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A novel cantharidin analog *N*-Benzylcantharidinamide reduces the expression of MMP-9 and invasive potentials of Hep3B via inhibiting cytosolic translocation of HuR

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ABSTRACT

Invasion and metastasis are major causes of malignant tumor-associated mortality. The present study aimed to investigate the molecular events underlying inhibitory effect of *N*-Benzylcantharidinamide, a novel synthetic analog of cantharidin, on matrix metalloproteinase-9 (MMP-9)-mediated invasion in highly metastatic hepatocellular carcinoma Hep3B cells. In this investigation, among six analogs of cantharidin, only *N*-Benzylcantharidinamide has the inhibitory action on MMP-9 expression at non-toxic dose. The MMP-9 expression and invasion of Hep3B cells were significantly suppressed by treatment of *N*-Benzylcantharidinamide in a dose-dependent manner. On the other hand, the transcriptional activity of MMP-9 promoter and nuclear levels of NF-κB and AP-1 as the main transcriptional factors inducing MMP-9 expression were not affected by it although the level of MMP-9 mRNA was reduced by treatment of *N*-Benzylcantharidinamide. Interestingly, the stability of MMP-9 mRNA was significantly reduced by *N*-Benzylcantharidinamide-treatment. In addition, the cytosolic translocation of human antigen R (HuR), which results in the increase of MMP-9 mRNA stability through interaction of HuR with 3′-untranslated region of MMP-9 mRNA, was suppressed by treatment of *N*-Benzylcantharidinamide, in a dose-dependent manner. Taken together, it was demonstrated, for the first time, that *N*-Benzylcantharidinamide suppresses MMP-9 expression by reducing HuR-mediated MMP-9 mRNA stability for the inhibition of invasive potential in highly metastatic Hep3B cells.

1. Introduction

Cancer invasion and metastasis are landmark events that transform a locally growing tumor into a systemic, metastatic, and life-threatening disease [1]. To invade surrounding tissue such as extracellular connective tissues and the basement membrane, cancer cells produce proteolytic enzymes, including Matrix metalloproteinases (MMPs) [2]. Among the various MMPs, MMP-9 has been focused in the studies of tumor metastasis because they are expressed in a variety of cancer cells and could degrade type IV collagen, a major component of the basement membrane [3]. Recently, MMP-9 was considered as modulators of signaling pathways that control cell growth, migration, and angiogenesis via in a non-enzymatic manner [2,4].

Cantharidin is a naturally occurring compound isolated from the medicinal insect blister beetle (*Mylabris phalerata* Pallas) [5], and has been used for topical medication for warts, furuncles, and skin ulcer [6]. Recently, it has been shown that cantharidin induces apoptosis in various types of tumor cells, including cervical, tongue, gingival, mucoepidermoid carcinoma, adenocystic carcinoma, neuroblastoma, bone, leukemia, ovarian, colon, and liver cancer [7] . However, the application of cantharidin is limited due to its severe side-effects such as dysphagia, dysuria, hematemesis, liver congestion and renal toxicity [8]. Therefore, many efforts

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to reduce toxicity while maintaining anti-tumor efficacy have been studies. Norcantharidin, a chemically demethylated analog of cantharidin, also possesses anti-cancer activity and less renal toxicity [5,7]. Recently, it was reported that norcantharidin and cantharidin has anti-metastatic effect via inhibiting expression of MMP-9 through Sp1 or p38/JNK pathways, respectively [9,10].

In present study, four novel synthetic analogs of norcantharidin and cantharidin were examined for suppressing MMP-9 expression in Hep3B cells, a highly metastatic hepatocellular carcinoma [11]. Among these compounds, *N*-Benzylcantharidinamide significantly inhibited the expression of MMP-9 at non-toxic dose. The results showed that *N*-Benzylcantharidinamide suppressed the expression of MMP-9 through the reduction of MMP-9 mRNA stability, but not through the inhibition of MMP-9 transcriptional activity. The cytosolic translocation of Human antigen R (HuR) was involved in regulating mRNA stability of MMP-9. To the best of our knowledge, this is the first report that *N*-Benzylcantharidinamide, a novel synthetic analog of cantharidin, has an anti-invasive activity on hepatocellular carcinoma.

