A novel derivative of decursin, CSL-32, blocks migration and production of inflammatory mediators and modulates PI3K and NF-κB activities in HT1080 cells

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Abstract

Decursin and related coumarin compounds in herbal extracts have a number of biological activities against inflammation, angiogenesis and cancer. We have analysed a derivative of decursin (CSL-32) for activity against inflammatory activation of cancer cells, such as migration, invasion and expression of pro-inflammatory mediators. The human fibrosarcoma cell line, HT1080, was treated with TNFα (tumour necrosis factor α) in the presence or absence of CSL-32. The cellular responses and modification of signalling adapters were analysed with respect to the production of pro-inflammatory mediators, as also migration, adhesion and invasion. Treatment of HT1080 cells with CSL-32 inhibited their proliferation, without affecting cell viability, and TNFα-induced expression of pro-inflammatory mediators, such as MMP-9 (matrix metalloproteinase-9) and IL-8 (interleukin-8). CSL-32 also suppressed phosphorylation and degradation of IκB (inhibitory κB), phosphorylation of p65 subunit of NF-κB (nuclear factor-κB) and nuclear translocation of NF-κB, which are required for the expression of pro-inflammatory mediators. In addition, CSL-32 inhibited invasion and migration of HT1080 cells, as also cellular adhesion to fibronectin, an ECM (extracellular matrix) protein. CSL-32 treatment resulted in a dose-dependent inhibition of PI3K (phosphoinositide 3-kinase) activity, required for the cellular migration. The analyses show that CSL-32 inhibits processes associated with inflammation, such as the production of pro-inflammatory mediators, as well as adhesion, migration and invasion in HT1080 cells.

Keywords: cytokine; inflammation; invasion; NF-κB; signal transduction

1. Introduction

Decursin is a coumarin compound that has strong anti-cancer activities along with its structural isomer decursinol angelate (Lee et al., 2003, 2009; Kim et al., 2006a; Yang et al., 2009). Recent reports demonstrated the underlying molecular mechanism of their anti-cancer activity. These included the induction of G1 arrest and apoptosis in prostate and breast cancer cells (Yim et al., 2005; Jiang et al., 2007); suppression of androgen-induced cellular proliferation (Jiang et al., 2006) and androgen-independent cellular proliferation through promotion of the degradation of β-catenin (Song et al., 2007) in prostate cancer cell lines; and suppression of angiogenesis through the inhibition of vascular endothelial growth factor-induced proliferation, migration and the tube formation of human umbilical vein endothelial cells (Jung et al., 2009; Kim et al., 2009; Son et al., 2009).

In addition to their anti-cancer activities, decursin and its derivatives have anti-inflammatory activities. Decursin and/or related compounds inhibited inflammation-associated production of pro-inflammatory mediators in cancer cells (Kim et al., 2010), macrophages (Kim et al., 2006a) and a mouse model of asthma (Yang et al., 2009). Since the onset and development of cancer is closely associated with inflammation (Philip et al., 2004; Mantovani et al., 2008), it is expected that both the anti-cancer and anti-inflammatory activities of these compounds will contribute to the successful treatment of cancer.

As a part of ongoing efforts in designing and synthesizing new decurcin analogues with potential anti-inflammatory activity, CSL-32 was generated. In vitro analysis of the effect of CSL-32 treatment showed that this compound can suppress various activities associated with inflammation in a fibrosarcoma cell line, HT1080. The activities inhibited by CSL-32 were secretion of inflammatory mediators, cell adhesion towards fibronectin-coated plates, migration and invasion through ECM (extracellular matrix). The molecular mechanisms of these anti-inflammatory activities were analysed with respect to the activation of cellular signalling adaptors that are involved in inflammation.

