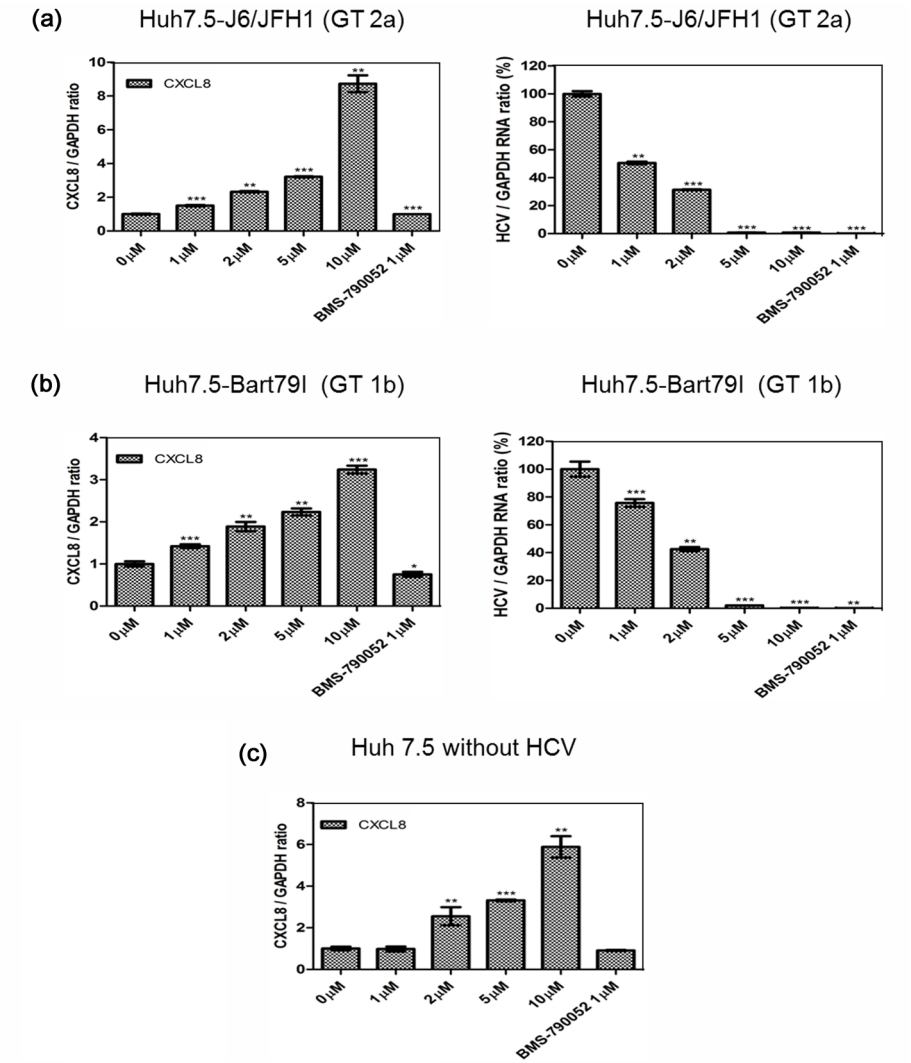


Fig. 4
Transcriptional induction of CXCL-8 by 12e

Dose-response graphs were determined by assaying relative CXCL-8, HCV as well as GAPDH RNA levels via qRT-PCR analysis of either J6/JFH1 (a) or Bart79I (b) RNA-transfected Huh7.5 cells treated with increasing concentrations of the compound 12e for 72 hr. NS5A inhibitor BMS-790052 (1 μmol/l) was used as a positive control for suppression of HCV replication. (c) A dose-response graph was determined by assaying relative CXCL-8 as well as GAPDH RNA levels via qRT-PCR analysis of Huh7.5 cells treated with increasing concentrations of the compound 12e for 72 hr. For error bars and statistical significance see above.

(ISGs) and CXCL-8 (Tokumoto *et al.*, 2012). In this paper, the authors measured mRNA levels of ISGs in T lymphocytes from patients with HCV infection, who were receiving IFN-α therapy with or without ribavirin. They found that average levels of CXCL-8 mRNA were much higher in IFN-α- and ribavirin-treated patients than in those treated with IFN-α alone. In addition, the up-regulation of CXCL-8 expression by ribavirin was confirmed in three HCV replicon cells regardless of IFN-α treatment (Tokumoto *et al.*, 2012). In this regard, compound 12e has a high degree of similarity to ribavirin in the antiviral mode of action, since both of them require enhanced transcription of CXCL-8 gene in order to exert their antiviral activity. It would be interesting to see what kind of effect a co-treatment with 12e and ribavirin would have on HCV replication. If the transcriptional activation of CXCL-8 gene is the major mechanism of action for both molecules, we expect that there would be no additive or synergistic down-regulation of HCV replication after the co-treatment with 12e and ribavirin.