2. Materials and methods

2.1. Materials

Antibodies for p65 subunit of NF-κB, c-jun, c-fos, hnRNP, HuR, and β-actin were purchased from Santz Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for MMP-9 was supplied by Calbiochem (San Diego, CA, USA). Cantharidin and norcantharidin was purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals and reagents were purchased from Sigma–Aldrich, unless otherwise stated.

2.2. Cell culture and viability assay

Hep3B cells were obtained from the ATCC (American Type Culture Collection, Rockville, MD, USA) and grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco–BRL) containing L-glutamine (200 mg/L) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Sigma–Aldrich) and 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator at 37 °C, 5% CO_2 prior to experimentation. The cytotoxicity caused by each cantharidin analogs was examined as a MTT assay. In brief, Hep3B cells were cultured in 24-well plates with each cantharidin analogs (at the indicated concentrations) for 24 h. Then, MTT solution (2.0 mg/mL) was added to each well containing cells. At 4 h after incubation at 37 °C in a CO_2 cell culture incubator, the supernatants were removed, and formazan crystals formed in viable cells were measured at 540 nm with a microplate reader. The percentage of living cells was calculated against untreated cells.

2.3. Gelatin zymography assay

The cells were grown in serum-free conditioned medium with the indicated concentrations of each cantharidin analogs for 24 h. The amount of secreted protein in the conditioned media was estimated by cell numbers. Conditioned media were prepared in a sample buffer containing 62.5 mM Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, and 0.00625% (w/v) bromophenol blue without boiling. The samples were loaded in an acrylamide/bisacrylamide (29.2:0.8) separating gel containing 0.1% (w/v) gelatin, and then electrophoresized at 4 °C. The gels were washed twice in 0.25% Triton X-100 for 30 min at room temperature and were incubated at 37 °C in the incubation buffer [50 mM Tris–HCl (pH 7.5), 5 mM $CaCl_2$, 3 mM NaN_3 and 1 μM $ZnCl_2$]. At 18 h after incubation, the gels were stained for 30 min in 0.5% (w/v) Coomassie blue R-250 solubilized in 30% methanol and 10% acetic acid, and then incubated in a destaining solution with 30% methanol and 10% acetic acid. The stained gel was photographed and bands corresponding to the activity of MMP-9 were quantified with the ImageJ (NIH, Bethesda, MD, USA) software.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Equal amounts of total RNA were subjected to reverse transcription with oligo-dT primers, using AccuPower RT-PreMix (Bioneer, Daejon, Korea). The cDNA was amplified by performing PCR with the following primers, using AccuPower PCR-PreMix (Bioneer). The oligonucleotide primer sequences were as follows: the forward and reverse primers of *MMP-9* were 5′-GTGCTCCTGGTGCTGGGCTG-3′ and 5′-GGTGCCACTTGAGGTCGCCC-3′, respectively (325 bp); and the forward and reverse primers of *GAPDH* were 5′-AACTTTGG-CATTGTGGAAGG-3′ and 5′-ACACATTGGGGGTAGGAACA-3′, respectively (223 bp). The reaction conditions were as follows: an initial denaturation at 95 °C for 5 min followed by 22 (for *GAPDH*) or 25 (for *MMP-9*) cycles of denaturation for 40 s at 95 °C, annealing for 40 s at 57 °C, and extension for 50 s at 72 °C with a final extension for 7 min at 72 °C. Amplified DNA was analyzed on 1.5% agarose gels under UV.

2.5. Western blot analysis

Total cell extracts were prepared using RIPA buffer (Cell Signaling) containing 1 mM PMSF. Cytosolic and nuclear fractions were isolated using an NE-PER nuclear extraction kit (Thermo Fisher Scientific, Waltham, MA, USA). The amounts of proteins were measured with the Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of proteins from each sample were size-fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred by electrophoresis to nitrocellulose membranes. The membranes were blocked for at least 1 h with 5% nonfat dry milk prior to incubation with target protein-specific primary antibodies at 4 °C overnight. The membranes were washed twice, and incubated with HRP-conjugated secondary antibodies. After incubation, specific bands of interest were detected using ECL Plus (GE Healthcare, Uppsala, Sweden).

