

## Binding Model of Fisetin and Human c-Jun NH<sub>2</sub>-Terminal Kinase 1 and Its Anti-inflammatory Activity

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Fisetin is a naturally occurring flavonoid with some anti-cancer and anti-inflammation capabilities. In this study, we perform docking studies between human c-Jun N-terminal kinase 1 (JNK 1) and fisetin and proposed a binding model of fisetin and JNK 1, in which the hydroxyl groups of the B ring and oxygen at the 4-position of the C ring play key roles in binding interactions with JNK. Fluorescence quenching and saturation-transfer difference (STD) NMR experiments showed that fisetin exhibits good binding affinity to JNK,  $1.32 \times 10^8 \text{ M}^{-1}$ . The anti-inflammatory activity of fisetin was also investigated. Fisetin significantly suppressed tumor necrosis factor, the NO production, and macrophage inflammatory cytokine release in LPS-stimulated RAW264.7 mouse macrophages. We found that the anti-inflammatory cascade of fisetin was mediated through the JNK, and cyclooxygenase (COX)-2 pathways. Our findings suggest the potential of fisetin as an anti-inflammatory agent.

**Key Words** : c-Jun N-terminal kinase 1, Fisetin, Anti-inflammatory activity, Docking model, STD-NMR

### Introduction

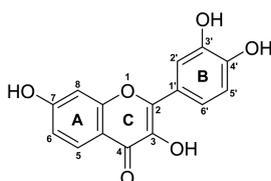
Inflammation is a natural biological response to injury or infection in the human body. Various factors, such as microbial infections, chemicals, and immunologic reactions can cause inflammation.<sup>1</sup> Prolonged inflammation can be harmful, contributing to the pathogenesis of many diseases, including rheumatoid arthritis, obesity, cardiovascular diseases, neurodegenerative diseases, diabetes, and cancer.<sup>2,3</sup> During the inflammatory process, many kinds of cells are activated, and these cells secrete various pro-inflammatory molecules, including cytokines and nitric oxide (NO). Macrophages are essential for the host defense system and the secretion of pro-inflammatory mediators in response to bacterial lipopolysaccharide (LPS).<sup>4-7</sup> LPS induces the expression of nitric oxide synthases (NOS), NO production, and the release of pro-inflammatory cytokines (*e.g.*, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1, and interleukin (IL)-6.<sup>8-10</sup> Inducible NOS (iNOS), which is responsible for the synthesis of NO, is highly expressed in macrophages. The increase in inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 causes cell damage following the increased production of NO and prostaglandins (PGs).<sup>11</sup> Suppressing the induction of COX-2 is a new strategy in the

prevention of inflammation and COX-2 expression is also related to the activities of many intracellular signaling proteins, such as extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK), and Jun-N terminal kinase (JNK).<sup>12</sup>

Inhibition of inflammatory cytokine and mediator production serves as a key mechanism in the control of inflammation. A number of anti-inflammatory molecules have already entered clinical trials for the treatment of inflammatory disorder such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), prostaglandin E2 (PGE2) or nitric oxide (NO).<sup>13</sup> Drugs that suppress the expression of these inflammatory mediators have therefore attracted significant interest as potential therapeutics for the treatment of inflammatory diseases.<sup>14</sup>

Flavonoids constitute a large group of low molecular weight polyphenolic compounds that are common components of plants. They have been studied as potential anti-inflammatory reagents because some of flavonoids inhibited pro-inflammatory cytokine-induced chemokine expression.<sup>15</sup> There are flavonoids known to interact with the ATP-binding sites of tyrosine kinase and serine kinase, resulting in the inhibition of these proteins.<sup>16</sup> Many lines of evidence suggest a direct role for these flavonoids in modulating the inflammatory response *in vitro* or in cellular models. Fisetin (3,7,3',4'-tetrahydroxyflavone) (Figure 1) is a naturally occurring flavonoid found in various vegetables and fruits such as strawberry, cucumber, persimmon, apple, and onion. Dietary fisetin possesses antioxidative,<sup>17</sup> anti-inflammatory,<sup>18</sup> and antiproliferative activities in a wide variety of cancer cells including hepatoma,<sup>19</sup> lung adenocarcinoma, and Burkitt lymphoma.<sup>20</sup>

