의학박사 학위논문

Mechanisms on Anti-cancer Effect of Cys-crotamine in Hepatocellular Carcinoma

간세포암에서 Crotamine 이 항암효과를 일으키는 기전에 관한 연구

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Mechanisms on Anti-cancer Effect of Cys-crotamine in Hepatocellular Carcinoma

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이 논문을 의학박사 학위 논문으로 제출함

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Abstract

Development of therapeutic modalities targeting the key molecules in cancer progression is crucial to improve the survival rates of hepatic cancer patients. Cys-crotamine (Cys-crot), a peptide toxin isolated from snake venom, has cytotoxicity effects on cells and anti-microbial activities. However, the effects of Cys-crot on cancer has not been extensively studied. We investigated the effect of Cys-crot on highly metastatic hepatic cancer cell line, primary HCC cells and Huh7 cell line. The study was focused on the effect of Cys-crot on invasion and migration of TPA-stimulated primary HCC cells and Huh7 cell line signaling pathways affected by Cys-crot. At 10 μM and 20 μM of concentration, Cys-crot significantly reduced the migration and invasion of TPA-stimulated primary HCC cells. Cys-crot treatment reduced the activity and expression of MMP-9 and expression of the active forms of AKT, ERK, c-JUN, and p65 in TPA-stimulated cells. Reporter assays showed Cys-crot attenuated the induction of NF-κB and AP-1-depenedent promoter activities and proximal MMP-9 promoter activity. Cys-crot repressed the invasion, migration, and activation of the signal pathways in Huh7 cells in the same way of primary HCC cells. Overall, these results suggest the anti-cancer effects of Cys-crot and provide the support for further application of Cys-crot and its derivatives in anti-cancer treatments.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of cancer-related deaths worldwide. Although there are several treatment options including surgery, trans-arterial chemoembolization (TACE), and radiofrequency ablation (RFA) available, the survival rates in liver cell carcinoma patients show no improvement. As a result of the fact that hepatitis B is endemic to East Asia, the Korean population experience high rates of liver cell cancer, resulting in a large financial burden for many families in the region.

In addition, less than 20% of patients are eligible for surgical intervention following diagnosis of HCC, and the 5-year survival rate is known to be much low. Various studies are currently underway to overcome the limitations of existing treatment strategies, including supplementation with various chemical agents, to improve the current survival rates, without much success, which is not like what has been reported for other cancers.

There has been a rapid increase in genetic research regarding solid organ malignancy since 2006, which has enabled the development of customized medicine for tumor- and patient-specific treatment strategies. This is a field that is expanding rapidly, and there are several large studies underway to evaluate their application in a clinical setting. One of the most promising fields is the development of immunotoxin-based treatments. Immunotoxin is a composite protein complex comprised of non-specific toxin protein and antibodies (scFv) with high affinity for certain molecules, which when combined with cancer cell-specific cell surface proteins can be used to deliver the toxin to the target cell and result in cancer cell death. Because toxins are protein compounds, they do not damage the normal cells around their target as they are degraded in the target cells during the cytotoxic event. This makes them an ideal treatment, and has resulted in rapid expansion of this field.

One of the potential target toxins used in these proteins is Cys-crotamine (Cys-crot),

a basic peptide composed of 42 amino acids, which is one of the major molecules in snake venom from *Crotalus durissus terrificus*.[1, 2] Cys-crot exhibits various biological activities including cytotoxic and neurotoxic properties, anti-microbial and anti-fungal activities, and anticancer activities. Structural similarity between Cys-crot and other antimicrobial peptides suggests that Cys-crot could have some of the common anti-cancer properties.[2] Cytotoxic activity of Cys-crot was investigated in melanoma cells, and was shown to be cytotoxic against B16-F10, Mia PaCa-2, and SK-Mel-28 melanoma cell lines and harmless to normal cell types. However, the effect of Cys-crot in any other cancers needs to be evaluated.[2, 3]

The aim of this study was to identify the effect of Cys-crot on primary HCC and Huh7 cells. This study was focused on the effect of Cys-crot on invasion and migration of TPA-stimulated primary HCC and Huh7 cells and tried to elucidate the signaling pathways that were affected by Cys-crot.

Materials and methods

Patient-derived xenograft (PDX) mouse model establishment by using primary HCC cells

HCC cells (1 mm^3) were implanted in 10-week-old NOD/SCID mice (Charles River Laboratories, Wilmington, MA). After 3 weeks, animals were separated into subgroups and treated by intradermal injection four times with various doses of Cys-crot (7.5 ng/g and 37.5 ng/g); this procedure was repeated every 10 days. Finally, the mice were anesthetized via intra-peritoneal injection of 40mg/kg Zoletil (Virbac, Virbac laboratories BP 27-06511 Carros, France) and 5 mg/kg Rumpum (Bayer Korea, South Korea), and the tumors were surgically removed.

