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A modulatory effect of novel kojic acid derivatives on cancer cell proliferation and macrophage activation

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We examined whether several newly synthesized derivatives of kojic acid, a compound with known anti-inflammatory, anti-proliferative, and anti-oxidative properties, were able to modulate glioma cell proliferation and Toll-like receptor (TLR) 4-mediated functional activation of macrophage-managed tumor microenvironments. Anti-cancer effects on C6 glioma and SYF cells were examined by cell proliferation assays, DNA laddering assays, nuclear staining experiments, and Western blot analysis. The anti-inflammatory activities of the derivatives were assessed by measuring the production of nitric oxide (NO) and cytokine expression in macrophages (RAW264.7 cells) stimulated with the TLR 4 ligand lipopolysacchride (LPS). Among the various derivatives tested, RHS-0110 exhibited the strongest inhibitory activity on the proliferation of C6 glioma cells, with an IC_{50} value of 4.7 μM. However, the inhibitory effect of this compound was abrogated with respect to the proliferation of SYF cells, a cell line lacking Src, Yes, and Fyn kinases, similar to effects observed with the Src kinase inhibitor PP2. In agreement with these findings, RHS-0110 decreased the expression of Src but not the activation of Yes and Fyn. Based on DNA laddering tests and nucleus staining experiments, the anti-proliferative effects of RHS-0110 appeared to be due to a necrotic pathway. Kojic acid derivatives also suppressed LPS-induced NO production and interleukin (IL)-6 expression in RAW264.7 cells under lowered or non-cytotoxic concentrations of compounds. Because of their anti-proliferative and anti-TLR4-mediated microenvironmental formation features, our results suggest that kojic acid derivatives, including RHS-0110, may be useful as novel anti-cancer drugs.

1. Introduction

The development of novel cancer drugs for the treatment of brain tumors is targeted particularly towards the tumorigenesis of glial cells, which are the homeostasis regulatory cells of the central nervous system (Silver et al. 1997). Unfortunately, no drugs proposed for the treatment of glial cell-derived tumors have been developed for clinical use. As a consequence, the survival rate of patients with malignant gliomas remains very low. Thus, the development of promising anti-cancer drugs for the treatment of glial cell-derived tumors remains a worthy endeavor (Jennings et al. 2002).

The proliferation, survival, and malignancy of cancer cells is tightly controlled at various levels of signaling and by the tumor microenvironment, which is composed of neighboring immune cells, such as macrophages (Schwartsburd 2003; Zumsteg and Christofori 2009). In particular, it is well known that oncogenic-activated tyrosine kinases, such as Src, and several survival- or proliferation-controlling kinases, such as phosphatidylinositide-3-kinase (PI3K) and Akt (protein kinase B), are highly active in various cancer cells (Cheng et al. 2008; Shim et al. 2009). Consequently, apoptotic and necrotic death pathways are greatly inhibited, allowing the proliferation of cancerous cells. Moreover, soluble factors, such as growth factors (transforming growth factor (TGF)-β and angiogenic factors), cytokines (interleukin(IL)-10 and IL-6), and pro-inflammatory mediators (nitric oxide (NO) and prostaglandins), released from environmental cells surrounding tumors are involved in modulating malignancy (de la Cruz-Merino et al. 2008; Pittet 2009; Schwartsburd 2003). These various enzymatic activities and complicated tumor microenvironmental conditions are, therefore, excellent targets for the development of novel anti-cancer drugs.