Abbreviations: ERK, extracellular signal-regulated kinase; iNOS, inducible nitric oxide synthase; JNK, Jun-N terminal kinase; LPS, lipopolysaccharide; GAPDH, glyceraldehyde 3-phosphate; MAPK, mitogen-activated protein kinase; MIP-1, macrophage inflammatory protein-1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; RT-PCR, reverse transcription-polymerase chain reaction; STD, saturation-transfer difference



**Figure 1.** Structure of fisetin.

In this study, we further investigated the anti-inflammatory activities of fisetin and its mechanism in mouse macrophages. The anti-inflammatory activity of fisetin was established by examining inhibition of nitrite production, tumor necrosis factor (mTNF)- $\alpha$  release, and macrophage inflammatory protein (mMIP)-1, and mMIP-2 cytokine release and mRNA expression in LPS-stimulated RAW264.7 cells and systematically present our understanding of the mechanisms by which it activated JNK, and COX-2 regulation. For the first time, we investigated the interactions between the fisetin and JNK by molecular docking and fluorescence quenching as well as saturation-transfer difference (STD)-NMR spectroscopy. These results suggest that fisetin could be a potent agent with anti-inflammatory activities.

### Materials and Methods

**Reagents.** Fisetin (Figure 1) was purchased from Sigma Aldrich and dissolved in DMSO at 10 mg/mL for the stock solution.

**Docking Study.** Using the X-ray crystallography structure of JNK (3v3v.pdb), we defined the ATP-binding site of JNK. Fisetin was docked to JNK using CDOCKER, a CHARMM-based molecular dynamics (MD) method for ligand-docking, in Discovery Studio modeling (Accelrys Inc., San Diego, USA). This algorithm assumes a rigid protein and permits only the ligand to be flexible. The Input Site Sphere parameter specifies a sphere around the center of the binding site, where the CDOCKER experiment is to be performed. The center of the sphere is used in the CDOCKER algorithm for initial ligand placement. The MD simulated annealing process is performed using a rigid protein and flexible ligand. The final minimization step is applied to the ligand's docking pose. The minimization consists of 50 steps of steepest descent followed by up to 200 steps of conjugated-gradient using an energy tolerance of 0.001 kcal/mol.<sup>21</sup>

**Fluorescence Quenching Analysis.** Experiments were performed at 25 °C on an RF-5301PC spectrofluorophotometer (Shimadzu, Kyoto, Japan). We titrated fisetin to 10  $\mu$ M JNK1 protein solution in 50 mM sodium phosphate buffer containing 100 mM NaCl at pH 8.0, with a final protein:inhibitor ratio of 1:10. The sample was placed in a 2 mL cuvette, with excitation and emission path lengths of 10 nm. Using tryptophan emission, we determined the fluorescence quantum yields of JNK and the ligand. The detailed methods are provided in a previous article.<sup>22</sup>

**Saturation Transfer Difference NMR (STD-NMR).** The protein was saturated on-resonance at  $-1.0$  ppm and off-resonance at 30 ppm, with a cascade of 40 selective Gaussian-

shaped pulses of 50 ms duration and 100 ms delay between each pulse in all STD-NMR experiments at 25 °C. The total duration of the saturation time was set to 2 s. For STD-NMR experiments, 10  $\mu$ M recombinant JNK in 50 mM sodium phosphate buffer, 100 mM NaCl, pH 8.0, and fisetin was mixed at a protein:ligand ratio of 1:100. In total, 1600 scans for each experiment were acquired, and a WATERGATE sequence was used to suppress the water signal. A spin-lock filter (5 kHz strength and 10 ms duration) was applied to suppress the protein background. All NMR spectra were recorded on a Bruker Avance 500 MHz NMR spectrometer at KBSI.

