Combined treatment with zingerone and its novel derivative synergistically inhibits TGF-β1 induced epithelial-mesenchymal transition, migration and invasion of human hepatocellular carcinoma cells

Young-Joo Kim, Youngsic Jeon, Taejung Kim, Won-Chul Lim, Jungyeob Ham, Young Nyun Park, Hyeonseok Ko

PII: S0960-894X(16)31311-7
DOI: http://dx.doi.org/10.1016/j.bmcl.2016.12.042
Reference: BMCL 24531

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 18 October 2016
Revised Date: 2 December 2016
Accepted Date: 15 December 2016

Please cite this article as: Kim, Y-J., Jeon, Y., Kim, T., Lim, W-C., Ham, J., Nyun Park, Y., Ko, H., Combined treatment with zingerone and its novel derivative synergistically inhibits TGF-β1 induced epithelial-mesenchymal transition, migration and invasion of human hepatocellular carcinoma cells, *Bioorganic & Medicinal Chemistry Letters* (2016), doi: http://dx.doi.org/10.1016/j.bmcl.2016.12.042

Combined treatment with zingerone and its novel derivative synergistically inhibits TGF-β1 induced epithelial-mesenchymal transition, migration and invasion of human hepatocellular carcinoma cells

Young-Joo Kim[a,b], Youngsic Jeon[b], Taejung Kim[c], Won-Chul Lim[d], Jungyeob Ham[c], Young Nyun Park[b] and Hyeonseok Ko[d,*]

[a] *Natural Products Research Center, Korea Institute of Science and Technology, Gangneung, Gangwon-do, South Korea*

[b] *Department of Pathology, Brain Korea 21 Plus Project for Medical Science, Yonsei University College of Medicine, Seoul, South Korea*

[c] *Natural Constituents Research Center, Korea Institute of Science and Technology, Gangneung, Gangwon-do, South Korea*

[d] *Laboratory of Molecular Oncology, Cheil General Hospital & Women's Healthcare Center, Dankook University College of Medicine, Seoul, South Korea*

* Corresponding author.

Hyeonseok Ko, Tel.: +82-10-5379-3365, +82-2-2000-7668.

E-mail: drug9054@naver.com or drug9054@dankook.ac.kr (H. S. Ko).

Abstract

The epithelial–mesenchymal transition (EMT) is an important cellular process during which polarized epithelial cells become motile mesenchymal cells, which promote cancer metastasis. Ginger, the rhizome of Zingiber officinale, is extensively used in cooking worldwide and also as a traditional medicinal herb with antioxidant, anti-inflammatory and anticancer properties. Several pungent compounds have been identified in ginger, including zingerone, which has anticancer potential. However, the role of zingerone in EMT is unclear. We investigated the synergistic effect of zingerone and its derivative on EMT. Transforming growth factor-beta 1 (TGF-β1) induces the EMT to promote hepatocellular carcinoma metastasis, including migration and invasion. To understand the repressive role of the combination of zingerone and its derivative (ZD 2) in hepatocellular carcinoma metastasis, we investigated the potential use of each compound of ginger, such as zingerone, ZD 2 and 6-shogaol, or the mixture of zingerone and ZD 2 (ZD 2-1) as inhibitors of TGF-β1 induced EMT development in SNU182 hepatocellular carcinoma cells *in vitro*. We show that ZD 2-1, but not zingerone, ZD 2 and 6-shogaol significantly increased expression of the epithelial marker E-cadherin and repressed Snail upregulation and expression of the mesenchymal marker N-cadherin during initiation of the TGF-β1 induced EMT. In addition, ZD 2-1 inhibited the TGF-β1 induced increase in cell migration and invasion of SNU182 hepatocellular carcinoma cells. Furthermore, ZD 2-1 significantly inhibited TGF-β1 regulated matrix metalloproteinase-2/9 and activation of Smad2/3. We also found that ZD 2-1 inhibited nuclear translocation of NF-κB, activation of p42/44 MAPK/AP1 signaling pathway in the TGF-β1 induced EMT. Our findings provide new evidence that combined treatment with ZD 2, novel zingerone derivative, and zingerone synergistically suppresses hepatocellular carcinoma metastasis *in vitro* by inhibiting the TGF-β1 induced EMT.

Keywords

Zingerone; Epithelial–mesenchymal transition (EMT); Transforming growth factor-beta 1 (TGF-β1); hepatocellular carcinoma cancer; metastasis.

Hepatocellular carcinoma (HCC) is the third leading cause of cancer related death globally, and is one of the most common causes of cancer morbidity and mortality in Asia and Africa. HCC occurs in association with viral infection,[1] extensive alcohol intake,[2] transgenic oncogenes,[3] and other causes of hepatic cirrhosis. It usually has poor prognosis due to resistance to chemotherapy and restricted efficacy of radiotherapy.[4] In spite of progress in understanding, diagnosis, and treatment, HCC remains a malignant tumor with a very low 5 year survival rate, partly because it is usually not detected at a curable time. Approximately 80% of HCC cases develop to an advanced malignancy.[5] Although surgery has been successfully used to control some cases of early HCC, recurrence remains in excess of 50% in 3 years.[6]

HCC is typically hypervascular, which means that new blood vessels grow rapidly to support the HCC cells with essential nutrients. Metastasis is the least understood aspect of cancer, and the primary reason for HCC high mortality rate. Accordingly, therapies to increase HCC patient survival should minimize tumor growth, and block cell invasion and metastasis.[7,8]

EMT is defined as the process where epithelial cells lose their epithelial phenotype while acquiring mesenchymal characteristics, including morphology, cellular structure, and biological function.[14] EMT usually occurs in the critical steps of embryonic development, as well tissue repair and remodeling in adults.[9,10] However, abnormal reactivation of developmental EMT programs also has an important role in tumor metastasis. Three major changes occur during the EMT in cancer: First, epithelial cells lose cell–cell adhesion and E-cadherin, the epithelial marker; Second, they acquire vimentin, the mesenchymal marker; Third, they undergo major changes in cytoskeleton rearrangement that enables them to acquire mesenchymal characteristics, such as cell migratory and invasive behaviors.[11]

Cancer metastasis proceeds by multiple genes and follows complex steps.[12] The first step is degradation of the extracellular matrix via matrix metalloproteinases (MMPs). MMPs are over-expressed during tumor growth and metastasis, and are associated with malignant tumors and a poor prognosis in cancer patients.[13,14] Multiple signaling pathways regulate expression of MMPs, including the MAPK and NF-κB signaling pathways.[15,16] Thus, targeting these signaling pathways could prevent tumor metastasis and consequently reduce mortality.