Kojic acid is a representative chelating compound isolated from *Aspergillus oryzae* (Wan et al. 2004). This compound is known as an inhibitor of pigment formation in plant and animal tissues (Han et al. 2002). Owing to this feature, kojic acid is used in various cosmetic biomaterials. In addition to its ability to inhibit tyrosinase activity, numerous studies revealed that kojic acid exhibits various biological activities, such as anti-oxidative, radioprotective, anti-proliferative, and anti-inflammatory effects (Hosseinimehr et al. 2009; Novotny et al. 1999). Efforts to improve the anti-cancer potential of kojic acid by introducing various chemical moieties to the compound have been attempted

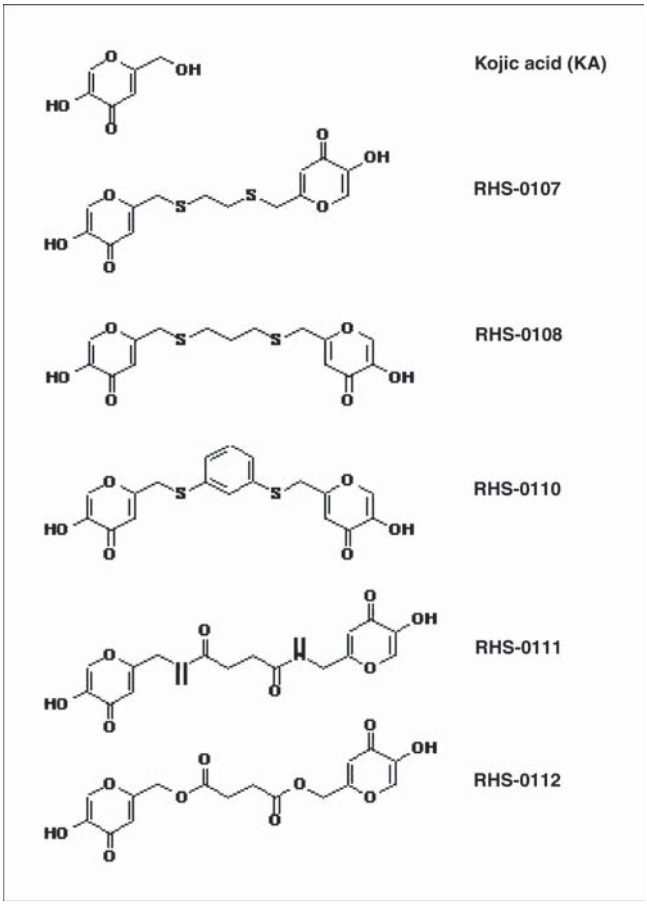

Fig. 1: Chemical structures of the kojic acid derivatives

by many research groups (Emami et al. 2007; Li et al. 2003; Uher et al. 2000; Vachalkova et al. 1996). We have also synthesized several novel derivatives of kojic acid for the same purpose. In the present study, the modulatory effects of these derivatives on cancer cell proliferation and management of the tumor microenviroment by macrophages were carefully examined using various cellular, pharmacological, and biochemical approaches.

2. Investigations, results and discussion

In this study, we explored the modulatory role of several kojic acid derivatives (Fig. 1), compounds with well-known anti-cancer and anti-inflammatory effects. As shown in Fig. 2, five derivatives at 100 μM displayed variable effects on C6 glioma cell proliferation. Specifically, RHS-107, −108, −0111, and −0112 exhibited only marginal inhibition of C6 cell proliferation, as compared to kojic acid (Fig. 2A); however, RHS-0110 more strongly suppressed the viability of C6 glioma cells, as compared with kojic acid (Fig. 2A). This result confirms that subtle differences in the chemical structure of kojic acid may be responsible for its anti-proliferative effects, as reported previously (Fickova et al. 2008).