Cells, Antibodies, and Reagents

Huh7 cells were purchased from American Type Culture Collection, (Manassas, VA) and maintained in complete DMEM media supplemented with 10 % fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, MD). Primary HCC cells were cultured in RPMI1640 medium supplemented with 5% FBS (Hyclone), hydrocortisol (10 μg/ml, Sigma-Aldrich, St. Louis, MO), transferrin (10 μg/ml, Sigma-Aldrich), and penicillin/streptomycin (Hyclone) after harvesting from PDX mouse model.[4]

All chemicals including inhibitors were purchased from Sigma-Aldrich (St. Louis, MO), otherwise specified. Specific antibodies against phosphorylated forms and the total of ERK, AKT, c-JUN and p65 were purchased from Cell Signaling Technology (Beverly, MA). β-actin antibody and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cys-crot was purchased from Jena Bioscience (Jena, Germany).

MTT and assay and Lactate dehydrogenase (LDH) assay

Cell viability assay was carried out with MTT. Cys-crot was dissolved in PBS and treated to the culture media directly. For MTT assay, 1×10^4 cells were seeded in 24-well plate and incubated for 24 h. Various concentrations of Cys-crot and 1 μM TPA were added and incubated for an additional 24 h. After the supernatants were carefully removed, the cells were subjected to the MTT assay. Cell cytotoxicity was examined by LDH assay with supernatants from cultured wells using LDH Cytotoxicity Assay Kit (Thermoscientific, Waltham, MA) according to manufacturer's protocol.

Invasion and migration assay

Invasion assays were carried out using a Matrigel-coated invasion chamber (Corning Inc., Corning, NY, USA). Briefly, cells were detached by trypsinization and counted. 2×10^4 cells were suspended in 100 μL serum-free media, and placed on the upper chamber of the invasion chamber. Upper chamber was assembled with lower chamber containing complete

DMEM supplemented with 10% FBS and incubate for 24 h. The migrated cells on bottom side of upper chamber were fixed with 100% methanol for 15 min and stained with 0.15% crystal violet in 10% ethanol form 30 min. The insert membranes were visualized under a light microscope and invaded cells were counted.

Wound healing migration assays were employed to assess cell migration. Cells were seeded and cultured for 16 h to produce a confluent monolayer, and then treated with mitomycin C (10 μg/mL) for 2 h. The monolayer was scratched to open a cell-free gap. The cells were treated with various concentration of Cys-crot and TPA for 24 h, and the cell migration was observed under a light microscope. The distance of migration was calculated using ImageJ software (v. 1.45) was used to calculate distance of migration.

Zymography

The enzymatic activity of MMP-9 was examined by gelatin zymography. The culture media from cells with Cys-crot treated and controls were subjected to non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 0.2% gelatin-containing gels. After electrophoresis, gels were washed in 2.5% Triton X-100 to remove the SDS and stained with 0.1% Coomassie blue staining solution

RNA preparation and real-time reverse transcription-PCR (RT-PCR)

Total RNA was extracted, reverse-transcribed and subjected with SYBR® Green Realtime PCR as described previously. The following primers were used: 5′-TGACAGCGACAAGAAGTG-3′ and 5′-CAGTGAAGCGGTACATAGG-3′ for MMP-9 and 5′-GGATTTGGTCGTATTGGG-3′ and 5′-GGAAGATGGTGATGGGATT-3′ for GAPDH.

Western blot analysis

Protein lysates were heated at 95 °C for 5 min, analyzed using SDS-PAGE and

electrophoretic ally transferred to an Immune-Blot™ polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). The membranes were incubated with primary antibodies and subsequently with appropriate secondary antibody conjugated with horse radish peroxidase. The blots were visualized by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL) and photographed by the ImageQuant™ LAS 4000 biomolecular imager (GE Healthcare Life Sciences, Waukesha, WI) and analyzed bundled Multi Gauge 3.0 software.

Transient transfection and luciferase assay

Transient transfection was carried out on 70 % confluent cells, which had been seeded 16 h before transfection, MMP-9 luciferase construct, its mutants at NF-κB or AP-1 binding sites, NF-κB or AP-1 luciferase reporter plasmids and pRL-SV40 plasmid (Promega, Madison, WI) were co-transfected using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Promoter-driven firefly luciferase activities were normalized with the level of luciferase activities. Relative promoter activities were generated relative to no treatment control.

Statistical analysis

All the statistical analysis was performed using Origin Software V. 8.1 (OriginLab Corporation, Northampton, MA). The results are expressed as the mean ± standard deviation from at least 3 times repeated experiments. The significance differences in experimental groups were determined using one-way analysis of variance followed by Student's *t*-test. A p value < 0.05 was considered statistically significant.

Results

Effect of Cys-crot on tumor growth in PDX mouse model.

The gross appearance of the PDX mouse model after treatment with different doses of Cys-crot is shown in **Fig.1**. Tumor size was measured as 120 mm^2 without Cys-crot administration, 81 mm^2 with 7.5 ng / g, and 32 mm^2 with 37.5 ng / g, indicating a significant decrease in tumor size (**Figure 1**).