EMT is a reversible procedure where epithelial cells transform into mesenchymal cells with changed adhesion and migratory capacity due to decrease of the epithelial cell-cell adhesion molecule E-cadherin and increase of mesenchymal markers vimentin and N-cadherin.[17] Downregulation of E-cadherin expression increases cell mobility and promotes tumor cell invasion due to several transcriptional repressors, including Snail, Slug, Zeb2, and Twist. Snail and Slug, which are zinc finger transcription factors, are overexpressed in

epithelial cell lines and induce E-cadherin downregulation during the EMT. The E-cadherin regulatory transcription factors are expressed in many malignant cancers.[18,19] It has been recently reported that various environmental factors may induce the release of inflammatory cytokines through increased expression of zinc-finger transcriptional factors.[20] Environmental factors, such as cytokines IL-6 and transforming growth factor-β1 (TGF-β1), also induce the EMT in cancer cells, which promotes tumorigenesis and metastasis.[21,22]

TGF-β1, an EMT stimulant, is produced by epithelial cells, eosinophils, macrophages, and fibroblasts; and is implicated in subepithelial fibrosis, airway smooth muscle (ASM) remodeling, increased mucus production, and microvascular changes.[23–25] It is a multifunctional cytokine that regulates a wide range of cellular functions, including tissue morphogenesis, development of mesenchymal cell phenotype, and synthesis of the extracellular matrix. TGF-β is a master regulator of EMT, which is responsible for the transformation of polarized epithelial cancer cells into highly motile mesenchymal phenotypes responsible for tumor invasion, intravasation, extravasation, and metastatic dissemination.[26] EMT has also been implicated in the acquisition of resistance to conventional therapies.[27]

TGF-β1 induces signaling pathways, such as the Smad and mitogen activated protein kinase (MAPK) pathways. In canonical TGF-β signaling, binding to the type I/II receptor (TβRI/TβRII) complex leads to phosphorylation of SMAD-2/3, their interaction with SMAD-4, and nuclear translocation.[28] In addition to the canonical TGF-β/Smad signaling pathway, TGF-β1 also activates non-Smad signaling pathways. Previous reports have shown that TGF-β1 directly activates SMAD-independent MAPK pathways, including c-Jun N-terminal kinase (JNK), extracellular-regulated kinase, and p38 MAPK, during signal transduction of TGF-β1-regulated cell migration and invasion.[29]

Ginger (*Zingiber officinale* Roscoe, family: Zingiberaceae) originated in South-East Asia and is a common spice, used all over the world. Ginger is used to treat a number of diseases throughout the world. Its varied phytochemistry contains numerous enzymes and vitamins as well as minerals, and various active compounds which vary significantly between plant varieties and regions in which it is grown. More than 60 active compounds are known to be present, commonly broadly divided into volatile and nonvolatile compounds. Volatile components include hydrocarbons, which impart ginger's distinct aroma and taste. Nonvolatile compounds include shogaols, gingerols, and zingerone.[30]

Zingerone is primarily present in dry ginger and is also produced by thermal degradation of gingerols or shogaols, comprising approximately 9.25% by mass.[31] Chemically, zingerone is vanillyl acetone, a member of the phenolic alkanone group, and is known to have potent pharmacological activities, including antioxidant,[32]

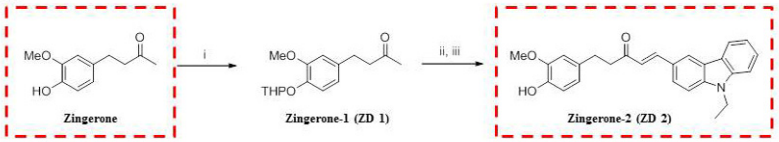
anti-inflammatory,[33] anticancer,[34] and antimicrobial activities.[35] Zingerones are the active components in ginger and possess anti-proliferative pharmacological activities on cancer cells. However, the effect of zingerone on the TGF-β1 induced EMT and of cancer cells has not been verified, and the effects of zingerone on inhibiting the TGF-β1 induced EMT of hepatoma cells have not been fully researched.

Therefore, to discover novel small molecule compounds inhibiting TGF-β1 induced EMT, we synthesized zingerone derivative 2 (ZD 2) (Fig. 1A), then mixed ZD 2 and zingerone at 1:1 ratio, and found the mixture synergistically inhibited the EMT more than each treatment alone, by suppressing E-cadherin and increasing N-cadherin expression in TGF-β1-activated SNU182 hepatocellular carcinoma cells. We also demonstrated that the mixture (ZD 2-1) reduced TGF-β1 induced increase in metastasis, including migration and invasion, and significantly inhibited TGF-β1 regulated matrix metalloproteinase-2 (MMP-2), MMP-9, and activation of Smad2/3. We also showed that ZD 2-1 repressed TGF-β1 induced degradation of IκB, nuclear translocation of NF-κB (p65), and activation of Smad 2 and p42/44 MAPK in SNU182 hepatocellular carcinoma cells. These results represent a novel anti-EMT effect of ZD 2-1 in human liver cancer cells.

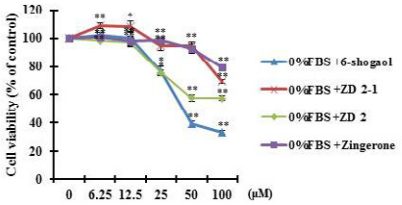
We examined whether each compound or mixture could inhibit TGF-β1 induced EMT in human liver cancer cells. To examine the anti-proliferative effect of 6-shogaol, ZD 2, and zingerone on SNU182 cell growth, SNU182 cells were treated with various concentrations (6.25, 12.5, 25, 50, and 100 μM) of 6-shogaol, ZD 2, and zingerone for 48 h, and then cell viability was measured by CCK-8 assay. CCK8 is more sensitive than 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitro-phenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] is reduced by dehydrogenases in cells to give a yellow colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. Concentrations of zingerone < 50 μM did not induce anti-proliferative effects. Similarly, concentrations of 6-shogaol and ZD 2 < 12.5 μM did not affect anti-proliferation of SNU182 cells and survival rates of the 12.5 μM were > 90%. However, concentrations of 6-shogaol and ZD 2 > 12.5 μM dose dependently inhibited SNU182 cell growth (Fig. 1B). ZD 2-1 (50 μM) did not induce anti-proliferative effect in SNU182 cells, although ZD 2 (50 μM) alone inhibited SNU182 cell growth to ~40% of the control. Thus, less than 50 μM zingerone and ZD 2-1 had no influence on cell proliferation, and less than 12.5 μM ZD 2 and 6-shogaol did not inhibited SNU182 cell proliferation when each compound or mixture of ginger was treated for 48 h. Therefore, cells were treated with selected doses (20 and 40 μM ZD 2-1) in subsequent experiments.