2. Materials and methods

2.1. Cell culture and reagents

mAbS (monoclonal antibodies) against p-p65 (clone 93H1) and phospho-ΙκB (inhibitory κB; Ser32/36) (clone 5A5), polyclonal

Abbreviations: CCK, cell counting kit; ECM, extracellular matrix; IκB, inhibitory κB; IL, interleukin-8; mAb, monoclonal antibody; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; PI3K, phosphoinositide 3-kinase; TNF, tumour necrosis factor.
antibodies against Iκ-B, phospho-Akt (Akt is also known as protein kinase B) and Akt were purchased from Cell Signaling Technology Inc. Mouse mAb against p65, phospho-p65 (clone F-6) and polyclonal antibody against actin were purchased from Santa Cruz. Recombinant TNFα (tumour necrosis factor α) was purchased from R&D systems. HT1080 cell line was purchased from A.T.C.C.

2.2. Measurement of cellular invasion and migration

These assays were done as previously described (Kim et al., 2010). Briefly, an invasion assay through Matrigel (Sigma) started with coating the upper part of Transwell (8 μm pore, Millipore) with 100 μg/cm² Matrigel for 15 min at 37°C. HT1080 cells (2 x 10⁵) were pretreated with CSL-32 for 3 h and added into the upper well and the lower wells were filled with a culture media. After 24 h incubation, cells on the upper side of the membrane were removed with cotton swabs and the membrane was fixed in methanol and the cells were stained with haematoxylin and eosin. Pictures (x 100) taken in 5 random fields of the membrane and the cell numbers were counted. For the measurement of cellular migration using the 48-well Boyden chamber (Probe Inc.), the lower wells were filled with 27 μl of RPMI 1640 (supplemented with 10% serum) and the upper wells were filled with 50 μl of cells (2 x 10⁵ cells/well) which had been pretreated with CSL-32 for 3 h. The two compartments were separated by a 8-μm-pored polyvinylpyrrolidone-free filter (Neuro Probe Inc.). After 24 h incubation at 37°C, the number of cells that had migrated into the lower wells was counted, pictures of the membrane were taken and cells were counted as described above. These experiments were performed in triplicate samples.

2.3. Gelatin zymogram, western blot and ELISA

HT1080 cells (1 x 10⁶/well) were incubated in 96-well plates in the presence or absence of 20–80 μM of CSL-32 and/or 3 ng/ml TNFα. The culture supernatants were collected 24 h after activation, and gelatin zymogram analyses were performed as described previously (Lee et al., 2001b; Kim and Lee, 2004). For the measurement of cytokine concentrations, culture supernatants were collected 24 h after activation and cytokines were measured by sandwich ELISA (Endogen Inc.); detection limits were <10 pg/ml. Cell lysis and Western blot analysis was performed as described previously (Kim et al., 2006b; Bae et al., 2007).

2.4. Cell adhesion assay and cell viability assay

For the measurement of cell adhesion, culture plates (96-well plates) were coated with 10 μg/ml fibronectin overnight. HT1080 cells pre-treated with or without CSL-32 for 3 h were added into each well (4 x 10⁵ cells/well). After 30 min, unattached cells were removed by PBS washing and the CCK-8 (cell counting kit-8) assay was performed to measure the amount of attached cells. For the measurement of cell viability, CCK-8 (Dojindo Laboratories) was used. HT1080 cells in 96-well plates (1 x 10⁶/100 μl/well) were incubated with CSL-32 for 24 h and 10 μl of CCK-8 solution was added into each well. After a 4 h incubation at 37°C in 5% CO₂, attenuation at 450 nm was measured using a microplate reader.