Since RHS-110 exhibited the strongest suppression of C6 glioma cell proliferation, the inhibitory mode of action of this compound was further characterized. The dose-dependency of RHS-110-mediated inhibition was first explored using C6 cells. As shown in Fig. 2B (left panel), this compound strongly diminished the viability of C6 cells at concentrations ranging from 3 to 200 μM, with an IC_{50} value of 4.7 μM. The inhibitory effect of RHS-0110 was higher than that of other kojic acid derivatives, such as 5-benzyloxy-2-selenocyanatomethyl-4-pyranone and 5-methoxy-2-selenocyanatomethyl-4-pyranone, which have IC_{50} values ranging from 15 to 30 μM, respectively (Fickova et al. 2008). Interestingly, however, the inhibitory effect of RHS-0110 was abrogated in proliferation assays performed with SYF cells, a cell line lacking the functional activity of the Src family of kinases, namely, Src, Yes, and Fyn (Laszlo and Nathanson 2003). Specifically, the viability of SYF cells was not affected by concentrations of RHS-0110 ranging from 3 to 12 μM (Fig. 2B right panel). Indeed, the IC_{50} value (56 μM) obtained in a proliferation test performed with SYF cells was almost 10-fold lower than that obtained for C6 glioma cells, suggesting that the Src kinase family signaling pathway may be the target of RHS-0110. This possibility was also supported by results upon treating cells with PP2, a strong inhibitor of the Src kinase family (Lee et al. 2009). Specifically, exposure of SYF cells to PP2 for 24 h weakly suppressed the viability of SYF cells (Fig. 2C left panel), whereas this compound exhibited significant cytotoxicity in C6 glioma cells, in which cell viability was diminished by up to 60% (Fig. 2C right panel).

These data led to the examination of whether RHS-0110 was able to suppress the expression or activation of the Src family kinases. Intriguingly, this compound strongly suppressed the expression of Src in C6 glioma cells after treatment for 24 or 48 h (Fig. 3A). In contrast, RHS-0110 altered neither the activation of Yes and Fyn, as assessed by measuring the levels of phosphorylation after immunoprecipitation (Fig. 3B), nor the abundance of these proteins (data not shown). Together, these data indicated that Src may be a prime target of RHS-0110. In accordance with this finding, the downstream signaling events mediated by Src activation were similarly suppressed in response to RHS-0110. Specifically, the phosphorylation of two representative enzymes (p85, a regulatory component of PI3K, and its downstream kinase, Akt) triggered by Src activation (Lee et al. 2008) were clearly suppressed by RHS-0110 (Fig. 3C). Hence, these data suggest that Src and its downstream signaling pathway mediate the anti-proliferative target pathway of RHS-0110. Whether RHS-0110 is able to induce apoptosis or necrosis of C6 glioma cells was assessed using DNA laddering experiments. To check these pathways, we employed control inducers of either necrosis (EtOH) (Cherian et al. 2008) or apoptosis (SNP) (Messmer and Brune 1996), and compared their cellular effects with that of RHS-0110 (Fig. 3D). The DNA laddering pattern in compound-treated cells was similar to that of cells exposed to EtOH but not SNP in both C6 glioma and SYF cells, indicating clearly that RHS-0110 induced the necrosis of the C6 glioma cells. Moreover, apoptotic bodies or nuclei were not found after RHS-0110 treatment, unlike SNP treated cells (Fig. 3E, white arrow). While stimulation of necrosis is a well-known and common anti-cancer feature of kojic acid and its derivatives (Fickova et al. 2008), necrotic cell death mediated primarily by a Src-mediated pathway has not been previously reported. Some anti-cancer agents, such as carbazolequinone, have reportedly induced both necrosis and apoptosis in $p60^{V\text{-}Src}$ transformed cells (Aouacheria et al. 2002). Therefore, data reported herein, showing Src kinase activity-related necrotic death by kojic acid derivatives, may suggest a novel form of anti-proliferative response by these derivatives that would prompt experimentation.

The microenvironment managed by immune cells, such as macrophages, is hypothesized to play a critical role in stimulating the malignancy of tumor cells in cancer tissue (Ch'ng and Tan 2009; Pittet 2009). Indeed, the tumor microenvironment contains a huge number of cellular and soluble factors, such as growth factors, cytokines, and inflammatory mediators, such as NO and prostaglandins (Pittet 2009). In particular,