2.6. Transwell invasion assay

For invasion assay, the filter inserts (8 μm pore size) that fit into 24-well invasion chambers (Becton Dickinson, Franklin Lakes, NJ) were used. The upper sides of the filters were coated with 100 μL of 0.2% collagen-fragmented gelatin. Serum free DMEM with or without *N*-Benzylcantharidinamide (indicated concentrations) was added to the lower compartment of the chamber. The chambers were incubated at 37 °C for 24 h in a 5% CO_2 atmosphere. After incubation, the filter inserts were removed from the chamber well, and the cells on the upper side of the filter were removed using cotton swabs. The filters were fixed, stained with hematoxylin and eosin, and mounted on microscope slides. The cells located on the underside of the filter were counted for invaded cells.

2.7. Confocal microscopic observation

Hep3B cells, which were seeded on 12 mm π-sterilized coverslips in 24-well tissue culture plates, were treated with or without *N*-Benzylcantharidinamide (indicated concentrations) for 24 h. After washed with PBS, the cells were fixed in 3.7% formalin for 10 min. The cells were washed with PBS, and then permeabilized with 0.1% Triton X-100 in PBS. After 1 h of incubation with blocking buffer (5% BSA in PBS), the cells were incubated with anti-HuR antibodies (1:100) for 1 h, followed by 1 h of incubation with

fluorescein Alexa 488-conjugated anti-mouse IgG (1:1000; Invitrogen, Carlsbad, CA, USA). The cells were washed with PBS again, and were mounted on glass slides using VECTASHIELD Mounting Medium with DAPI (1.5 μg/mL) (Vector Lab., Burlingame, CA, USA). Fluorescent images were obtained using a ZEISS LSM700 laser scanning confocal device attached to an Axio Observer Z1 microscope (Carl Zeiss, Oberkochen, Germany).

3. Results and discussion

3.1. N-Benzylcantharidinamide inhibits MMP-9 activity at a non-toxic dose

To avoid strong cytotoxic side effect of cantharidin [8], four novel analogs were synthesized (Fig. 1A). Initially, the cytotoxic effect of each cantharidin analogs on Hep3B cells was measured using a MTT assay. The results showed that only cantharidin had significant cytotoxic activity at 50–100 μM concentrations. The other analogs did not display any significant cytotoxic activity up to 100 μM (Fig. 1B). Because cantharidin had no significant cytotoxicity at 30 μM (data not shown), levels of cantharidin analogs at 30 μM were used for subsequent experiment. Previously, norcantharidin and cantharidin were reported as an anti-metastatic agent through suppressing MMP-9 expression [9,10]. However, in present study, results from gelatin zymography showed that only cantharidin and *N*-Benzylcantharidinamide displayed significant inhibitory effect on MMP-9 activity (Fig. 1C). The inhibitory effect of norcantharidin on MMP-9 expression may be controversial because it was also reported as a facilitator of MMP-9 expression in presence of LPS via promoting the phosphorylation of Akt/p65 and transcriptional activity of NF-κB [12]. Regardless on the previous reports, the results from this study certainly show that *N*-Benzylcantharidinamide, a novel synthetic analog of cantharidin, has low cytotoxicity and significant inhibitory effect on MMP-9 activity.

Next, it was confirmed that *N*-Benzylcantharidinamide inhibited the activity of MMP-9 secreted in culture medium of Hep3B cells in a dose-dependent manner (Fig. 2A and B). The inhibition of *N*-Benzylcantharidinamide on MMP-9 activity in cultured medium can be regulated by two main ways: direct enzymatic inhibition and expressional regulation [13]. To elucidate the mechanism underlying inhibitory effect of *N*-Benzylcantharidinamide on MMP-9 activity in culture medium, the expressions of MMP-9 mRNA and proteins were examined. The results showed that *N*-Benzylcantharidinamide inhibited the expression of MMP-9 mRNA and proteins in a dose-dependent manner (Fig. 2B). These results suggest that the inhibitory effect of *N*-Benzylcantharidinamide on MMP-9 is mainly achieved through down-regulation of MMP-9 expression at mRNA levels.