Fig. 1

A.



B.

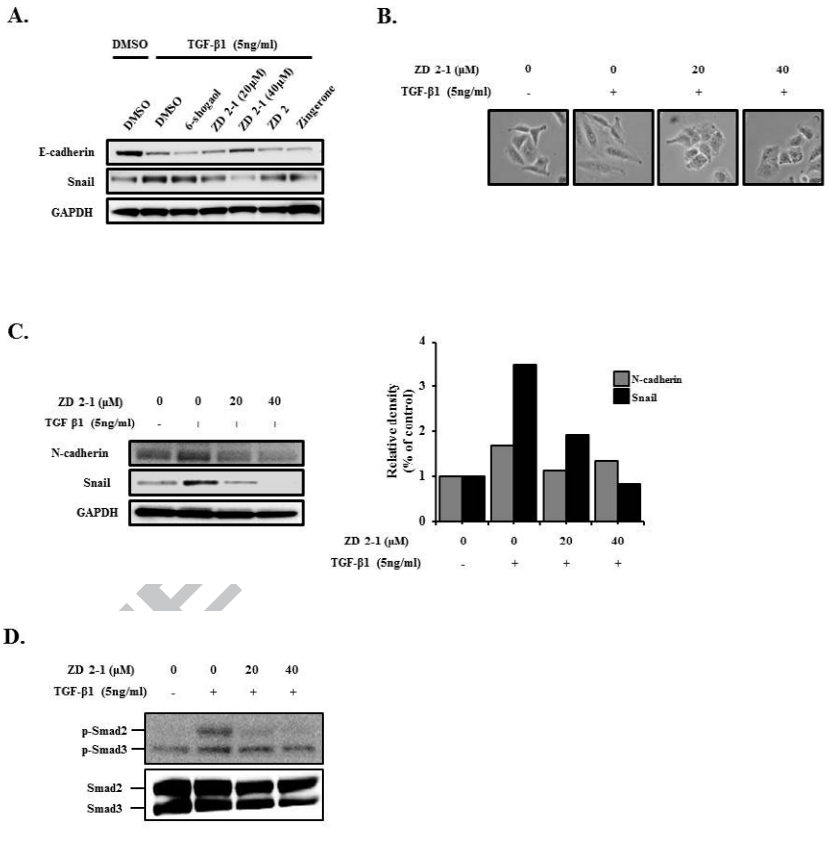


Downregulation of E-cadherin is considered an important step in the TGF-β1 induced EMT process, and can be induced by transcriptional repression mediated by the transcription factor Snail.[36] To determine the inhibitory effect of each compound or mixture of ginger during the TGF-β1 induced EMT, we examined the effect of 6-shogaol, zingerone, ZD 2, and ZD 2-1 on expression of E-cadherin and Snail in TGF-β1 induced SNU182 cells by Western blotting. Figure 2A shows that E-cadherin expression significantly decreased, whereas Snail expression significantly increased in TGF-β1 treated SNU182 cells compared with control cells. We also found that 6-shogaol, zingerone, or ZD 2 alone could not mitigate the decrease in E-cadherin expression and they could not inhibit the increase in Snail expression (Fig. 2A). However, ZD 2-1 treatment significantly mitigated the decrease in E-cadherin and increase in Snail expression in a dose dependent manner (Fig. 2A, lines 4 and 5). Therefore, ZD 2-1 inhibited the expression of TGF-β1 induced Snail, suggesting that ZD 2-1 reduces TGF-β1-mediated E-cadherin expression through Snail. TGF-β1 significantly decreased E-cadherin expression and hence increased N-cadherin expression.[37,38]

To confirm the suppressive role of ZD 2-1 during the TGF-β1 induced EMT, we analyzed morphological changes, which are regulated by ZD 2-1 in TGF-β1 treated SNU182 cells. SNU182 cells were preincubated with ZD 2-1 (20 μM and 40 μM) in serum-free media. After 2 h, these cells were incubated with 5 ng/ml TGF-β1 for 48 h. Figure 2B shows that treatment with TGF-β1 significantly induced the mesenchymal phenotype, including spindle-like shapes. However, TGF-β1 induced mesenchymal phenotype was significantly suppressed by pretreatment with 40 μM ZD 2-1 (Fig. 2B). Therefore, TGF-β1 induced EMT was inhibited by ZD 2-1.

We then examined the effect of ZD 2-1 on expression of N-cadherin and Snail in TGF-β1 induced SNU182 cells. Figure 2C shows that N-cadherin and Snail expression increased in TGF-β1 treated SNU182 cells compared with control cells. In contrast, ZD 2-1 treatment dose dependently inhibited the increase in N-cadherin and Snail expression (Fig. 2C). These results strongly suggest that ZD 2-1 suppressed the effect of the TGF-β1 induced EMT in SNU182 cells.

Fig. 2



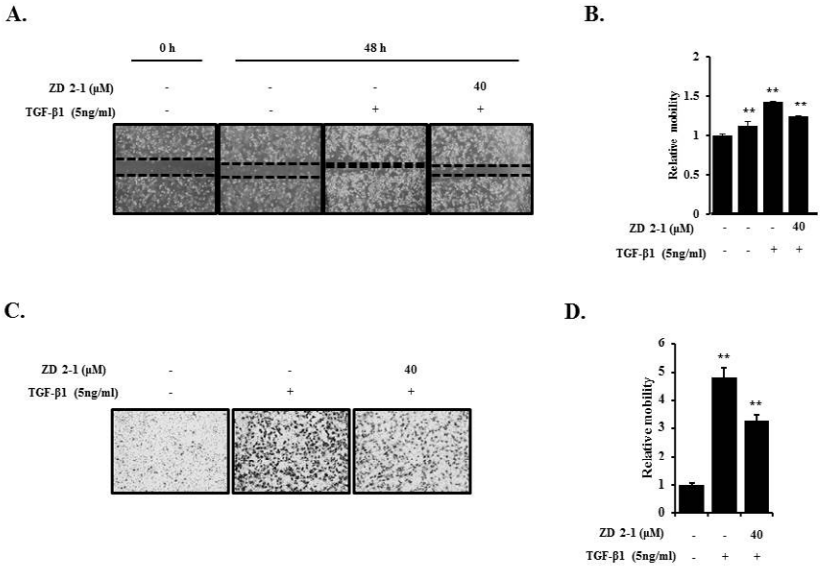
The TGF-β1 induced EMT is mediated through Smad and non-Smad signaling pathways. After TGF-β1 stimulation, in the canonical TGF-β signaling pathway, Smad2 and Smad3 are phosphorylated, and then induce the EMT.[18] Therefore, we examined whether ZD 2-1 suppresses TGF-β1 induced phosphorylation of Smad 2/3. Figure 2D shows that TGF-β1 treatment promoted phosphorylation of Smad 2/3, whereas it was significantly decreased only phosphorylation of Smad 2 in the presence of ZD 2-1. These results suggest that ZD 2-1 inhibits phosphorylation of Smad 2 increased by the TGF-β1 induced EMT in SNU182 cells.