Regulation of CXCL-8 expression is a complicated process working at both transcriptional and post-transcriptional levels (Koo *et al.*, 2006). CXCL-8 induction requires either transcriptional activation of CXCL-8 promoter or stabilization of CXCL-8 mRNA (Holtmann *et al.*, 1999). Therefore, an increase in CXCL-8 mRNA levels after the treatment with 12e might be due to the direct up-regulation of either transcription factors involved in CXCL-8 promoter activation or other host factors involved in regulation of CXCL-8

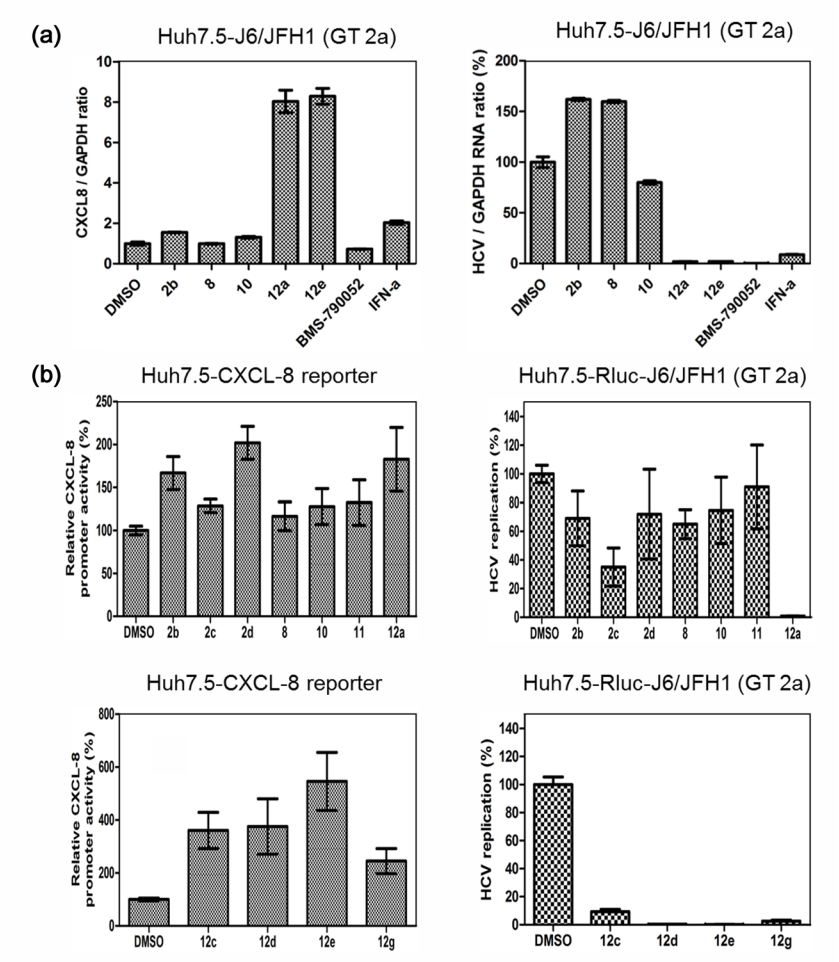


Fig. 5
Inverse correlation between transcriptional induction and HCV replication by 12e
(a) Dose-response graphs were determined by assaying relative CXCL-8, HCV as well as GAPDH RNA levels via qRT-PCR analysis of J6/JFH1 RNA-transfected Huh7.5 cells treated with 10 μmol/l of compounds 2b, 8, 10, 12a and 12e for 72 hr. (b) Dose-response graphs were determined by assaying relative CXCL-8 promoter activity and HCV replication levels via dual luciferase analysis of both CXCL-8 promoter-linked firefly luciferase reporter DNA and Rluc-J6/JFH1 RNA-transfected Huh7.5 cells treated with 10 μmol/l of compounds 2b, 2c, 2d, 8, 10, 11, 12a, 12c, 12d, 12e, and 12g for 72 hr. For BMS-790052 and error bars see above.

mRNA stability. A number of transcription factors including NF-kB, IRFs, AP-1, and C/EBP have been showed to be necessary for transcription activation of CXCL-8 gene (Casola *et al.*, 2000). Therefore, in order to answer which transcription factor is targeted by 12e, a series of CXCL-8 promoter-linked luciferase reporters lacking binding sites for each transcription factor need to be constructed in the future. On the other hand, CXCL-8 expression also involves AU-rich elements in 3'-untranslated region that regulates mRNA stability (Green *et al.*, 2006). In this regard, it would be also interesting to see whether 12e exerts any positive effect on regulation of CXCL-8 mRNA stability through altered binding of host factors on these AU-rich elements in 3'-untranslated region of CXCL-8 mRNA.