**Expression and Purification of JNK1 Protein.** JNKs have numerous isoforms, including JNK1, JNK2, and JNK3, which have been identified in mammals. JNK1 and JNK2 are expressed in the whole cells and tissues of mammals while JNK3 is found primarily in the brain. To express JNK1, the C-terminal truncated form of human JNK1 (residues 1-364) was cloned into the pET21b expression vector (Novagen) and expressed in *Escherichia coli* as a 6 His-tagged form at the C-terminus. JNK1 was then purified as reported previously.<sup>23</sup>

**Cytotoxicity in Mammalian Cells.** The mouse macrophage-derived RAW264.7 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The cytotoxicity of fisetin in RAW264.7 was determined as reported previously.<sup>23,24</sup>

**Quantification of Nitrite Production in LPS-stimulated RAW264.7 Cells.** Nitrite accumulation in culture media was used as an indicator of NO production (25). Raw264.7 cells were plated at a density of  $1 \times 10^5$  cells/mL and stimulated with LPS (20 ng/mL) from *E. coli* O111:B4 (Sigma) in the presence or absence of 3,6-fisetin for 24 h. Isolated supernatant fractions were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid), and nitrite production was determined by measuring absorbance at 540 nm and converted to nitrite concentrations by reference to a standard curve generated with NaNO<sub>2</sub>.<sup>25</sup>

**Quantification of Inflammatory Cytokines (mTNF- $\alpha$  and mMIP-2) in LPS-stimulated RAW264.7 Cells.** Antibodies against mTNF  $\alpha$  and mMIP-2 were used for immobilization on immune plates as reported previously.<sup>26</sup>

**Reverse Transcription-polymerase Chain Reaction.** RAW264.7 cells were stimulated without (negative control) or with 20 ng/mL LPS in the presence or absence of fisetin for 3 h. Competitive RT-PCR was performed as described previously.<sup>27</sup> The targets were amplified from the resulting cDNA by PCR using the following specific primers: mMIP-1, 5'-ATG AAG CTC TGC GTG TCT GC-3' (sense) and 5'-TGA GGA GCA AGG ACG CTT CT-3' (antisense); mMIP-2, 5'-ACA CTT CAG CCT AGC GCC AT-3' (sense), and 5'-CAG GTC AGT TAG CCT TGC CT-3' (antisense). The primers for glyceraldehyde 3-phosphate (GAPDH), used as an internal control, were 5'-ACC ACA GTC CAT GCC ATC AC-3' (sense) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (antisense). PCR was performed using the

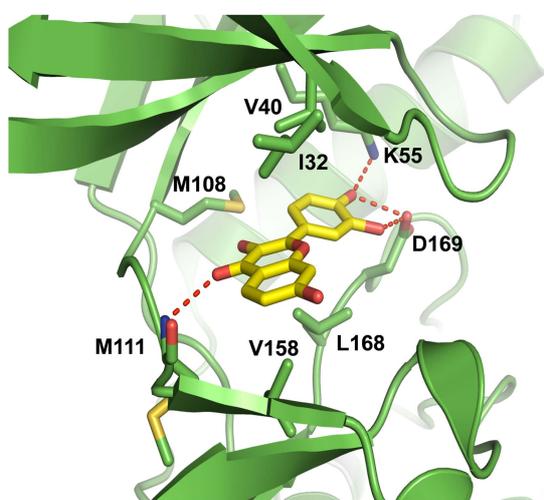
following cycling conditions: 94 °C for 5 min, followed by 25 cycles of 94 °C for 1 min, 55 °C for 1.5 min and 94 °C for 1 min, and a final extension step of 72 °C for 5 min.

**Western Blotting.** The proteins were isolated from LPS-stimulated RAW264.7 cells with or without fisetin, and signals were detected as reported previously.<sup>28</sup> Antibodies were used specifically for phospho-ERK (1:2000; Cell Signaling Technology, Beverly, MA, USA), JNK and COX-2 (1:1000, Cell Signaling Technology), and  $\beta$ -actin (1:5000, Sigma-Aldrich, St. Louis, MO, USA). The relative amount of protein associated with each antibody was quantified using ImageJ (NIH, Bethesda, USA).