Accumulating evidence suggests that the EMT contributes to increased metastatic progression in various

processes, including increased motility and invasiveness.[18,39,40] First, to determine whether ZD 2-1 inhibits TGF-β1 induced motility, a wound healing assay was performed by scratching the cell layer prior to TGF-β1 treatment with or without ZD 2-1 40 μM, and imaging subsequent cell migration after 48 h. Figures 3A and 3B shows that migration ability increased in TGF-β1 treated SNU182 cells compared with control cells. ZD 2-1 also suppressed TGF-β1 promoted SNU182 cell migration, since the migration ability of TGF-β1 treated cells was significantly inhibited by ZD 2-1 (Figs. 3A, and 3B). Thus, ZD 2-1 inhibited TGF-β1 induced migration.

Second, to examine whether ZD 2-1 prevents TGF-β1 induced invasion in SNU182 cells, SNU182 cells were cultured in an invasion chamber. Subsequently, groups of cells were treated in Transwells (inner chamber) with TGF-β1, and TGF-β1 and ZD 2-1 for 48 h. The invasive cells, presented on the lower surface of the chamber, were then stained and imaged, and invasion ability measured. Figures 3C and 3D show that TGF-β1 treated cells increased cell invasion ability by approximately 4.8 fold compared with control cells. In contrast, TGF-β1 treated SNU182 cells with ZD 2-1 showed impaired invasion ability (Figs. 3C and 3D). Thus, ZD 2-1 prevented TGF-β1 induced migration and invasion.

Fig. 3

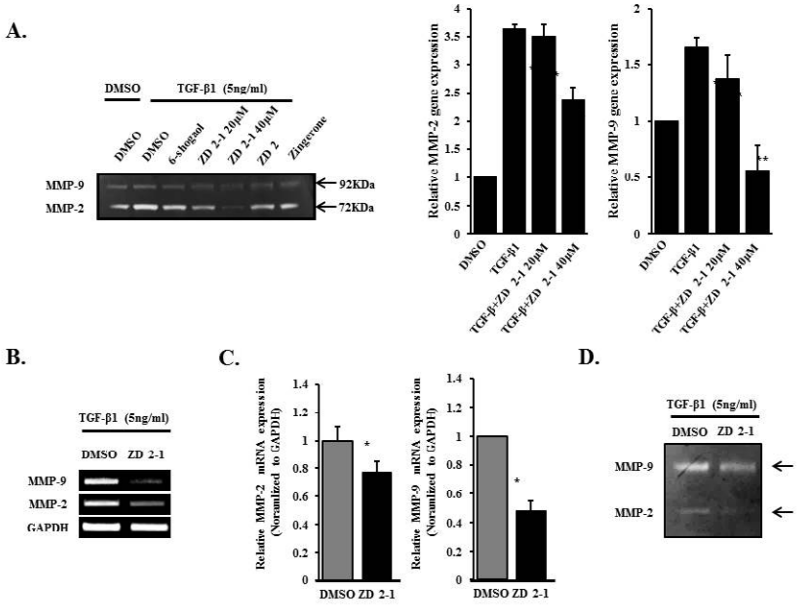


MMPs play critical functions in cancer progression. MMP-2 and MMP-9 play an important role in tumor cell migration and invasion.[41] TGF-β1 also increases production of MMP-2 and MMP-9, and enhances their activity in various tumor cells.[42,43] Therefore, to determine whether ZD 2-1 inhibits TGF-β1 induced expression of MMP-2 and MMP-9 in SNU182 cells, we performed RT-PCR, qRT-PCR, and densitometric analysis to

measure MMP expression at the transcriptional level. Figures 4A and 4B show that TGF-β1-activated cells had increased MMP-2 and MMP-9 expression compared with control cells. In contrast, MMP-2 and MMP2-9 expression by TGF-β1 treated cells were not significantly prevented by 6-shogaol, ZD 2, or zingerone alone, but were significantly prevented by ZD 2-1 treatment in a dose dependent manner (Figs. 4A–4C).

Subsequently, we performed gelatin zymography to measure MMP-2 and MMP-9 activity in cell culture medium. Figure 4D shows that ZD 2-1 significantly inhibited increased MMP-2 and MMP-9 activity by TGF-β1. Thus, ZD 2-1 prevents TGF-β1 induced MMP-2 and MMP-9 expression and activity in SNU182 cells.