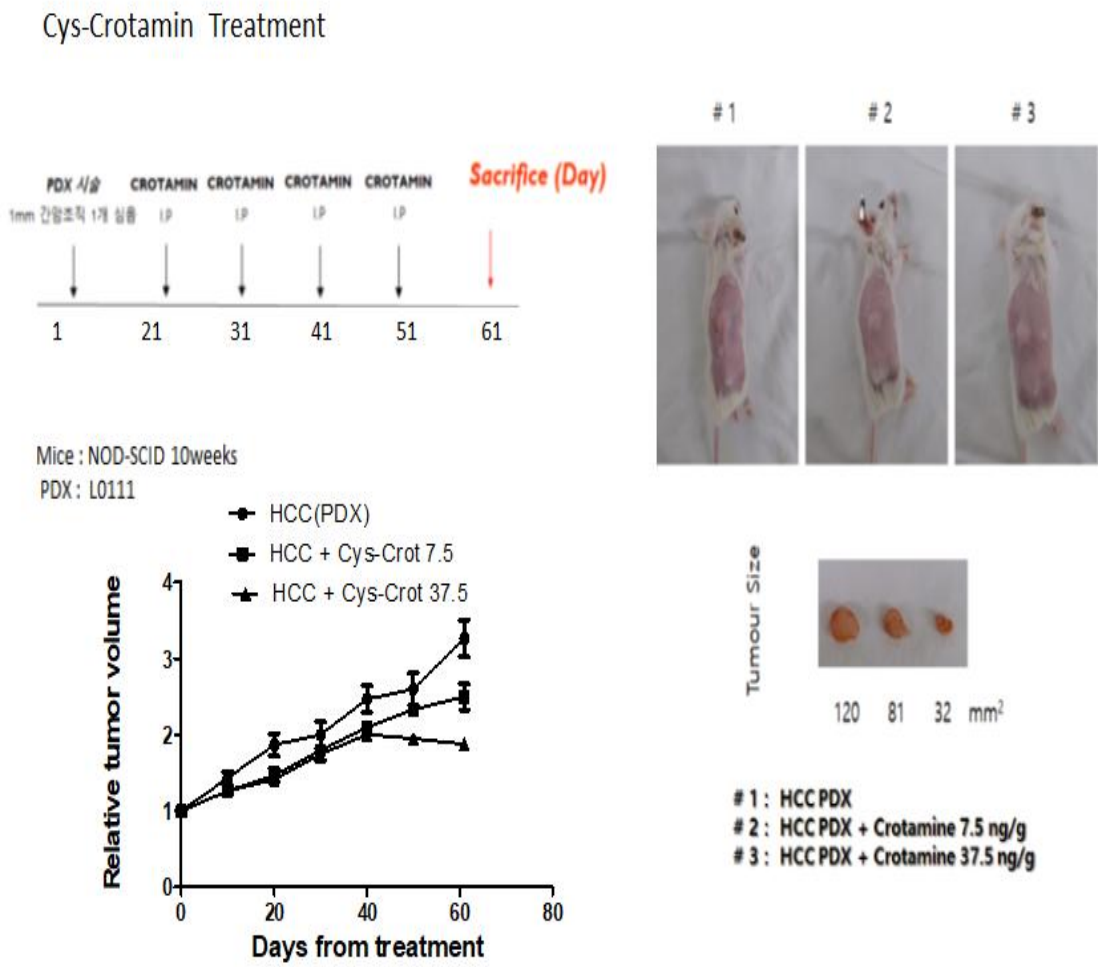

Figure 1. PDX mouse model on Cys-crot treatment schedule and Tumor size after various doses of Cys-crot treatment

Cys-crot attenuates invasion and migration of primary HCC cell in non-cytotoxic concentration.

Cys-crot is reported to be lethal to melanoma cell lines at 5 μg/ml (approximately 1 μM) final concentration in culture media[3]. The cytotoxicity of Cys-crot in primary HCC cells was initially investigated.

Viability was significantly reduced at 40 μM and higher concentrations of Cys-crot as established by an MTT assay. In the LDH assay, cytotoxicity was increased only a small amount at 30 μM and significantly increased at 40 μM and higher (**Figure 2A**). The activities of MMP-9 in primary HCC cells were reduced at 20 μM final concentration of Cys-crot (**Figure 2B**). In addition, invasion and migration of TPA-stimulated primary HCC cells were both reduced by Cys-crot treatment at 10 μM and 20 μM (**Figure 2C and 2D**). Overall, these data show that Cys-crot reduces invasion and migration and expression of MMP-9 in TPA-stimulated primary HCC cells even at non-cytotoxic concentrations.