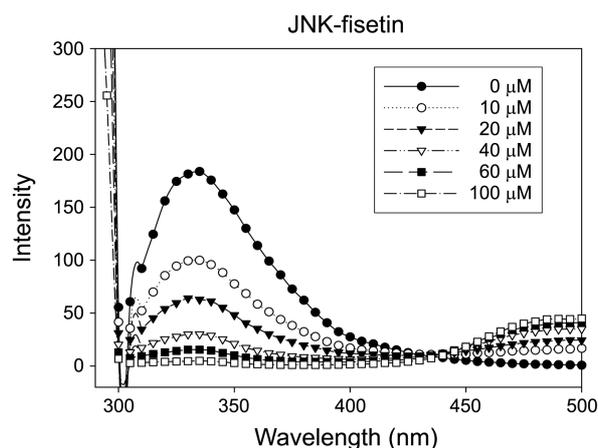
## Results and Discussion

**Docking Study.** To know the binding model of fisetin and JNK, we carried out docking study with fisetin and JNK. Fisetin was docked into the ATP-binding active site of JNK. We investigated the importance of the hydrogen bonds that participate in the interaction between fisetin and JNK. The side chains of Lys55, Glu73 and Asp169 form a network of hydrogen bonds with the 4'-hydroxy group of the catechol moiety of the ligand, while the benzopyran portion forms a hydrogen bond interaction with the backbone amide of Met111. The ligand forms additional hydrophobic interactions with both nonpolar faces of the binding cleft (Ile32, Val40, Ala53, Met108, Val158, and Leu168 from the N- and C-terminal lobes), as shown in Figure 2.

**Fluorescence Quenching.** The JNK protein contains four tryptophan residues at positions 209, 234, 324, and 352. Among these, Trp352 is in closest proximity to the substrate binding site of JNK and protein fluorescence should be decreased upon ligand binding to JNK. Fluorescence quenching was used to estimate the binding constant,  $K$ . The binding (or dissociation) constant,  $K$ , is defined as  $[\text{complex}]/[\text{free protein}][\text{free inhibitor}]$ .<sup>29</sup> We successfully analyzed the binding affinities between JNK and fisetin using this method.



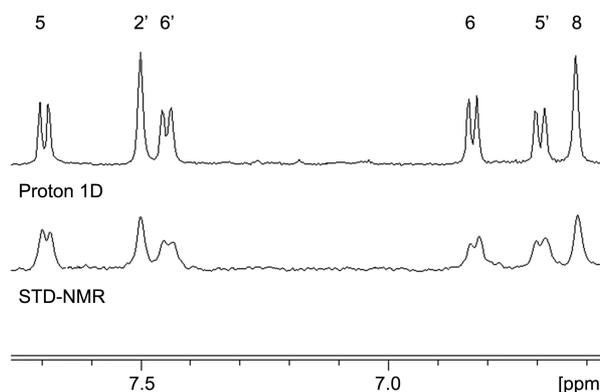
**Figure 2.** Binding model of fisetin and JNK determined by docking study. Red dotted line presented hydrogen bonds between JNK and inhibitor.



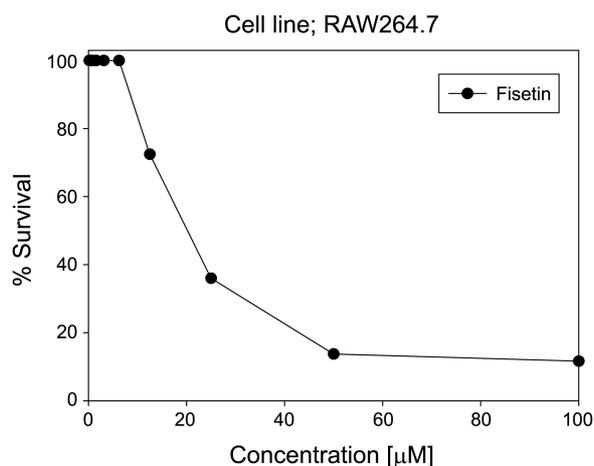
**Figure 3.** Fluorescence spectra of an aqueous solution of 10  $\mu\text{M}$  JNK in the presence of different concentrations of fisetin (0, 10, 20, 40, 60, and 100  $\mu\text{M}$ ) at pH 7.0. Samples were excited at 290 nm, and emission spectra recorded for light scattering effects from 290 to 500 nm.