3.2. N-Benzylcantharidinamide inhibits invasive potential of Hep3B cells

For invasion and metastasis of cancer, MMPs play key role in cell migration through the ECM and basement membrane for intra and extravasation [14,15]. MMP-9 is well known to degrade type IV collagen, a major constituent of the basement membrane [15]. Thus, a transwell invasion assay was performed to investigate whether *N*-Benzylcantharidinamide represses the invasive potential of highly metastatic Hep3B cells. As shown in Fig. 2C, *N*-Benzylcantharidinamide displayed a significant inhibition in the number of invaded Hep3B cells, in a dose-dependent manner. To evaluate the inhibition of invasiveness was due to inhibition of MMP-9 expression, the Hep3B cells were transfected with negative control or MMP-9 siRNA. The knockdown of MMP-9 expression was estimated by RT-PCR (Fig. 2D). The invasive potential of Hep3B cells was significantly reduced by transfection of MMP-9 siRNA (Fig. 2E). These results suggest that *N*-Benzylcantharidinamide has an inhibitory effect on invasiveness of cancer cells by suppressing expression of gelatinolytic enzyme MMP-9.

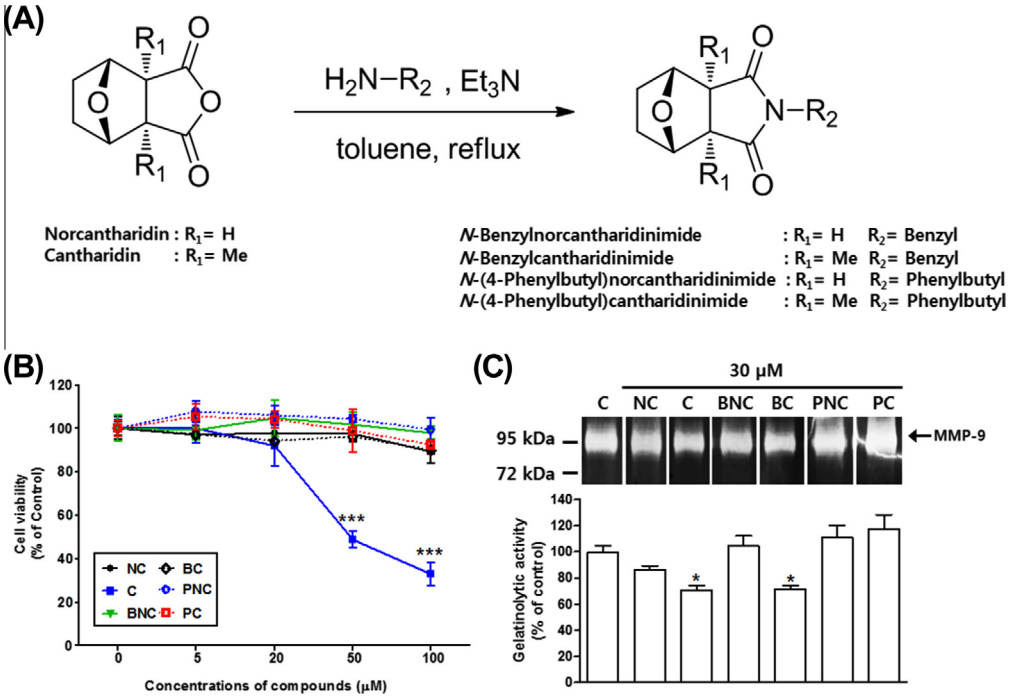

Fig. 1. Effect of cantharidin analogs on cytotoxicity and MMP-9 inhibition. (A) Schematic presentation of synthetic methods. (B) Hep3B cells were cultured with each cantharidin analogs (at the indicated concentrations) in culture medium containing 10% FBS for 24 h. Cell viability was estimated using a MTT assay (***$p < 0.001$ in comparison with the control group). (C) Hep3B cells were cultured with 30 μM of each cantharidin analogs in serum-free medium for 24 h, and the culture media were collected. The inhibitory effects of each cantharidin analogs on MMP-9 activities were measured by gelatin zymography. The intensities of bands from gelatin zymography were estimated by densitometric analysis and calculated as means ± SD of three independent experiments (*$p < 0.05$ in comparison with the control group).