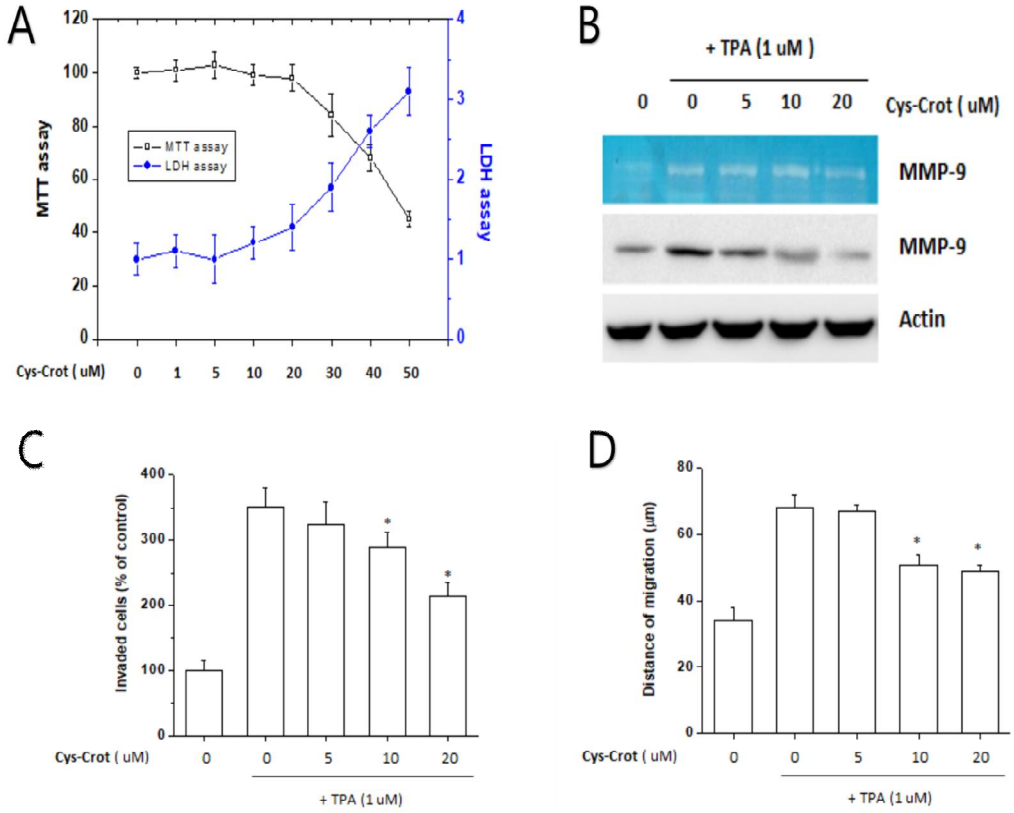

Figure 2. Identification of effect on TPA-stimulated primary HCC cells at various Cys-crot doses. Viability was significantly reduced at 40 μM and higher concentration of Cys-

crot in MTT assay. In LDH assay, cytotoxicity was a little increased at 30 μM and significantly increased at 40 μM and higher concentration (**A**). Activities of MMP-9 of primary HCC cells were reduced at 20 μM of final concentrations of Cys-crot (**B**). Invasion and migration of TPA-stimulated primary HCC cells were reduced by Cys-crot treatment at 10 μM and 20 μM (**C** and **D**).

Cys-crot *attenuates invasion and migration of Huh7 cell in non-cytotoxic concentration.*

To characterize the effects of Cys-crot on Huh7 cells, we examined the effects of Cys-crot treatment on invasion and migration of TPA-stimulated Huh7 cells, as this experimental approach has been shown to be effective in these cells.[5, 6] We treated cells with various concentrations of Cys-crot ranging from 1 ~ 50 μM. Cys-crot significantly reduced viability and induced cytotoxicity at 40 and 50 μM final concentration (**Figure 3A** and **3B**). However, no significant cytotoxicity was observed at 30 μM and lower concentrations. Western blot confirms that as the Cys-crot dose increases up to 50 μM, so the cleavage of PARP increases leading to increased activated Caspase-3, which results in cell death (**Figure 3C**).

Cys-crot treatment reduced invasion and migration of TPA-stimulated Huh7 cells significantly at non-lethal concentrations (10 μM and 20 μM) (**Figure 3D**). Taken together, these data suggest that Cys-crot represses the TPA-stimulated invasion and migration of Huh7 cells at sublethal concentrations.