Fluorescence titration curves for JNK with fisetin are depicted in Figure 3. The fluorescence intensity changed with increasing concentrations of fisetin. This spectral change was attributed to the formation of protein-fisetin complexes. As shown in Figure 3, a significant decrease was evident in the quenching of JNK tryptophan fluorescence in the presence of fisetin. The binding affinity of the fisetin was estimated to be  $1.32 \times 10^8 \text{ M}^{-1}$ .

**STD-NMR Experiment.** To investigate the mode of fisetin interaction with JNK, we performed STD-NMR experiments to support the site-specific binding information of the ligand in the active site of JNK. In the STD-NMR spectra of fisetin, <sup>1</sup>H signals of the B-ring (2', 5' and 6') were reduced by 46%, 44% and 51%, respectively, and <sup>1</sup>H spectra of the A-ring (5, 6 and 8) were decreased by 36%, 54% and 45%, respectively after saturation transfer (Figure 4). <sup>1</sup>H signal of the 5-position at A-ring has largest STD effects; obviously, this hydrogen has more and tighter contacts with the protein surface. The large STD effect was found for 6-position at C-ring, implying that 7-OH group of fisetin display less interactions with the protein. These STD-NMR spectra, showing that reson-



**Figure 4.** Representative result of the STD-NMR binding assay. <sup>1</sup>H NMR spectra of fisetin and its STD effects.



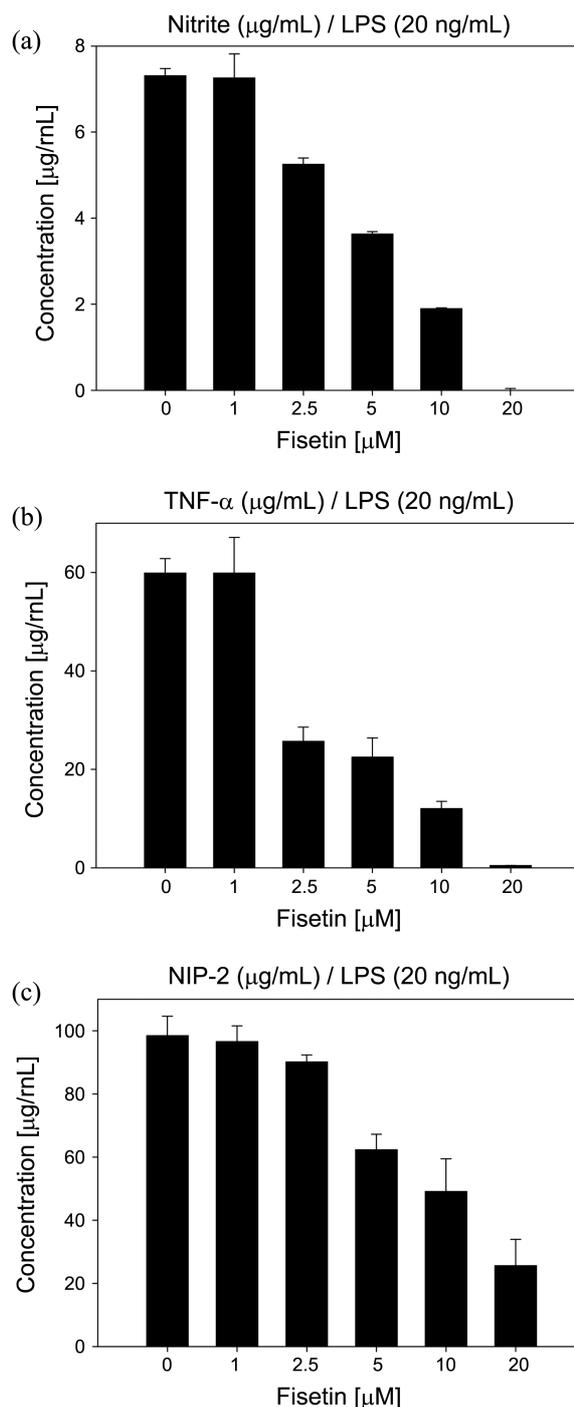
**Figure 5.** Effect of fisetin on the cell viability of RAW264.7 cells. RAW264.7 cells were treated with fisetin (0–100  $\mu\text{M}$ ) for 24 h. Cell viability was determined by the MTT assay.

ances of fisetin interact with JNK1, imply that binding of the fisetin occurs mainly *via* hydroxyl groups at the B and C rings. The STD-NMR spectra imply that fisetin binds to JNK1 tightly and agree well with its binding affinity. Also, results obtained from STD-NMR experiment are in good agreement with the results from the binding model of fisetin and JNK.