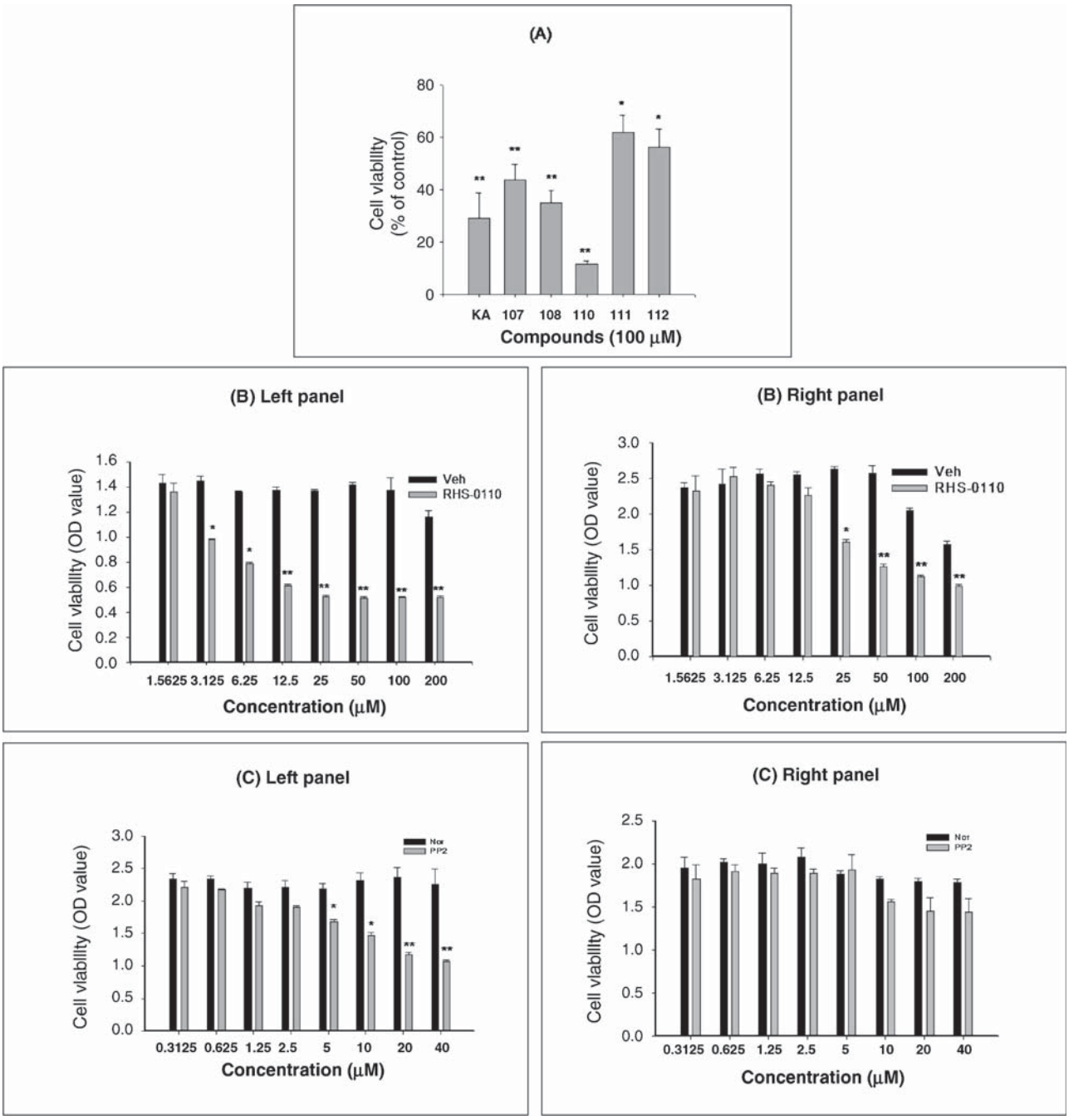

Fig. 2: The effects of kojic acid derivatives on cell proliferation of C6 glioma cells. (A, B left panel, and C left panel) C6 cells (1×10^6 cells/ml) were incubated with kojic acid derivatives or PP2 for 24 h. (B right panel and C right panel) SYF cells (1×10^6 cells/ml) were incubated with RHS-0110 or PP2 for 24 h. Cell viability was determined by conventional MTT assay, as described in the Materials and Methods section. *: $p < 0.05$ and **: $p < 0.01$, as compared to the vehicle control