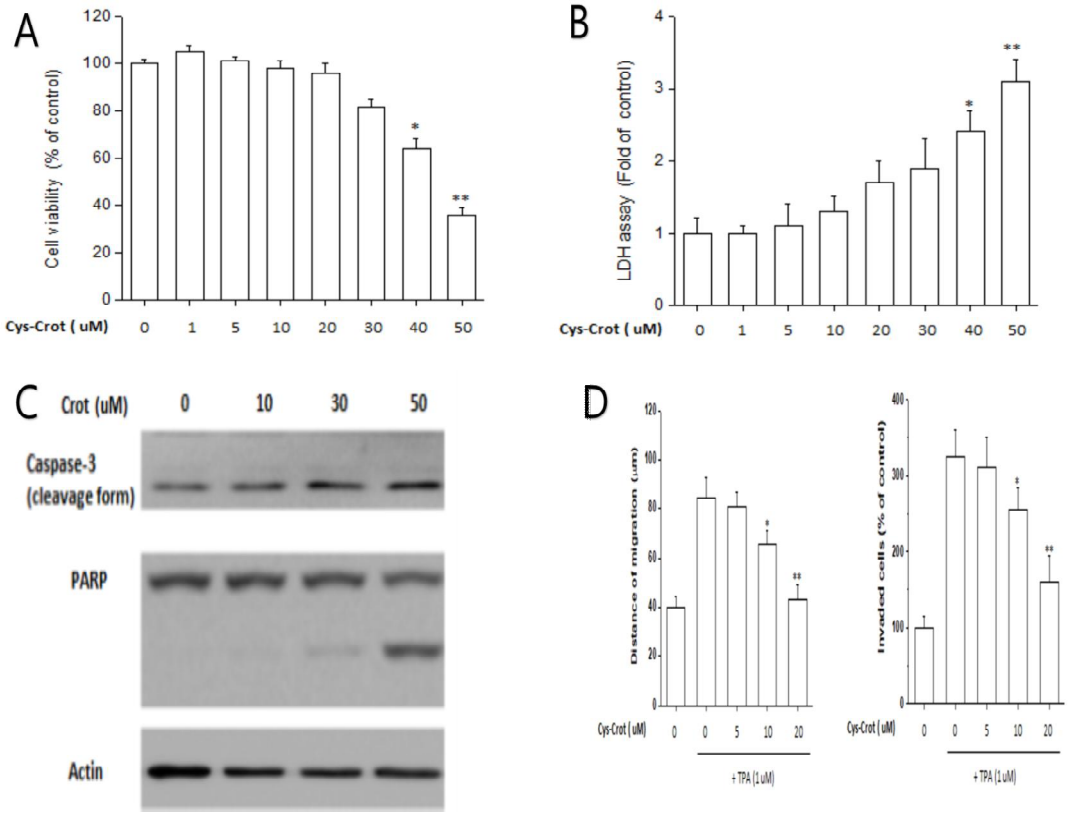

Figure 3. Effect of Cys-crot treatment on cellular signaling of TPA-stimulated Huh7 cells. Cys-crot significantly reduced viability and induced cytotoxicity at 40 and 50 μM final concentration (**A** and **3**). However, no significant cytotoxicity was observed at 30 μM and lower concentrations. The Western blot confirms that as the Cys-crot dose increases up to 50 μM, the cleavage of PARP occurs more and this leads to an increase in the cleavage form of Caspase-3, which results in the death of cancer cells (**C**). Cys-crot treatment reduced invasion and migration of TPA-stimulated Huh7 cells significantly at non-lethal concentrations (10 μM and 20 μM) (**D**).

Cys-crot suppresses the signal pathways in TPA-stimulated hepatic cancer cells.

Invasion and migration of hepatic cancer cells were regulated by multiple signal transduction pathways induced by stimulating growth factor like TPA. We investigate the effect of Cys-

crot treatment on cellular signaling of TPA-stimulated Huh7 cells. Corresponding the repressing activity of Cys-crot on invasion and migration, MMP-9 activities, which is crucial to invasion and migration of most solid tumors, was suppressed by Cys-crot treatment (**Fig. 4A**). These results suggested that Cys-crot regulates activity of MMP-9 by transcriptional control. Western blotting results showed that reduced expression of MMP-9 protein by Cys-crot treatments. Cys-crot treatment suppressed expression of phosphorylated AKT and ERK, where total amount of kinases was not changed at 10 μM and 20 μM (**Figure 4B** and **4C**). Cys-crot also suppressed activation of transcription factors c-JUN and p65, a subunit of NF-κB. Taken together, these results showed Cys-crot attenuates the TPA-stimulating cellular signaling related with invasion and migration in hepatic cancer cells.

Cys-crot suppresses MMP-9 promoter-driven transcriptional activity through AP-1 and NF-κB binding sites in TPA-stimulated hepatic cancer cells

Next, we investigated the role of the AP-1 and NF-κB pathways in the mechanism of Cys-crot mediated suppression of MMP-9 in TPA-stimulated Huh7 cells. Western blot showed decreased expression of activated AP-1 subunit c-JUN and NF-κB subunit p65 following Cys-crot treatment in TPA-stimulated hepatic cancer cells. These results suggest that Cys-crot induces suppression of the AP-1 and NF-κB pathways in TPA-stimulated hepatic cancer cells. Treatment with Cys-crot significantly attenuated the induction of NF-κB and AP-1 transcriptional activity even after TPA treatment (**Figure 5A**). Expression of MMP-9 in cells is regulated by both the AP-1 and NF-κB pathways through their binding sites which are located at the transcriptional start site of the MMP-9 promoter.[7-9] To investigate the contribution of AP-1 and NF-κB pathway suppression in Cys-crot induced downregulation of MMP-9 expression, we analyzed the proximal promoter of the MMP-9 gene. To evaluate the effect of the AP-1 and NF-κB binding sites in MMP-9 promoter activity, we used wild-type and mutant MMP-9 promoters.