**Cytotoxicity in RAW264.7 Cell.** In this study, we first determined the cytotoxicity of fisetin by treating mouse macrophage-derived RAW264.7 cell line with various concentrations (0, 3.12, 6.25, 12.5, 25, 50, and 100  $\mu\text{M}$ ) for 24 h followed by MTT assay, as shown in Figure 5. Compared to 0  $\mu\text{M}$ , after 24 h treatment with fisetin at a concentration between 0 and 6.25  $\mu\text{M}$  was not significantly altered (Figure 5), indicating fisetin was not toxic to RAW264.7 cells at these dosages. When cells were treated with 12.5–100  $\mu\text{M}$  fisetin for 24 h, cell viability was significantly decreased. These results demonstrated treating fisetin with doses higher than 12.5  $\mu\text{M}$  for 24 h resulted in dose- and time-dependent loss of cell viability in RAW264.7 cells but doses lower than 12.5  $\mu\text{M}$  for 24 h did not cause cytotoxicity.

**Quantification of Nitrite Production in LPS-stimulated RAW264.7 Cells.** The effects of fisetin on LPS-induced NO production in RAW264.7 cells were investigated by measuring the accumulated nitrite at 1  $\mu\text{M}$ , 2.5  $\mu\text{M}$ , 5  $\mu\text{M}$ , 10  $\mu\text{M}$ , and 20  $\mu\text{M}$  fisetin. The 5  $\mu\text{M}$  fisetin resulted in more than twice the inhibition compared to cells that were not treated with fisetin. Fisetin completely inhibited NO production at 20  $\mu\text{M}$  (Figure 6(a)).

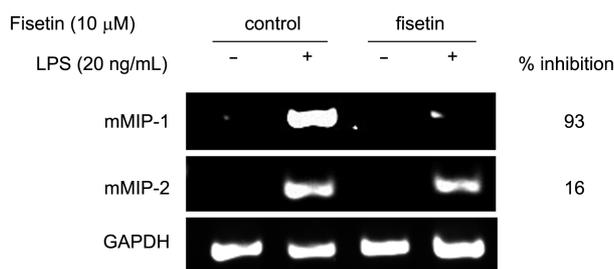
**Quantification of Inflammatory Cytokines (mTNF- $\alpha$  and mMIP-2) in LPS-stimulated RAW264.7 Cells.** We also studied the effects of fisetin on TNF- $\alpha$  and mMIP-2 secretion in RAW264.7 cells (Figure 6(b) and (c)). The inflammatory-induced cytokines that were directly measured in this study was mTNF- $\alpha$ . At 10  $\mu\text{M}$ , fisetin inhibited 72.1% mTNF- $\alpha$  production in 20  $\text{ng}\cdot\text{mL}^{-1}$  of LPS-stimulated RAW264.7 cells compared with cells that were not treated with fisetin (Figure 6(b)) and showed good anti-inflam-



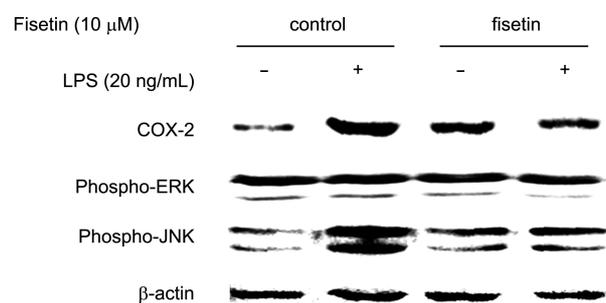
**Figure 6.** (a) Inhibition of NO production, (b) Inhibition of mTNF- $\alpha$  (inflammatory cytokine) production and (c) Inhibition of mMIP-2 production by fisetin in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated with fisetin (1, 2.5, 5, 10 and 20  $\mu\text{M}$ ) in the presence of LPS (20  $\text{ng}\cdot\text{mL}^{-1}$ ) for 24 h. The error bars represent standard deviation values of mean determined from three independent experiments.

matory activity. In fisetin-treated macrophages, the levels of mMIP-2 decreased 51.2% at the 10  $\mu\text{M}$  concentration (Figure 6(c)).