Fig. 4

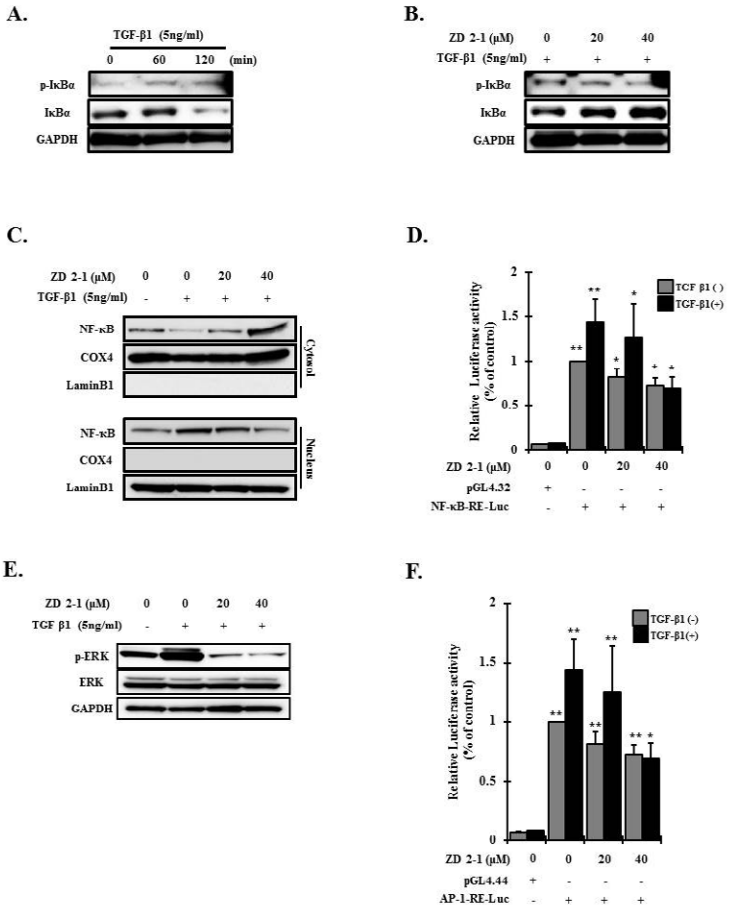


It has been reported that promoter activity of MMP-2/9 is enhanced by cis-acting regulatory elements, including NF-κB.[44] In the cytoplasm, NF-κB is bound to a group of inhibitory proteins (IκB). Accumulation of non-phosphorylated IκB prohibits translocation of NF-κB from the cytoplasm to nucleus, resulting in inactivation of NF-κB and its downstream targets.[45] Hence, we examined whether ZD 2-1 suppresses TGF-β1 induced nuclear translocation of NF-κB. Figure 5A shows that SNU182 cells treated with TGF-β1 for various times induced significant phospho-IκB expression, which subsequently induced degradation of IκB (Fig. 5A). However, treatment of SNU182 cells with ZD 2-1 attenuated TGF-β1 induced phosphorylation of IκBα, and consequently increased IκB stability (Fig 5B). Figure 5C and 5D also show that ZD 2-1 dose dependently suppressed TGF-β1 induced nuclear translocation of NF-κB and subsequently suppressed NF-κB binding activity. Thus, ZD 2-1 antagonizes the nuclear translocation of NF-κB permitted by the TGF-β1 induced EMT in

SNU182 cells.

Previous studies have reported that the MAPK signaling pathway is involved in modulating the cancer metastasis and regulated MMPs in tumor cells.[46] Hence, we examined whether ZD 2-1 suppresses TGF-β1 induced phosphorylation of p42/44 MAPK. Figure 5E shows that ZD 2-1 reduces the expression of p-p42/44 MAPK in SNU182 cells in a dose dependent manner. The p-42/44 MAPK/AP-1 signaling pathway is the most important transcription factor in MMPs expression.[47] Therefore, SNU182 cells transfected with luciferase reporter plasmids, involving AP-1 binding element, were pretreated with ZD 2-1, and then stimulated by TGF-β1 or DMSO. Figure 5F shows that ZD 2-1 suppressed TGF-β1 induced AP-1 binding activity, which indicates that ZD 2-1 can decrease AP-1 binding activity. Thus, ZD 2-1 could decrease TGF-β1 induced cancer invasiveness via inhibiting MMP-2/9 expression through the suppression of the NF-κB translocation and p42/44 MAPK/AP-1 signaling pathways.

Fig. 5



HCC remains a malignant tumor with a very low 5-year survival rate, predominantly attributed to recurrence and metastasis.[48] Metastasis occurs through a complex multistep process consisting of cell invasion from a primary tumor into the circulation, emigration of these cells to distant organs, adhesion to endothelial cells and infiltration into tissue. Active metastasis contributes to poor survival rate and high mortality of HCC, accompanied by the EMT, where typical epithelial phenotype cells transform into mesenchymal phenotype cells. The EMT involves loss of E-cadherin expression and gain of mesenchymal marker, such as N-cadherin and vimentin, and is triggered by transcription factors (Snail1, Snail2, Twist1, and Zeb2), inflammatory cytokines, chemokines, growth factors (TGF-β1 and epidermal growth factor), and other enzymes or proteins (ID1, PRL3, and Bmi-1).[21,49–52]

TGF-β1 plays significant roles in pathogenesis of chronic fibroses, including those of the liver. These roles include regulation of proliferation and apoptosis, and stimulation of the EMT. Moreover, the TGF-β1 family regulates MMP expression during TGF-β1 induced EMT in tumor cells. MMP-2 (gelatinase A) mainly degrades gelatins; non-fibrillar collagens IV, V, VII; fibronectin; laminin; and elastin, which enhances motility and invasiveness of tumor cells during metastasis.[53] TGF-β1 also activates the Smads, NF-κB nuclear translocation, and MAPK/AP-1 pathways (non-Smad signaling pathway) during signal transduction of TGF-β1 regulated metastasis. In particular, TGF-β1 induced activation of p42/44 MAPK through non-Smad signaling pathway, and then activation of p42/44 MAPK promotes cell metastasis by activating the different downstream transcription factors, which mediates induction of MMPs and other pro-inflammatory cytokines. It has also been reported that AP-1 is the positive transcriptional regulator of MMPs.[28,29,54]

Naturally occurring compounds with anti-cancer activities interfere with tumor development[55–62] and cancer progression by inhibiting various mechanisms including cell migration, invasion, and metastasis. Supplementation with zingerone in DMH (dimethyl hydrazine) treated rats produced a significant decrease in tumor incidence and aberrant crypt foci formation with simultaneous modulation in the levels of tissue lipid peroxidation and antioxidant status.[34] However, the effect of zingerone in preventing cancer invasion or metastasis has not been previously verified, and the effects of zingerone on inhibiting the TGF-β1 induced EMT of hepatoma cells have not been reported.

Our results showed that 6-shogaol (12.5 μM), ZD 2 (12.5 μM), and zingerone (40 μM) alone did not inhibit TGF-β1 induced EMT development. However, ZD 2-1, a mixture of ZD 2 and zingerone inhibited TGF-β1 induced EMT development in SNU182 hepatocellular carcinoma cells at concentrations of 20 μM and 40 μM, respectively, although these concentrations exerted no influence on cell proliferation (Figs. 1 and 2). Therefore,

we speculate that ZD 2-1 has dual anti-cancer effects via synergistic effect in SNU182 cells. At low concentrations, ZD 2-1 inhibits the TGF-β1 induced EMT, whereas at high concentrations it prevents the anti-proliferative effect. Dual anti-cancer effects of natural compound have been reported in various research articles. For example, ginsenoside 20-Rg has dual anti-cancer effects via stereospecificity in A549 cells. At low concentrations, ginsenoside 20-Rg inhibited TGF-β1 induced EMT via 20(R)-Rg3, whereas at high concentrations it prevented the anti-proliferative effect via 20(S)-Rg3.[63]

The transcriptional factors Snail, Twist1, Slug and Zeb2 play a critical role in the EMT. The Snail transcriptional factor binds to the E-box domain in the E-cadherin promoter, and suppresses E-cadherin transcription.[52] TGF-β1 induced Snail expression and Smad 2/3 activation during EMT development.[37] Figure 2 shows that the increase in Snail expression and phosphorylation of Smad 2 via the TGF-β1 induced EMT was also inhibited by ZD 2-1. Resveratrol, which is polyphenolic component, reduces the TGF-β1 induced Snail level in a dose dependent manner in A549 cells.[38]