2.5. Immunofluorescence assay

For the analysis of subcellular location of NF-κB (nuclear factor-κB), HT1080 cells (2 x 10⁵) were grown on a cover glass, washed in PBS and fixed with 4% (w/v) formaldehyde. Cells were then permeabilized with 1% Triton X-100 in PBS for 10 min and incubated with anti-p65 mAb (clone F-6, Santa Cruz) (10 μg/ml in PBS containing 3% BSA) at 37°C for 45 min. Cells were incubated with the Alexa Fluor® 488-labelled goat anti-mouse antibody (Molecular Probes) (10 μg/ml) at 37°C for 45 min and counterstained with DAPI (4',6-diamidino-2-phenylindole; Molecular Probes). The slides were then mounted in a 1:1 ratio mixture of Xylene and Malinol (Muto Pure Chemicals) for observation with a fluorescence microscope. Pictures (x100) were taken in 5 random fields and the cell numbers with nuclear p65 were counted.

2.6. ELISA-based measurement of NF-κB binding activity

NF-κB binding activity was measured following a previously described method (Rosenau et al., 2004). Briefly, streptavidin-coated 96-well culture plates were used to immobilize biotin-labelled double-stranded oligonucleotides containing a consensus NF-κB binding site (5'-cagaggttgaggggcttcaggc-3') (0.02 nM/well). Whole cell lysates or nuclear lysates were then added into the NF-κB oligonucleotide plates and incubated at room temperature for 1 h with mild agitation in 100 μl/well of PBS. Utilization of either whole cell lysate or nuclear lysate resulted in the same results. The plates were sequentially incubated with antibodies specific to NF-κB p65 subunit, HRP-labelled secondary antibody and tetramethylbenzidine (chromogen). Attenuance (450–540 nm) was then measured and the values were normalized by subtracting the background values. For blocking, cell lysates were pre-incubated with 1 nM per sample of double-stranded oligonucleotides containing wild-type NF-κB binding sequence or a mutant sequence (5'-cagaggttgaggggcttcaggc-3') before adding them to the NF-κB oligonucleotide plates.

2.7. Statistical analysis

The statistical significance of differences was evaluated by means of two-sided Student’s t test assuming equal variances. The differences were considered significant when P < 0.05.

3. Results and discussion

3.1. CSL-32 inhibits the cellular invasion, migration and adhesion in HT1080 cells

Inflammatory activation involves activation of adhesion, migration and invasion potential. In order to test whether CSL-32 (Figure 1A) affects invasion of HT1080 cells, a Matrigel invasion assay was
performed. The addition of increasing amounts of CSL-32 resulted in a dose-dependent inhibition of cellular invasion through the ECM (Figure 1B). The effect of CSL-32 on the migration of HT1080 cells was tested using a Boyden chamber. Treatment of CSL-32 resulted in a significant inhibition of cellular migration (Figure 1C). Since migration and invasion of cancer cells are accompanied by the adhesion of cells to the ECM, HT1080 cells were incubated in fibronectin-coated plates in the presence of CSL-32. Treatment with CSL-32 resulted in the inhibition of cellular adhesion in a dose-dependent manner (Figure 1D).

Invasion and migration require cytoskeletal movement regulated by the PI3K (phosphoinositide 3-kinase)/Akt pathway. PI3K is a family of enzymes with complex multifunctional roles that involves a variety of cellular activities including migration, chemotaxis and cellular trafficking (Katsu et al., 2001; Teranishi et al., 2009). One of the major downstream targets of PI3K is serine/threonine kinase Akt. Stimulation of PI3K/Akt pathway results in an increase of cell survival and actin cytoskeleton reorganization (Shiojima and Walsh, 2002; Tu et al., 2009; Yan et al., 2009). Activated Akt also plays a role in a variety of biological functions, including cell migration (Lee et al., 2001a; Kumar et al., 2009).

Inhibition of PI3K activity resulted in the suppression of invasion and migration in HT1080 cells (Kim et al., 2010). To analyse whether the activity of PI3K was affected by CSL-32, the phosphorylation levels of Akt were tested using Western blot in HT1080 cells stimulated with TNF-α in the presence of CSL-32. TNF-α-induced phosphorylation of Akt within 30 min and pretreatment with CSL-32 resulted in a dose-dependent inhibition of Akt phosphorylation (Figures 1E and 1F). This indicates that CSL-32-mediated inhibition of PI3K activity could be one of the mechanisms that are responsible for the inhibition of cellular invasion and migration.