**Inhibition of Cytokine mRNA Expression by Fisetin in LPS Stimulated RAW264.7 Cells.** To know the gene



**Figure 7.** Effect of fisetin on inflammatory-induced cytokine mRNA expression by LPS-stimulated RAW264.7 cell. GAPDH was used as a control. Total RNA was analyzed for the expression of mMIP-1, mMIP-2, and GAPDH (loading control) mRNA by RT-PCR.



**Figure 8.** Effects of fisetin on COX-2, phospho-ERK, JNK and β-actin. COX-2, phospho-ERK, phospho-JNK and β-actin protein levels were determined by western blot analysis using specific antibodies. The relative protein expression was quantified using ImageJ (NIH, Bethesda, MD, USA).

expression of inflammatory-induced cytokine, we analyzed the mRNA expression of mMIP-1, and mMIP-2 by RT-PCR. The expression level of inflammatory-induced cytokine gene was decreased by fisetin as shown in Figure 7. The expression of mMIP-1, and mMIP-2 mRNA was effectively suppressed 93% and 16%, respectively, compared to the mRNA levels in non-treated cells. These data indicate that fisetin can be a potent anti-inflammatory agent.

**Western Blotting.** RAW264.7 cells were treated with fisetin in the presence or absence of LPS, and a western blot was performed to detect COX-2, JNK, and ERK. The fisetin suppressed 58% phosphorylation of JNK in LPS stimulated RAW264.7 cells while the phosphorylation of ERK was not significantly changed by fisetin treatment compared to LPS-treated RAW264.7 cells (Figure 8). Many studies have shown that COX-2 expression is crucially related with inflammation and suppression of COX-2 is one of the novel strategies in the prevention of inflammation. In our study, LPS treatment significantly causes the COX-2 expression compared to non-stimulated control and COX-2 expression is inhibited 44% in fisetin treated RAW264.7 cells (Figure 8). Thus, we demonstrated that fisetin exhibits anti-inflammatory activities through JNK and COX-2 signaling cascade in LPS-stimulated RAW264.7 cells.

### Conclusion

Fisetin is widely distributed in many fruits and plants, and

it has been shown that fisetin has anti-inflammatory activity; however, the mechanism of how the fisetin exhibit their anti-inflammatory activity has not been clarified, yet.<sup>30</sup> We investigated fisetin inhibitory effects on inflammatory responses in LPS-activated RAW 264.7 macrophages. Here, we demonstrated the inhibitory effect of fisetin on the production of the pro-inflammatory mediators NO in the LPS-activated macrophage culture system and found that fisetin thoroughly inhibits NO production in LPS-stimulated macrophages at nontoxic concentrations. In order to characterize whether fisetin inhibits LPS signaling and the inflammatory cascade, we further investigated gene expression of mMIP-1, and mMIP-2 cytokines by RT-PCR and the expression of mRNA was suppressed by 93% and 16%, respectively in LPS-stimulated macrophages by fisetin. Data from STD-NMR and fluorescence quenching experiments correlated well with the ligand docking results. Fisetin, with good anti-inflammatory activities, showed good binding affinity to JNK at  $1.32 \times 10^8 \text{ M}^{-1}$ . From the docking study, a binding model of fisetin and JNK was proposed, and the 3'-OH and 4'-OH of the B-ring as well as oxygen at the 4-position at C-ring of fisetin are responsible for hydrogen bonding with the residues in JNK active site. A-ring of fisetin formed a hydrophobic interaction with V158 and L168. If we can modify hydrophobic group in A-ring, there may increase binding affinity to JNK, so that we can develop more potent compound.

In conclusion, we proposed that fisetin is a potent anti-inflammatory inhibitor of JNK without cytotoxicity to mammalian cells. The JNK signaling pathway plays an important function in anti-inflammatory activities, and we have shown that fisetin targets JNK. The results suggest that the inhibitory effect of fisetin on the pro-inflammatory cytokines is likely dependent on signaling through JNK and COX-2.

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