Recent studies have also demonstrated that EGCG inhibits the TGF-β1 mediated EMT by suppressing acetylation of Smad2 and Smad3 in A549 cells.[37] Also, geraniin, a polyphenolic component, prevents TGF-β1 induced activation of Smad 2, Snail, and N-cadherin expression level in a dose dependent manner in A549 cells.[22] Sanguiin H6 also suppresses lung cancer migration and invasion in vitro by inhibiting TGF-b1 induction of the EMT.[64] Cerastrol, derived from *T. wilfprdii* Hook F., inhibits expression of TGF-β1 induced Snail in MDCK and A549 cells.[65] The EMT is able to increase metastasis, including migration and invasion in cancer cells. Thus, ZD 2-1 significantly inhibits the TGF-β1 induced increase in migration and invasion in SNU182 hepatocellular carcinoma cells when the concentration of ZD 2-1 reached 40 μM (Fig. 3). ZD 2-1 also inhibited nuclear translocation of NF-κB and activation of p42/44 MAPK/AP1 signaling pathways in the TGF-β1 induced EMT, suggesting that ZD 2-1 probably prevents invasion in the TGF-β1 induced EMT by inhibiting activation of MMP-2/9 and p42/44 MAPK, and nuclear translocation of NF-κB. This is consistent with previous evidence that p42/44 MAPK promotes cell metastasis by activating the different downstream transcription factors, e.g. NF-κB, which mediates much of the downstream effects of IL-1, including induction of MMPs and other pro-inflammatory cytokines.[66] It has also been reported that AP-1 is the positive regulator of MMPs. In the MMP-9 promoter upstream regulation sequence, two AP-1 binding sites (-533 and -79) play essential roles in MMP-9 expression in human Caski cells.[67]

In summary, we have demonstrated that combined treatment with ZD 2, novel zingerone derivative, and zingerone synergistically suppressed SNU182 hepatocellular carcinoma cells migration and invasion *in vitro* by

inhibiting TGF-β1 induced development. Our study provides new evidence that ZD 2 and zingerone synergistically inhibited the TGF-β1 induced EMT and suppressed hepatocellular migration and invasion.

Acknowledgements

This work was supported by Basic Science Research Programs through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (NRF-2015R1D1A3A01017660), and the Korea Institute of Science and Technology Institutional Program (2Z04690).

Conflict of Interest

All the authors confirm there is no potential conflict of interest regarding this publication.

References

1. J.H. Kao, D.S. Chen. *Liver Int.* **2005**, 25, 696.

2. S.S. Thorgeirsson, J.W. Grisham. *Nat. Genet.* **2002**, 31, 339.

3. M.A. Feitelson; B. Sun; N.L. Satiroglu Tufan; J. Liu; J. Pan, Z. Lian. *Oncogene* **2002**, 21, 2593.

4. M.A. Avila; C. Berasain; B. Sangro, J. Prieto. *Oncogene* **2006**, 25, 3866.

5. M.B. Thomas, J.L. Abbruzzese. *J. Clin. Oncol.* **2005**, 23, 8093.

6. M.F. Mulcahy. *Curr. Treat. Options Oncol.* **2005**, 6, 423.

7. A.S. Chung; J. Lee, N. Ferrara. *Nat. Rev. Cancer* **2010**, 10, 505.

8. C.J. Weng; C.P. Chou; C.T. Ho, G.C. Yen. *Mol. Nutr. Food Res.* **2012**, 56, 1304.

9. R. Kalluri, R.A. Weinberg. *J. Clin. Invest.* **2009**, 119, 1420.

10. J.P. Thiery. *Bull Acad. Natl. Med.* **2009**, 193, 1969.

11. D. Xie; C. Gore; J. Liu; R.C. Pong; R. Mason; G. Hao; M. Long; W. Kabbani; L. Yu; H. Zhang; H. Chen; X. Sun; D.A. Boothman; W. Min, J.T. Hsieh. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, 107, 2485.

12. B.H. Hong; C.H. Wu; C.T. Yeh, G.C. Yen. *Mol. Nutr. Food Res.* **2013**, 57, 886.

13. C.M. Overall, O. Kleifeld. *Nat. Rev. Cancer* **2006**, 6, 227.

14. C. Mehner; A. Hockla; E. Miller; S. Ran; D.C. Radisky, E.S. Radisky. *Oncotarget* **2014**, 5, 2736.

15. P. Garg; D. Sarma; S. Jeppsson; N.R. Patel; A.T. Gewirtz; D. Merlin, S.V. Sitaraman. *Cancer Res.* **2010**, 70, 792.

16. K.B. Reddy; J.S. Krueger; S.B. Kondapaka, C.A. Diglio. *Int. J. Cancer* **1999**, 82, 268.

17. H. Ko; S. Kim; C.H. Jin; E. Lee; S. Ham; J.I. Yook, K. Kim. *Mol. Cancer Res.* **2012**, 10, 1032.

18. P.O. Humbert; N.A. Grzeschik; A.M. Brumby; R. Galea; I. Elsum, H.E. Richardson. *Oncogene* **2008**, 27, 6888.

19. D. Medici; E.D. Hay, B.R. Olsen. *Mol. Biol. Cell* **2008**, 19, 4875.

20. D. Zappulla. *J. Cardiometab. Syndr.* **2008**, 3, 30.

21. A. Yadav; B. Kumar; J. Datta; T.N. Teknos, P. Kumar. *Mol. Cancer Res.* **2011**, 9, 1658.

22. H. Ko. *Bioorg. Med. Chem. Lett.* **2015**, 25, 3529.

23. J.Y. Cho. *Korean J. Intern. Med.* **2011**, 26, 367.

24. R. Halwani; S. Al-Muhsen; H. Al-Jahdali, Q. Hamid. *Am. J. Respir. Cell Mol. Biol.* **2011**, 44, 127.

25. Z.H. Qu; Z.C. Yang; L. Chen; Z.D. Lv; M.J. Yi, N. Ran. *Int. J. Mol. Med.* **2012**, 29, 564.

26. C.H. Heldin; M. Landstrom, A. Moustakas. *Curr. Opin. Cell Biol.* **2009**, 21, 166.

27. B.G. Hollier; K. Evans, S.A. Mani. *J. Mammary Gland Biol. Neoplasia* **2009**, 14, 29.

28. A.L. Smith; T.P. Robin, H.L. Ford. *Clin. Cancer Res.* **2012**, 18, 4514.

29. G.J. Sawyer; M. Rela; M. Davenport; M. Whitehorne; X. Zhang, J.W. Fabre. *Curr. Gene Ther.* **2009**, 9, 128.

30. Y. Choi; Y. Park; J.M. Storkson; M.W. Pariza, J.M. Ntambi. *Biochem. Biophys. Res. Commun.* **2002**, 294, 785.

31. B. Ahmad; M.U. Rehman; I. Amin; A. Arif; S. Rasool; S.A. Bhat; I. Afzal; I. Hussain; S. Bilal, M. Mir. *ScientificWorldJournal* **2015**, 2015, 816364.