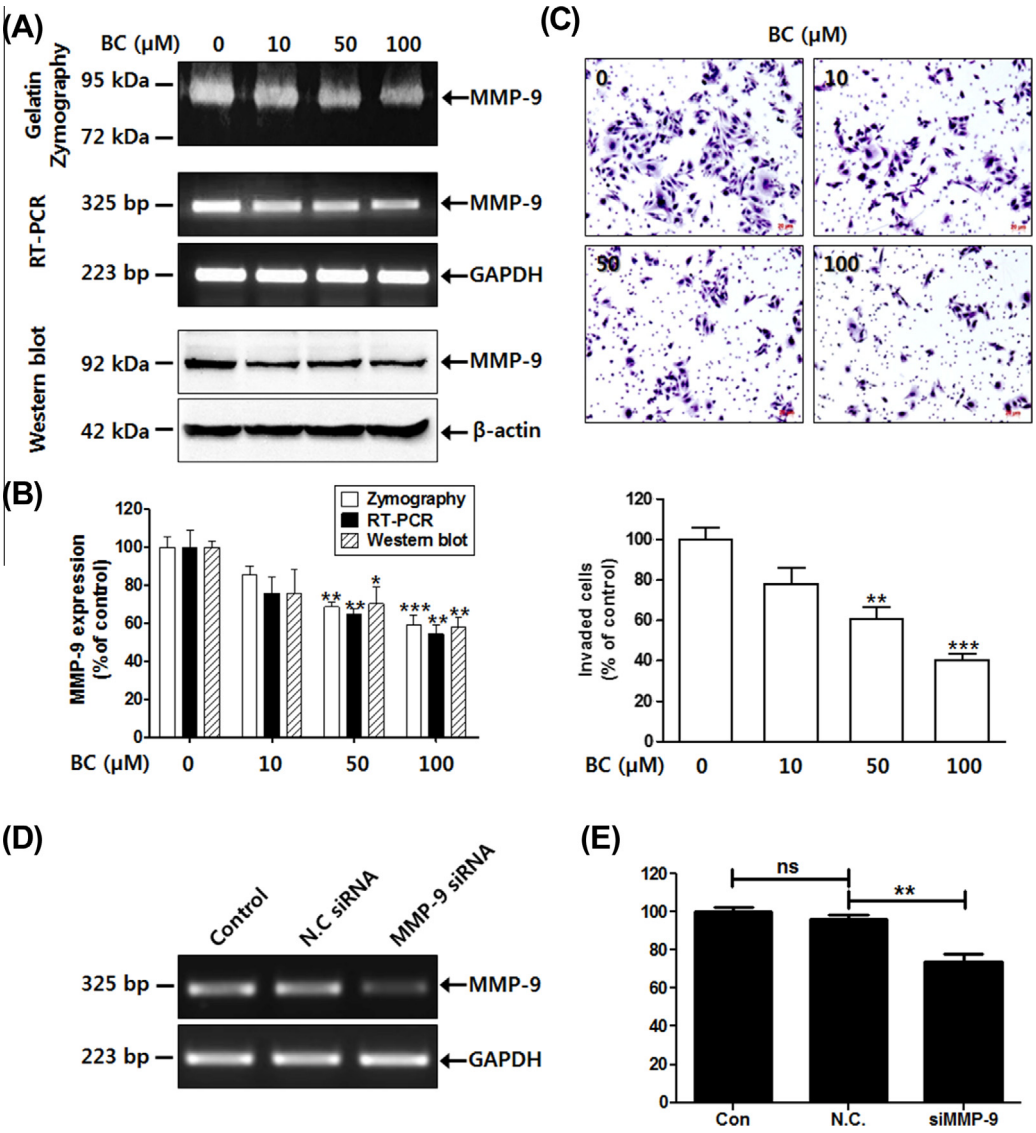

Fig. 2. *N*-Benzylcantharidinamide inhibits the MMP-9 expression and invasiveness of Hep3B cells. (A) Hep3B cells were cultured with *N*-Benzylcantharidinamide (at the indicated concentrations) in serum-free medium for 24 h. The culture media were collected for gelatin zymography and total RNA and proteins were extracted from the cell lysates. Dose-dependent inhibition of *N*-Benzylcantharidinamide on the activity and expression of MMP-9 was determined by gelatin zymography, RT-PCR, and Western blot analysis. (B) The intensities of bands from gelatin zymography, RT-PCR, and Western blot analysis were estimated by densitometric analysis and calculated as means ± SD of three independent experiments ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ in comparison with the control group). (C) *N*-Benzylcantharidinamide inhibits the Hep3B cells were treated with the indicated concentrations of *N*-Benzylcantharidinamide for 24 h and invasion assay was performed. The invaded cells were counted. Data represent the means ± SD of three independent measurements ($^{**}p < 0.01$, $^{***}p < 0.001$ in comparison with the control group). (D) The Hep3B cells were transfected with negative control or MMP-9 siRNA, and the expression of MMP-9 was measured by RT-PCR. (E) The effect of MMP-9 knockdown on invasion of Hep3B cells was examined by invasion assay. Data represent the means ± SD of three independent experiments ($^{**}p < 0.01$ in comparison with each group and ns means no significance).