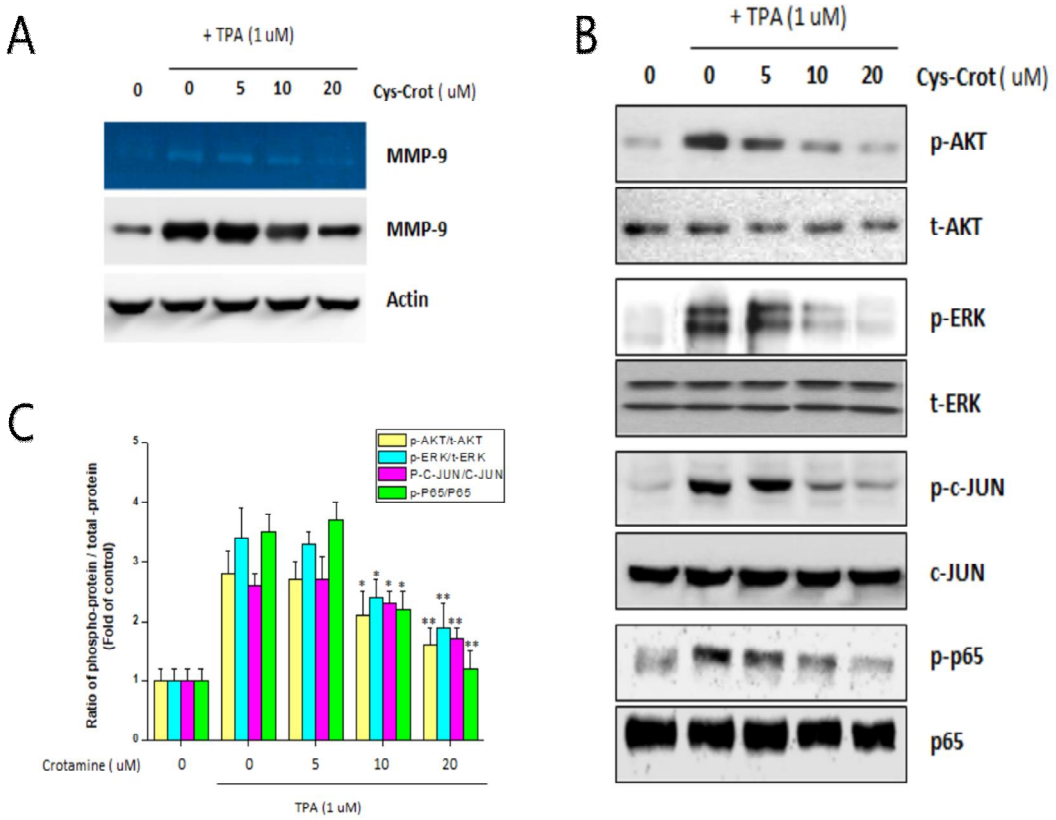

Figure 4. Cys-crot suppresses the signal pathways in TPA-stimulated Huh7 cells. MMP-9 activities, which is crucial to invasion and migration of most solid tumors, was suppressed by Cys-crot treatment (**A**). Cys-crot treatment suppressed expression of phosphorylated AKT and ERK, where total amount of kinases was not changed at 10 μM and 20 μM. Cys-crot also suppressed activation of transcription factors c-JUN and p65, a subunit of NF-κB (**B** and **C**)

The mutant promoters had a point mutation disrupting either the NF-κB or AP-1 binding site. While the activity of the wild-type MMP-9 promoter and mutant harboring the AP-1 binding site mutation was up to four-fold higher in EGF-stimulated cells when compared to untreated cells, the activity of the promoter harboring the NF-κB mutation only increased around 2.5 fold following EGF treatment (**Figure 5B** and **5C**). These results suggest a critical role for

NF-κB in EGF-stimulated MMP-9 promoter activity, rather than AP-1. However, Cys-crot treatment reduced the wild-type and both mutant promoters significantly at 20 μM which suggested that the inhibitory effects of Cys-crot on MMP-9 promoter is independent of NF-κB and AP-1 binding sites. However, the promoter activities of the mutant constructs were reduced less than 2 folds compared to the TPA-only treated samples, while that of the wild-type promoter reduced more than 2.5 folds. Overall, these results suggest that Cys-crot regulates MMP-9 proximal promoter activity through suppression of the AP-1 and NF-κB pathways in TPA-stimulated hepatic cancer cells.