32. I. Rajan; N. Narayanan; R. Rabindran; P.R. Jayasree, P.R. Manish Kumar. *Biol. Trace Elem. Res.* **2013**, 155, 455.

33. M.K. Kim; S.W. Chung; D.H. Kim; J.M. Kim; E.K. Lee; J.Y. Kim; Y.M. Ha; Y.H. Kim; J.K. No; H.S. Chung; K.Y. Park; S.H. Rhee; J.S. Choi; B.P. Yu; T. Yokozawa; Y.J. Kim, H.Y. Chung. *Exp. Gerontol.* **2010**, 45, 419.

34. R. Vinothkumar; R. Vinothkumar; M. Sudha, N. Nalini. *Eur. J. Cancer Prev.* **2014**, 23, 361.

35. L. Kumar; S. Chhibber, K. Harjai. *Fitoterapia* **2013**, 90, 73.

36. H. Ikushima, K. Miyazono. *Nat. Rev. Cancer* **2010**, 10, 415.

37. H. Ko; Y. So; H. Jeon; M.H. Jeong; H.K. Choi; S.H. Ryu; S.W. Lee; H.G. Yoon, K.C. Choi. *Cancer Lett.* **2013**, 335, 205.

38. H. Wang; H. Zhang; L. Tang; H. Chen; C. Wu; M. Zhao; Y. Yang; X. Chen, G. Liu. *Toxicology* **2013**, 303, 139.

39. W. Birchmeier, J. Behrens. *Biochim. Biophys. Acta* **1994**, 1198, 11.

40. K. Vleminckx; L. Vakaet, Jr.; M. Mareel; W. Fiers, F. van Roy. *Cell* **1991**, 66, 107.

41. T.V. Do; L.A. Kubba; H. Du; C.D. Sturgis, T.K. Woodruff. *Mol. Cancer Res.* **2008**, 6, 695.

42. S.W. Lin; M.T. Lee; F.C. Ke; P.P. Lee; C.J. Huang; M.M. Ip; L. Chen, J.J. Hwang. *Clin. Exp. Metastasis* **2000**, 18, 493.

43. L. Han; H.W. Zhang; W.P. Zhou; G.M. Chen, K.J. Guo. *Chin. Med. J. (Engl)* **2012**, 125, 2032.

44. E. Hadler-Olsen; J.O. Winberg, L. Uhlin-Hansen. *Tumour Biol.* **2013**, 34, 2041.

45. M.S. Hayden, S. Ghosh. *Semin. Immunol.* **2014**, 26, 253.

46. H.L. Hsieh; H.H. Wang; W.B. Wu; P.J. Chu, C.M. Yang. *J. Neuroinflammation* **2010**, 7, 88.

47. U. Benbow, C.E. Brinckerhoff. *Matrix Biol.* **1997**, 15, 519.

48. S. Hatakeyama. *Nat. Rev. Cancer* **2011**, 11, 792.

49. M.K. Asiedu; J.N. Ingle; M.D. Behrens; D.C. Radisky, K.L. Knutson. *Cancer Res.* **2011**, 71, 4707.

50. R.I. Fernando; M.D. Castillo; M. Litzinger; D.H. Hamilton, C. Palena. *Cancer Res.* **2011**, 71, 5296.

51. T. Onoue; D. Uchida; N.M. Begum; Y. Tomizuka; H. Yoshida, M. Sato. *Int. J. Oncol.* **2006**, 29, 1133.

52. J.P. Thiery; H. Acloque; R.Y. Huang, M.A. Nieto. *Cell* **2009**, 139, 871.

53. I. Sehgal, T.C. Thompson. *Mol. Biol. Cell* **1999**, 10, 407.

54. A.M. Grau; P.K. Datta; J. Zi; S.K. Halder, R.D. Beauchamp. *Cell Signal* **2006**, 18, 1041.

55. Y.J. Kim; K.S. Kang; K.C. Choi, H. Ko. *Bioorg. Med. Chem. Lett.* **2015**, 25, 2559.

56. H. Ko; J.M. Kim; S.J. Kim; S.H. Shim; C.H. Ha, H.I. Chang. *Bioorg. Med. Chem. Lett.* **2015**, 25, 4191.

57. H. Ko; S.J. Kim; S.H. Shim; H. Chang, C.H. Ha. *Biomol. Ther. (Seoul)* **2016**, 24, 501.

58. D. Lee; H. Ko; Y.J. Kim; S.N. Kim; K.C. Choi; N. Yamabe; K.H. Kim; K.S. Kang; H.Y. Kim, T. Shibamoto. *J. Agric. Food Chem.* **2016**, 64, 801.

59. J.M. Kim; H. Ko; S.J. Kim; S.H. Shim; C.H. Ha, H.I. Chang. *J. Biochem. Mol. Toxicol.* **2016**, 30, 45.

60. H. Ko; M.H. Jeong; H. Jeon; G.J. Sung; Y. So; I. Kim; J. Son; S.W. Lee; H.G. Yoon, K.C. Choi. *Oncotarget* **2015**, 6, 9970.

61. M.H. Jeong; H. Ko; H. Jeon; G.J. Sung; S.Y. Park; W.J. Jun; Y.H. Lee; J. Lee; S.W. Lee; H.G. Yoon, K.C. Choi. *Oncotarget* **2016**.

62. Y.J. Kim; W.I. Choi; H. Ko; Y. So; K.S. Kang; I. Kim; K. Kim; H.G. Yoon; T.J. Kim, K.C. Choi. *Life Sci.* **2014**, 95, 101.

63. Y.J. Kim; W.I. Choi; B.N. Jeon; K.C. Choi; K. Kim; T.J. Kim; J. Ham; H.J. Jang; K.S. Kang, H. Ko. *Toxicology* **2014**, 322, 23.

64. H. Ko; H. Jeon; D. Lee; H.K. Choi; K.S. Kang, K.C. Choi. *Bioorg. Med. Chem. Lett.* **2015**, 25, 5508.

65. H. Kang; M. Lee, S.W. Jang. *Biochem. Biophys. Res. Commun.* **2013**, 437, 550.

66. A. Santoro; J. Conde; M. Scotece; V. Abella; A. Lois; V. Lopez; J. Pino; R. Gomez; J.J. Gomez-Reino, O. Gualillo. *PLoS One* **2015**, 10, e0135979.