Interaction with ECM is required for the cell survival as well as cellular migration. The acquisition of the ability to move through the ECM environment is required for cancer metastasis. When the cells were allowed to interact with fibronectin, one of the ECM proteins, in the presence of CSL-32, cellular adhesion was inhibited. These data suggest that CSL-32-mediated inhibition of cellular adhesion could be an additional mechanism responsible for its inhibition of cell migration and invasion.

3.2. CSL-32 inhibits the expression of inflammatory mediators through suppression of NF-κB activation

HT1080 cells are well known to respond to TNF-α treatment by secretion of pro-inflammatory mediators. When HT1080 cells were stimulated with TNF-α in the presence of CSL-32, the expression of MMP-9 and IL-8 (interleukin-8; Figure 2A) was inhibited in a dose-dependent manner. The expression levels of MMP-2, which is known to be independent on the inflammatory activation, were unaffected by TNF-α and CSL-32. Since decursin causes cell cycle arrest and apoptotic cell death in some cancer cell lines (Yim et al., 2005; Jiang et al., 2006, 2007; Song et al., 2007), it is possible that the observed reduction in MMP-9 and IL-8 expression levels by CSL-32 may have caused by its growth suppressive effects or cytotoxicity. To test this possibility, the viability of the cells was measured using CCK-8-based metabolic activity test. CSL-32 did not affect the viability of HT1080 cells at the time and concentration ranges used (data not shown).

NF-κB is the major transcription factor that is involved in the expression of pro-inflammatory mediators such as cytokines, matrix degrading enzymes and adhesion molecules. NF-κB, present in the cytoplasm in complex with IκB, can be activated by...
CSL-32 suppresses inflammation in cancer cells

Figure 2  CSL-32 inhibits the production of pro-inflammatory mediators and phosphorylation and degradation of IκB
(A) HT1080 cells were pretreated with CSL-32 for 3 h and then stimulated with 3 ng/ml TNF-α. The culture supernatants were collected 24 h after activation and subjected to gelatin zymogram for the measurement of MMP-9 and MMP-2 activities or ELISA for the measurement of IL-8 concentration. (B) Cells were stimulated with TNF-α in the presence or absence 80 μM CSL-32. Total cell lysates obtained at indicated times and the level of phospho-IκB or actin in the cellular lysates were analysed using Western blotting. The p-IκB band intensity values were measured and normalized with corresponding actin band intensity values. (C) Cells were stimulated with TNF-α in the presence of CSL-32. The level of IκB and actin in the cellular lysates were analysed using Western blotting. The IκB band intensity values were measured and normalized with corresponding actin band intensity values. Data points are represented as means ± S.E.M. of 3 independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared with the corresponding positive control.

activation-induced phosphorylation and degradation of IκB. Liberated NF-κB then translocates to the nucleus for the transcriptional activation of its target genes. TNFα-induced expression of MMP-9 and IL-8 is being suppressed by pharmacological inhibitors of NF-κB (Kim et al., 2010). Since CSL-32 inhibits the TNFα-induced expression of MMP-9 and IκB, the phosphorylation status of IκB was tested in HT1080 cells that have been stimulated with TNFα in the presence of CSL-32. Treatment with TNFα-induced rapid phosphorylation of IκB and CSL-32 pretreatment resulted in a partial inhibition of it (Figure 2B). Accordingly, degradation of IκB was also blocked by CSL-32 pretreatment (Figure 2C). Nuclear translocation of NF-κB was then tested using immunofluorescence analysis with a mAb specific for p65 subunit of NF-κB. As shown in Figure 3(A), CSL-32 dose-dependently inhibited nuclear translocation of NF-κB that contains p65 subunits.