TLR4 is reported to be preferentially involved in regulating the macrophage-mediated microenvironment in tumor tissue (Fukata et al. 2009). Based on these findings, the modulatory functions of kojic acid derivatives on macrophage activation were explored using LPS, a strong TLR4 agonist. Interestingly, RHS-0107, -0108, and -0110 strongly diminished NO production in RAW264.7 cells induced by LPS by up to 70 to 95% (Fig. 4A, left panel). Moreover, RHS-0107 and -0108 exhibited similar cytotoxic levels (50 to 60%) in RAW264.7 cells during LPS exposure, as compared to LPS alone (60%) (Fig. 4A, right panel), implying that the NO inhibitory effect by these compounds was potentially irrelevant to their cytotoxic activity. In the case of RHS-0110, its NO inhibitory activity was investigated at concentrations below 25 μM, in which case it significantly suppressed LPS-induced NO production in RAW264.7 cells by up to 63% (Fig. 4B, right panel), while 35% more cells died than with LPS alone (Fig. 4B, left panel). These compounds were also able to diminish the expression of IL-6 in RAW264.7 cells under LPS stimulation (Fig. 4C). Further studies will be necessary to understand the molecular mechanisms of the anti-inflammatory effects under lowered or non-cytotoxic conditions and to expand our knowledge regarding the regulatory role of these inhibitors in tumor microenvironments managed by TLR4-activated macrophages.

In conclusion, we identified several kojic acid derivatives (in particular, RHS-0110) that were able to suppress proliferation and induce necrosis of C6 glioma cells via a Src-dependent pathway. Furthermore, these compounds were found to modulate TLR4-mediated functional activation of macrophages (RAW264.7 cells), as assessed by NO production under lowered or non-cytotoxic concentrations of compounds. Therefore, these results suggest that kojic acid derivatives, including RHS-0110, may