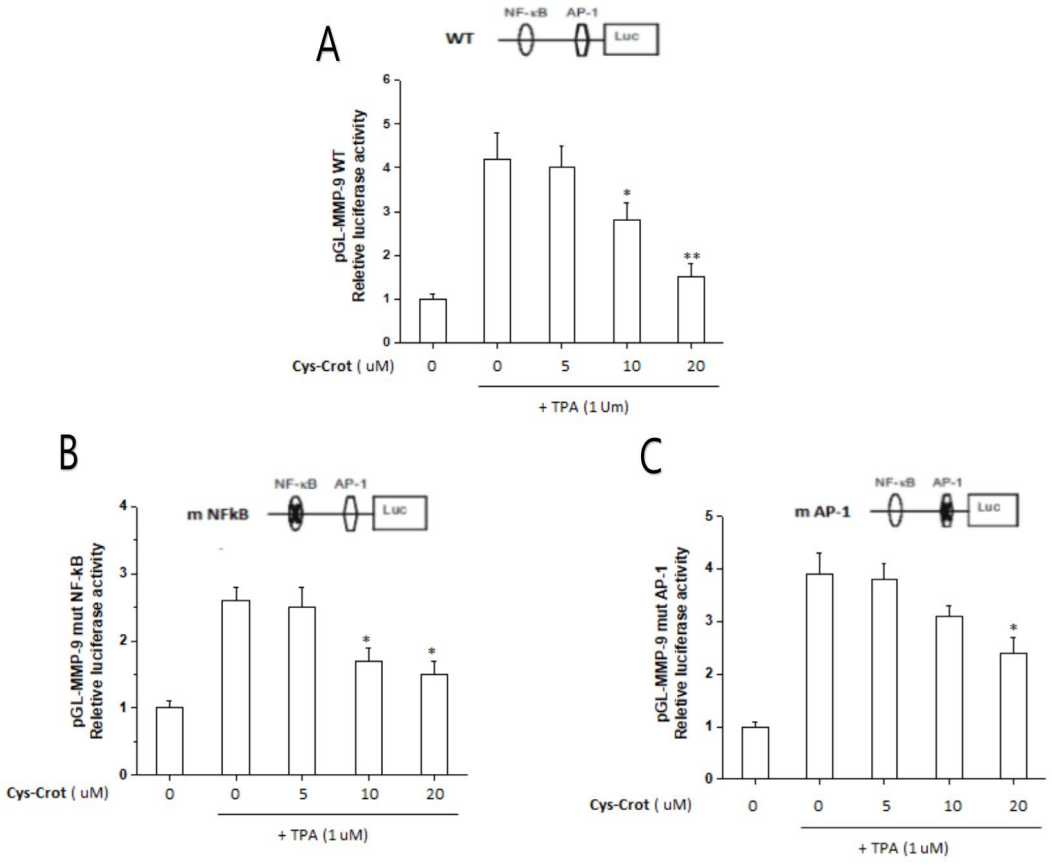

Figure 5. Cys-crot suppresses MMP-9 promoter-driven transcriptional activity through AP-1 and NF-κB binding sites in TPA-stimulated Huh7 cell. Treatment of Cys-crot significantly attenuated the induction of NF-κB and AP-1 transcriptional activity by EGF treatment (**A**). Luciferase assay by using wild-type MMP-9 promoter and mutant MMP-9 promoters with a point mutation disrupting the NF-κB binding site or AP-1 binding site (**B** and **C**).

Discussion

The development of a PDX model provides better tool for personalized cancer research than the well-known cancer cell lines that have been widely used. In 2009, by Hidalgo et al., a PDX model was developed for the establishment of an incurable disease treatment strategy, such as pancreatic cancer, and a disease-specific library consisting of patient-specific cancer tissues could be established[10]. This model of incurable cancer PDX has been proven in several papers to have a high degree of predictability in clinical treatment effects. We also established a PDX model using a primary tumor cell. We achieved a 70% success rate in establishing these primary cultures. By comparing the results of TP53, CerbB2, DPC4 immunoassay and analyses of DNA methylation and SNP mutation, the genetic homology between the primary tumor tissue and the PDX model of liver cell cancer patients were verified. Cys-crot is a promising anticancer, antibacterial, antifungal protein and potential nanocarrier. It is classified as a small basic myotoxin and has specific cytotoxicity in vitro against various neoplastic cells and was shown to inhibit tumors.[12, 13]

This toxin induces cell death in a dose-dependent manner in several actively proliferating cancer cells. Cys-crot contains many basic amino acids, so it is possibly charged and has a binding affinity for DNA. Due to these potential biomedical applications, the efficient

production of Cys-crot is desirable for further clinical research**.** Cys-crot cytotoxicity effects has been shown to be mediated by the disruption of lysosome, subsequent dysregulation of proteases and increased concentration of calcium.[13] In this study, we also observed the cytotoxicity of Cys-crot on primary HCC cell and its cell line Huh7 at higher concentration than previously reported on melanoma cell lines. We focused on the effect of Cys-crot on invasion and migration of breast cancer, which are critical properties causing bad outcomes of highly metastatic HCCs. TPA pathway is the major therapeutic target for the cure of HCCs.[5, 14] Overexpression of TPA and TPA receptors are correlated with high incident rates of metastasis in hepatic cancers. In our results, we also confirmed the induction of invasion and migration in the presence of TPA in Huh7 and primary HCC cells. Cys-crot suppressed the TPA-induced invasion and migration. Specially Cys-crot targeted TPA-induced intracellular signaling pathways including AKT-NK-κB and ERK-AP-1 pathways.[14] These results suggest that Cys-crot could be one of the anti-breast cancer agents by attenuating TPA stimulation.[5, 6, 16] MMP-9 is required for invasion in breast cell culture and for pulmonary metastasis in a mouse model of basal-like breast cancer.[16] In addition, expression of MMP-9 is highly associated with the metastasis of breast cancers, overall survival, and recurrence rate.[8, 17, 18]