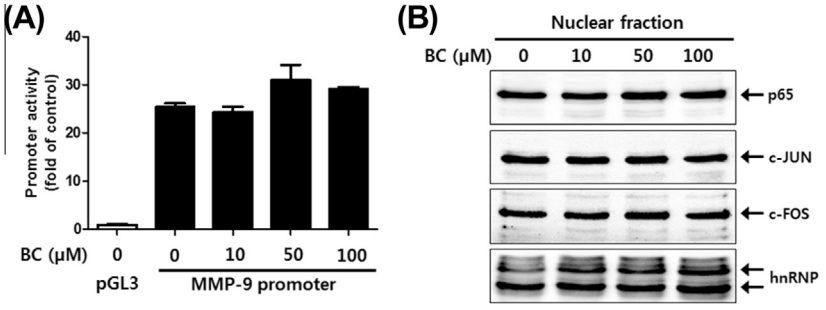

Fig. 3. *N*-Benzylcantharidinamide does not affect transcriptional activity of *MMP-9* promoter and nuclear translocation of NF-κB and AP-1. (A) Hep3B cells were co-transfected with WT-MMP9pro and 1 μg of β-galactosidase reporter plasmid. Transfected cells were treated with *N*-Benzylcantharidinamide (at the indicated concentrations) for 24 h. Transcriptional activation of *MMP-9* promoter was estimated using a luciferase assay, and the results were normalized by β-galactosidase activities. (B) Hep3B cells were treated with the indicated concentrations of *N*-Benzylcantharidinamide for 24 h and nuclear proteins were extracted. The levels of p65, c-jun, and c-fos in nuclear fraction were measured by Western blot analysis. The levels of hnRNP were used for internal control purpose.

3.3. N-Benzylcantharidinamide does not inhibit the transcriptional activity of MMP-9 promoter

The mRNA levels of MMP-9 are regulated by diverse pro-inflammatory signaling pathways, including the MAPK pathway, IKK pathway, and STAT pathway [16]. Resulting from these signal pathways, the MMP-9 transcription is mainly controlled by binding of essential transcriptional factors such as NF-κB and AP-1 to the promoter region [13,15]. Thus, the promoter assay was applied to investigate whether suppression of *N*-Benzylcantharidinamide on the MMP-9 expression was conducted by transcriptional regulation. Interestingly, the results revealed that *N*-Benzylcantharidinamide did not inhibit the transcriptional activity of MMP-9 promoter (Fig. 3A). In addition, the nuclear translocation of p65, a subunit of NF-κB, and nuclear levels of c-jun and c-fos, proteins composing AP-1, were not suppressed by treatment of *N*-Benzylcantharidinamide (Fig. 3B). These results clearly display that the inhibition of MMP-9 expression by *N*-Benzylcantharidinamide is not associated with transcriptional activity of MMP-9 promoter, which are activated by NF-κB or AP-1.

3.4. N-Benzylcantharidinamide suppressed the stability of MMP-9 mRNA by inhibiting cytosolic translocation of HuR

In present study, the mRNA level of MMP-9 was clearly decreased by treatment of *N*-Benzylcantharidinamide (Fig. 2A and B), but the transcriptional activity of MMP-9 promoter was not affected (Fig. 3A). Thus, from these results, it is possible that *N*-Benzylcantharidinamide may decrease the stability of MMP-9 mRNA. To certify this possibility, the Hep3B cells were treated with