CXCL-8 serves as one of the major chemokines that send a chemical signal to attract neutrophils to the site of inflammation (Van Damme *et al.*, 1989). One possible benefit of 12e-induced transcriptional activation of CXCL-8 gene would be to stimulate neutrophils, which are attracted by CXCL-8, to digest HCV antigens, which in turn results in increased IFN-α and ISG production *in vivo*. Interestingly, we were not able to see an increase in secretion of CXCL-8 protein in the macrophage cell line (RAW 264.7) treated with 12e (data not shown). This suggests that there is an

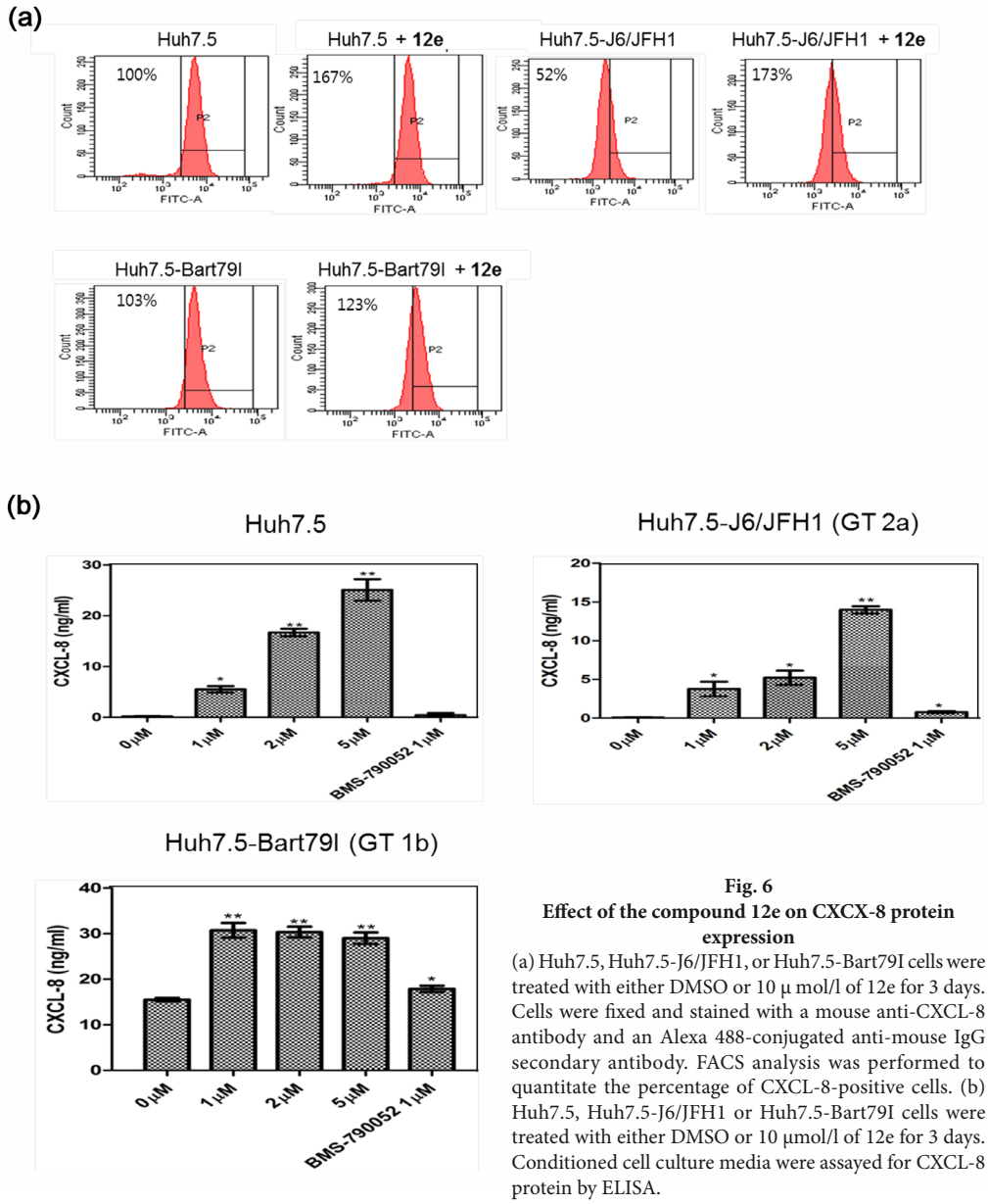


Fig. 6
Effect of the compound 12e on CXCX-8 protein expression

(a) Huh7.5, Huh7.5-J6/JFH1, or Huh7.5-Bart79I cells were treated with either DMSO or 10 μ mol/l of 12e for 3 days. Cells were fixed and stained with a mouse anti-CXCL-8 antibody and an Alexa 488-conjugated anti-mouse IgG secondary antibody. FACS analysis was performed to quantitate the percentage of CXCL-8-positive cells. (b) Huh7.5, Huh7.5-J6/JFH1 or Huh7.5-Bart79I cells were treated with either DMSO or 10 μmol/l of 12e for 3 days. Conditioned cell culture media were assayed for CXCL-8 protein by ELISA.