67. J.H. Woo; J.H. Lim; Y.H. Kim; S.I. Suh; D.S. Min; J.S. Chang; Y.H. Lee; J.W. Park, T.K. Kwon. *Oncogene* **2004**, 23, 1845.

Figure Legends

Figure 1. Anti-proliferative effect of 6-gingerol, ZD 2, zingerone, and ZD 2-1 on the viability of SNU182 HCC cells. (A) Synthetic scheme of zingerone derivative 2 and chemical structure: (i) DHP, PPTS, CH_2Cl_2, 99% (ii) LDA, THF, -78℃, 1 h, then 9-ethyl-3-carbazolecarboxaldehyde, 2 h; (iii) TsOH, benzene, 80℃, 1 h, 87% (2 steps). DPH = dihydropyran, PPTS = pyridinium p-toluenesulfonate, THP = tetrahyddropyran, LDA = lithium diisopropylamide, THF = tetrahydrofuran, TsOH = p-toluenesulfonic acid. (B) Anti-proliferative effect of each compound in SNU182 cells. The cells were treated with different concentration of indicated compounds for 48 h on serum free DMEM media. Cell viability was measured using CCK-8 assay. $^{**}p < 0.01$, $^{*}p < 0.05$ versus vehicle. The data are expressed as mean±SD for triplicates.

Figure. 2. ZD 2-1, but not 6-gingerol, ZD 2, or zingerone, suppresses TGF-β1 induced EMT. (A) SNU182 cells were pretreated with 6-shogaol (12.5 μM), ZD 2 (12.5 μM), zingerone (40 μM) or ZD 2 (20 and 40 μM) for 2 h and then stimulated with TGF-β1 (5 ng/ml) for 48 h. Changes in protein expression of E-cadherin and Snail were analyzed by Western blotting using GAPDH loading control. (B, C and D) SNU182 cells were pretreated with ZD 2 (20 and 40 μM) for 2 h and then stimulated with TGF-β1 (5 ng/ml) for 48 h. (B) TGF-β1 treatment induces more elongated morphological shape and increases scattering, while ZD 2-1 suppresses these process in a dose dependent manner. (C) Changes in protein expression of E-cadherin and Snail were analyzed by Western blotting and densitometric quantification using GAPDH as loading control. (D) The phosphorylation of Smad 2/3 was checked by Western blotting using Smad 2/3 as loading control.

Figure 3. Effect of ZD 2-1 on SNU182 cell migration and invasion during inhibition of TGF-β1-induced EMT. (A and B) ZD 2-1 prevented TGF-β1-induced migration. (A) ZD 2-1 antagonized the TGF-β1-induced migration of SNU182 cells. When cell confluence was approximately 90%, the cells were scratched with a 200 μl pipette tip and washed with culture media to remove any free floating cells and debris. Culture media was then added, and the culture plates were incubated with TGF-β1 alone or TGF-β1 and ZD 2-1. Cells were photographed

under a microscope at 0 h and 48 h after scratching. (B) Relative motility was quantified by measuring the cell surface area using the Image J program. (C and D) ZD 2-1 inhibited TGF-β1-induced invasion. (C) Effect of ZD 2-1 on SNU182 cell invasion using 200× light microscope after staining by Matrigel invasion assay. (D) In vitro invasiveness of SNU182 cells was measured by counting cells that migrated through the extracellular matrix layer of invasion chambers. $^{**}p < 0.01$ versus control. The data are expressed as mean±SD for triplicates.

Figure 4. Effects of ZD 2-1 on protein expression and activity of MMP-2 and MMP-9 activated by TGF-β1. (A) SNU182 cells were pretreated with 6-shogaol (12.5 μM), ZD 2 (12.5 μM), zingerone (40 μM) or ZD 2 (20 and 40 μM) for 2 h, and then stimulated with TGF-β1 (5 ng/ml) for 48 h. The expression of MMP-2 and MMP-9 mRNA was analyzed by RT-PCR and densitometric quantification. (B) SNU182 cells were pretreated with ZD 2 (40 μM) for 2 h and then stimulated with TGF-β1 (5 ng/ml) for 48 h. The expression of MMP-2 and MMP-9 mRNA was analyzed by RT-PCR. (C) The expression of MMP-2 and MMP-9 mRNA was analyzed by qRT-PCR. Experiments were repeated at least 3 times. Fold change was calculated by $2^{-\Delta\Delta Ct}$ relative quantitative analysis. (D) SNU182 cells were serum starved in medium containing 0.2% FBS overnight. Cells were then left untreated, or treated with TGF-β1 (5 ng/ml) and/or ZD 2-1 for 48 h. MMP-2 and MMP-9 activity in cell culture medium was examined by gelatin zymography. $^{**}p < 0.01$, $^{*}p < 0.05$ versus vehicle. The data are expressed as mean±SD for triplicates.

Figure 5. Effects of ZD 2-1 on translocation of NF-κB and activation of p42/44 MAPK/Ap-1 pathway by TGF-β1. (A) NF-κB translocation related to phosphorylation and expression of IκB was assessed using Western blotting in TGF-β1 induced SNU182 cells for 0, 60 and 120 min. (B) SNU182 cells were pre-treated with ZD 2-1 (20 and 40 uM) for 2 h and treated with TGF-β1 for 120 min. Protein levels of IκB were assessed using Western blotting using GAPDH loading control. (C) SNU182 cells were pre-treated with ZD 2-1 for 2 h and treated with TGF-β1 for 12 h. Protein levels of NF-κB translocation from cytosol to the nucleus was assessed using Western blotting using COX4 and LaminB1 as cytosolic and nucleus markers, respectively. (D) Reporter assay of NF-κB binding activity in SNU182 cells. After cells were transfected with pGL4.32-NF-κB-RE (Response Element)-Luc or pGL4.32, ZD 2-1 and/or TGF-β1 were treated in SNU182 cells. Reporter assay was performed under the same conditions. (E) SNU182 cells were incubated with TGF-β1 (5 ng/ml) or with TGF-β1 (5 ng/ml) and ZD 2-1 (20 and 40 μM) for 48 h. The cells were harvested and then lysed. Phosphorylation of

p42/44 MAPK was analyzed by Western blotting using p42/44 MAPK as loading control. (F) Reporter assay of AP-1 binding activity in SNU182 cells. After cells were transfected with pGL4.44-AP-1-RE (Response Element)-Luc or pGL4.44, ZD 2-1 (20 and 40 μM) and/or TGF-β1 were treated in SNU182 cells. Reporter assay was performed under the same conditions. The data are expressed as mean±SD for triplicates. $^{**}p < 0.01$, $^{*}p < 0.05$ versus vehicle.

*Graphical Abstract