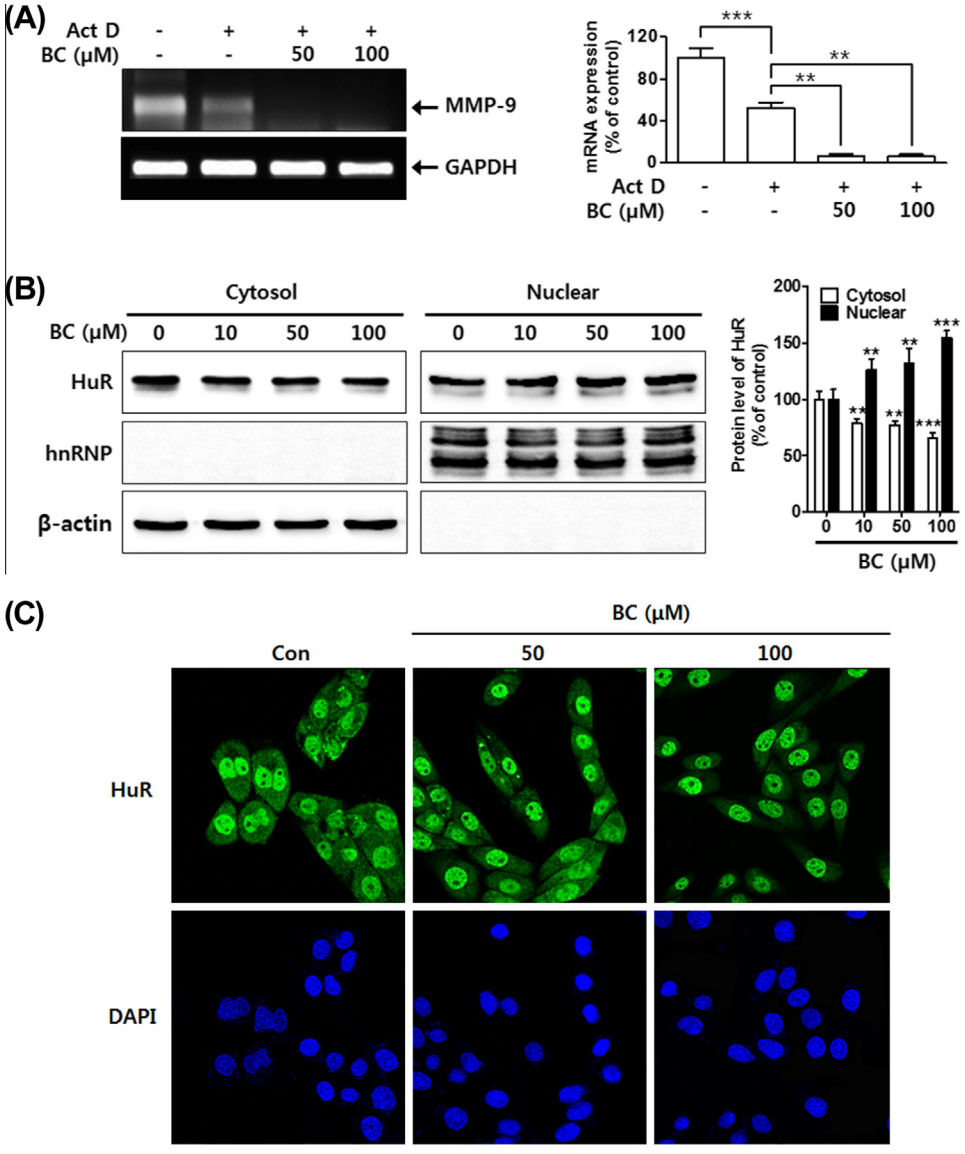

Fig. 4. *N*-Benzylcantharidinamide reduces the stability of *MMP-9* mRNA via suppressing cytosolic translocation of HuR. (A) Hep3B cells were treated with actinomycin D (2 μg/mL) and/or *N*-Benzylcantharidinamide (at the indicated concentrations) for 24 h and total mRNA were extracted. The levels of *MMP-9* mRNA were examined by RT-PCR. The intensities of bands from RT-PCR were estimated by densitometric analysis and calculated as means ± SD of three independent experiments ($^{**}p < 0.01$, $^{***}p < 0.001$ in comparison with the control group). (B) Hep3B cells were treated with *N*-Benzylcantharidinamide (at the indicated concentrations) for 24 h and proteins from nuclear and cytosolic fraction were extracted. The levels of HuR were estimated by Western blot analysis. The levels of hnRNP and β-actin were used for internal control of nuclear and cytosolic fraction, respectively. The intensities of bands from Western blot analysis were estimated by densitometric analysis and calculated as means ± SD of three independent experiments ($^{**}p < 0.01$, $^{***}p < 0.001$ in comparison with the control group). (C) Hep3B cells were treated with *N*-Benzylcantharidinamide (at the indicated concentrations) for 24 h. The translocation of HuR was observed by confocal microscopy. To detect HuR, the cells were permeabilized and incubated with anti-HuR antibodies and then an Alexa 488-conjugated secondary antibody (green). Nucleus was stained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