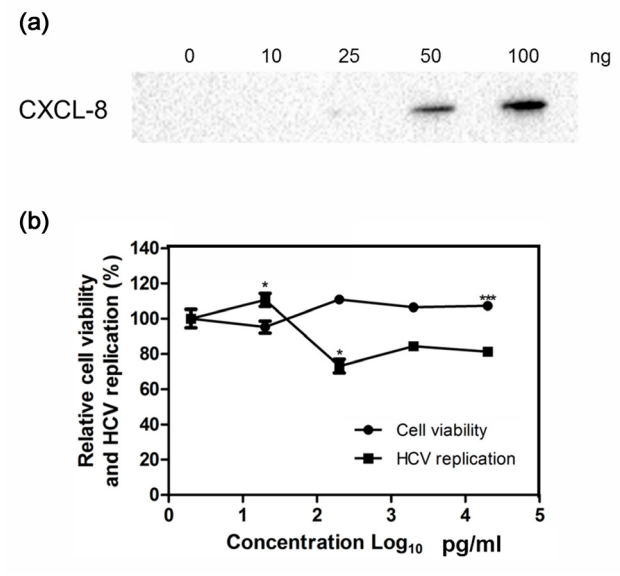


Fig. 7
Effect of the recombinant CXCX-8 protein on HCV replication
(a) A fixed amount of recombinant CXCX-8 protein (0, 10, 25, 50, and 100 pg) was tested for its reactivity with anti-CXCL-8 antibody by Western blot analysis. (b) Huh7.5-Rluc-J6/JFH1 cells were treated with an increasing concentration of the recombinant CXCL-8 protein for 3 days. Relative cell viability and HCV replication were determined by MTT and renilla luciferase assays.

intrinsic difference in the way of transcriptional activation of CXCL-8 gene in the liver and in immune cells.

It has been well known that there is a positive relationship between HCV replication and CXCL-8 production (Koo *et al.*, 2006). In this regard, the up-regulation of CXCL-8 induced by 12e would not help elimination of HCV infection. In the worst scenario, this might lead to the weakening of the antiviral efficacy of other co-administered anti-HCV drugs. Moreover, CXCL-8 was shown to play a major role in induction of inflammatory response such as viral hepatitis. Our observation of a negative relationship between HCV replication and transcriptional activation of CXCL-8 gene seems to be a sharp contrast to the previous observations. One plausible explanation for this discrepancy might be based on the following hypothesis. HCV may need to intentionally increase the amount of CXCL-8 up to a certain level to support its optimal RNA replication. Excessive up-regulation of CXCL-8 induced by the treatment with 12e might surpass this optimal concentration of CXCL-8 required for HCV replication and this might lead to a deleterious outcome to the HCV replication. We are now in the process of performing relevant experiments to test this hypothesis. In addition, confirming the antiviral efficacy of 12e using a humanized liver mouse model would help us to understand a detailed antiviral mechanism of action for 12e in the context of more *in-vivo-like* setting.

We were only able to see a partial reduction of HCV replication after the treatment with the recombinant CXCL-8 protein. We were not able to increase the concentration of the recombinant CXCL-8 protein to more than 20 ng/ml due to the restrictions resulting from the original concentration of the recombinant CXCL-8 protein. Treatment with 5 μmol/l of 12e increased the extracellular concentration of CXCL-8 to up to 25 ng/ml. These data indicate that CXCL-8 protein alone may not be enough to suppress the HCV replication by itself. CXCL-8 may rather need to work together with other pro-inflammatory cytokines such as IL-1α, TNF-α, IL-3, IRAK-1, and DDX58 in order to achieve the full repression of HCV replication. It would be interesting to test the combined effect of these cytokines on HCV replication in the future.

In conclusion, we showed a set of specific pro-inflammatory cytokine and chemokine proteins induced by 12e that play an important role in the 12e-dependent anti-HCV activity. Especially, the up-regulation of CXCL-8 protein together with other pro-inflammatory cytokines may play a critical role in suppression of HCV replication by 12e. Based on these results, we anticipate that indole derivatives could be further developed in the near future to serve as a new class of anti-HCV therapeutics with a novel mode of action.

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