The role of MMP-9 in tumor growth, metastasis, and angiogenesis has been the focus of intense investigation for many years.[18,21] Overexpression of MMP-9 in tumor cells will enhance degradation of the basement membrane to facilitate invasion of nearby blood vessels (intravasation), followed by extravasation to the distant tissue to seed new metastatic sites. MMP-9 show high activity against basement membrane as it is composed primarily of type IV collagen.

According to the study published in 2012 by Chen et al., the authors demonstrated that MMP-9 was superior to MMP-2 for prediction of HCC recurrence and survival of

patients after surgical resection.[23] In HCC, MMP-2 was expressed predominantly in tumor stroma cells and only to a lesser extent in carcinoma cells, while MMP-9 was expressed mainly by tumor cells. Stromal cells produce regulatory factors that modulate MMP-2 expression of tumor cells. When tumor cells are separated from stromal cells, as occur in the circulation after vascular intravasation, MMP-2 expression of tumor cells might be downregulated.[24,25] In contrast, tumor cells may still express MMP-9 and so retain invasive capability for extravasation and metastasis. Moreover, MMP-9 can directly cleave osteopontin to promote HCC metastasis and can colocalize with F-actin at the front of extending pseudopodia in HCC cells. As previously reported, increased MMP-9 expression was associated with capsular infiltration of HCC, and levels of plasma MMP-9 were frequently elevated in patients with macroscopic portal vein invasion and metastasis by upregulating MMP-9 expression.

Therefore, suppression of MMP-9 by Cys-crot has the important clinical meanings for the care of hepatic cancer.[18-22] In the data of this study, we provided the evidences for the inhibitory effects of Cys-crot on the transcriptional activity of MMP-9 driven by proximal promoters possibly via the suppression of NF-κB and AP-1 transcription factors. Considering the role of MMP-9 in breast cancers, inhibition of MMP-9 activity may be the part of molecular mechanisms resulting the suppression of invasion and migration by Cys-crot treatment.

Conclusions

In this study, we identified the effects of Cys-crot on TPA-stimulated hepatic cancer cells. Cys-crot inhibited TPA-driven invasion and migration on HCCs and activation of intracellular signaling pathways. Given the critical roles of TPA stimulation, these results

suggest the potential use of Cys-crot and its derivatives in the development of novel therapeutic modality in the cure of HCCs.

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국문 요약

간세포암은 전세계적으로 많은 국가에서 암중에 5번째로 사망률이 높은 암으로 한국에서도 매우 큰 사회적 비용을 발생시키는 암이다. 암 진행에서 key molecule을 표적으로 하는 치료 방법의 개발은 간암 환자의 생존율을 향상시키는 데 중요하다.

뱀 독에서 분리한 펩타이드 독소 인 Cys-crotamine (Cys-crot)은 세포 및 세균의 활동에 대한 세포독성 효과가 있는 것으로 알려져 있으나 Cys-crot가 암에 미치는 영향은 광범위하게 연구되지 않았다.

이에 이 연구에서는 전이 특성이 매우 강한 간암 세포주인 primary hepatocellular carcinoma (HCC) cell 과 Huh-7 cell line 을 이용해 Cys-crot 의 항암 효과에 대해 알아보고자 하였다. PDX 쥐 모델을 이용한 Cys-crot 실험에서 높은 용량의 Cys-crot을 투여한 PDX 쥐 모델에서 유의한 종양세포 용적의 감소를 확인할 수 있었으며, 이러한 항암효과의 작용기전을 확인하기 위해 primary HCC cell 과 Huh-7 cell line을 이용한 in-vitro 실험을 시행하였다.

10 μM 과 20 μM 용량의 Cys-crot에서 TPA-stimulated Huh-7 와 primary HCC cell 모두 migration 와 invasion 이 유의하게 감소하는 것으로 나타났다. Cys-crot 처리는 TPA-stimulated HCC cell에서 MMP-9의 활동과 발현 및 phosphorylated AKT, ERK, c-JUN, p65의 발현이 억제되는 것이 관찰되었다. Reporter assay를 시행한 결과 Cys-crot에 의해 NF-κB and AP-1-depenedent promoter의 활성이 억제됨을 확인할 수 있었다.

결론적으로, 이러한 결과들은 Cys-crot의 간세포암에 대한 항암 효과를 증명하는데 중요한 이론적 배경을 제공하며, 이를 토대로 Cys-crot의 다양한 적용을 통한 암 치료의 저변을 확대할 수 있을 것이다.