N-Benzylcantharidinamide in presence of actinomycin D, an established transcription inhibitor [17]. The mRNA levels of MMP-9 were almost not identified when the cells were treated together with *N*-Benzylcantharidinamide and actinomycin D (Fig. 4A). The result suggests that *N*-Benzylcantharidinamide decreased the mRNA level of MMP-9 by reducing the stability of MMP-9 mRNA.

Previously, HuR, a member of ELAV-like RNA-binding protein family, was reported as a posttranscriptional regulator for the expression of the proteins related with cancer progression, including EGF, VEGF, cyclin A, bcl-2, SIRT-1, uPA and MMP-9 [18]. HuR is a factor of constitutive nuclear protein, participating in the splicing and export processing of pre-mRNA [19]. When translocated into the cytoplasm, HuR increase the stability of target mRNA and induce their translation through its binding to 3′-untranslated region of mRNA, so-called adenylate-uridylate-rich elements (AREs) [20]. As MMP-9 mRNA has four AREs in its 3′-untranslated region, cytoplasmic HuR enhances the mRNA stability of MMP-9 [21,22]. Therefore, to evaluate whether the decreased stability of MMP-9 mRNA is related with HuR in *N*-Benzylcantharidinamide-treated Hep3B cells, the location of HuR was examined by Western blot analysis and confocal microscopic observation. The results show that the levels of cytosolic HuR were significantly decreased by treatment of *N*-Benzylcantharidinamide. On the contrary, the levels of nuclear HuR were increased (Fig. 4B and C).

Since MMP-9 expression is closely related with lethality of malignant tumor, including hepatocellular carcinoma [23], many efforts have been directed toward developing MMP-9 inhibitors [24]. Many natural products, including caffeic acid phenethyl ester, curcumin, acacetin, and baicalein, are reported as an inhibitor of MMP-9 through suppression of signal pathways involved in its transcription [15,25–27]. Recently, green tea polyphenols having 3′-galloyl group, sulforaphane, and kalopanaxsaponin A were reported as inhibitors of HuR-mediated MMP-9 expression [28–30]. Regulation of mRNAs could be achieved through binding of RNA-binding proteins and non-coding RNAs toward the mRNA *cis*-acting elements and thereby through determining the degradation of the mRNA [31]. In addition, variable target mRNAs stabilized by HuR, including cyclin A, cyclin D1, c-myc, EGF, VEGF, HIF-1α, uPA, and MMP-9, are coordinately influence in tumor progression [18]. Thus, suppressing mRNA stability by regulation of HuR may be a good strategy for MMP-9 inhibition. The results from present study clearly showed that *N*-Benzylcantharidinamide has anti-MMP-9 action through regulating HuR-mediated stability of MMP-9 mRNA.

In conclusion, it was demonstrated for the first time that *N*-Benzylcantharidinamide, a novel cantharidin analog, suppresses metastatic potential of Hep3B hepatocellular carcinoma cells through inhibition of MMP-9 expression. In addition, the inhibition of MMP-9 expression is achieved through posttranscriptional regulation via reducing cytosolic translocation of HuR, but not through transcriptional suppression. Therefore, it is suggested that *N*-Benzylcantharidinamide could be value in preventing MMP-9-mediated cancer invasion and metastasis.

Authorship

J.Y.L., T.W.C., H.S.J. and K.T.H. designed research; J.Y.L., T.W.C. and H.J.C. performed research; C.H.L., J.S.E. and Y.T.H synthesized of novel compounds; T.W.C., J.Y.C., C.W.H., H.S.J. and K.T.H. analyzed data; T.W.C. and K.T.H. wrote paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.035.

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