In addition to the association with IκB, other mechanisms including phosphorylation of p65 subunit regulate NF-κB activities. Phosphorylation of p65 subunit is mediated by IκB kinase complex during LPS (lipopolysaccharide) stimulation (Yang et al., 2003) and Ser536 phosphorylation is responsible for the recruitment of co-activators such as p300, promoting the activating potential of NF-κB and the subsequent production of inflammatory cytokines (Chen et al., 2004). In an effort to find out whether CSL-32 affects phosphorylation of NF-κB p65 subunit, Western blot analysis was performed using mAb specific for the phosphorylated form of p65. TNFα treatment induced phosphorylation of

Figure 3  CSL-32 inhibits the nuclear translocation of NF-κB, phosphorylation of p65 subunit and NF-κB DNA-binding activity
(A) HT1080 cells were stimulated with 3 ng/ml TNFα in the presence of CSL-32. Total cell lysates obtained 20 min after activation and the subcellular location of NF-κB was analysed using immunofluorescence analysis with mAb against p65 subunit. Stained cells were then analysed using a fluorescence microscope and the percentage of cells with nuclear translocation of p65 was counted. (B) Cells were stimulated with TNFα for 15 min in the presence of CSL-32. The levels of phospho-p65, p65 and actin in the cell lysates were analysed using Western blotting. The p-p65 band intensity values were measured and normalized with corresponding actin band intensity values. Data points are represented as means ± S.E.M. of 3 independent experiments. *P<0.05 and ***P<0.001 when compared with positive control.
p65. When the cells were treated with CSL-32, TNF-α-induced phosphorylation of p65 subunit was attenuated in a dose-dependent manner (Figures 3B and 3C).

To investigate the inhibitory effect of CSL-32 on the activation of NF-κB at the functional level, ELISA-based NF-κB binding assay was performed. The plates were coated with consensus NF-κB binding sequences and the amount of DNA-bound NF-κB was detected with mAb specific for NF-κB p65 subunit. DNA-binding activity peaked at 20 min after stimulation with TNFα (Figure 3D). The binding was inhibited by pre-incubation of cell lysates with consensus NF-κB binding sequences, but not with mutant sequences (Figure 3D), indicating that the interaction was specific. When the cells were pre-treated with CSL-32, DNA-binding activity of NF-κB was significantly inhibited in a dose-dependent manner (Figure 3E). Decursin also inhibited the DNA-binding activity of NF-κB at 80 μM, but the inhibitory activity was lower than 20 μM CSL-32. Decursinol anglate, a closely related derivative of decursin, tended to lower the DNA-binding activity of NF-κB in a dose-dependent manner (Figure 3F). When the cells were pre-treated with CSL-32, DNA-binding activity of NF-κB was lower than 20 μM CSL-32. Decursinol anglate, a closely related derivative of decursin, tended to lower the DNA-binding activity, but the extent of reduction was not statistically significant.

4. Conclusion

CSL-32 inhibits TNF-induced expression of pro-inflammatory mediators and cellular invasion (or migration) through ECM in HT1080 cells. CSL-32 also suppresses activation and nuclear translocation of NF-κB required for the expression of pro-inflammatory mediators. Additionally, CSL-32-inhibited TNF-induced activation of PI3K, cellular adhesion towards ECM proteins and the production of a matrix degrading enzyme (MMP-9), known to be associated with cell migration and/or invasion.

Author contribution

Seung-Hee Lee and Eun-Ju Kim analysed the activities of CSL-32 in the cell culture system. Won-Jung Kim was involved in the initial screening of CSL-32 in a chemical library. Jee Hyun Lee, Joo-Hwan Kim and Gyu Yong Song were involved in chemical synthesis of CSL-32. Kyoungho Suk helped with paper preparation and setting the experimental scheme. Won-Ha Lee is the corresponding author, involved in the overall process of research and paper writing.

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