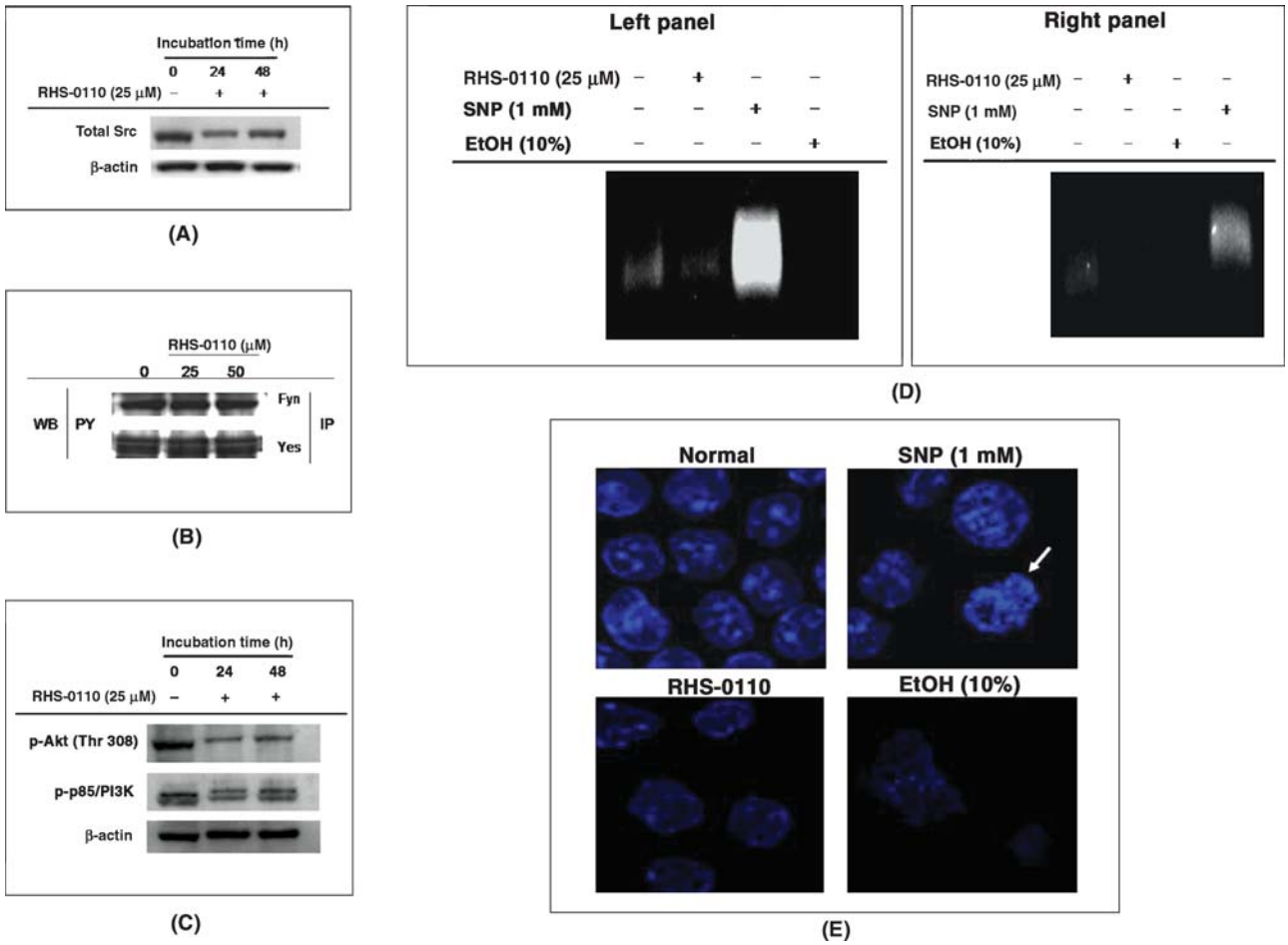

Fig. 3: The effect of RHS-0110 on the expression and activation of Src kinase family members and their downstream signaling enzymes, as well as apoptotic or necrotic events. (A and C) C6 glioma cells (5×10^6 cells/ml) were incubated with RHS-0110 for the indicated times. After immunoblotting, total or phosphorylated levels of Src, p85, Akt, and β-actin were identified with total or phospho-specific antibodies, respectively. (B) C6 glioma cells (5×10^6 cells/ml) were incubated with RHS-0110 for 24 h. Immunoprecipitated Yes or Fyn prepared from whole lysates were assayed for tyrosine phosphorylation levels using a phosphotyrosine antibody. (D) C6 glioma or SYF cells (5×10^6 cells/ml) were incubated with RHS-0110, EtOH (10%), or SNP (1 mM) for 24 h. After preparing DNA extracts, DNA laddering patterns were evaluated by agarose gel electrophoresis. (E) C6 glioma cells (5×10^5 cells/ml) were incubated with RHS-0110, EtOH (10%), or SNP (1 mM) for 24 h. After nuclear staining, images were obtained using confocal microscopy

be useful as novel anti-cancer drugs with anti-proliferative and anti-TLR4-mediated microenviromental formation features. Additional molecular mechanism studies and *in vivo* efficacy testing will be the focus of future studies.

3. Experimental

3.1. Materials

RHS-0107, -0108, -0110, -0111, and -0112 were obtained from Amore-Pacific Co. R&D Center (Yongin, Korea) (Kang et al. 2009). The purity of these compounds was more than 95%, as assessed by HPLC analysis. PP2 was obtained from Calbiochem (La Jolla, CA). Rat C6 glioma and RAW264.7 cells were purchased from the American Type Culture Collection (Manassas, VA). SYF cells were obtained from Professor Seong-Jin Kim (Gachon University of Medicine and Science, Incheon, Korea). Phospho- or total antibodies to phosphotyrosine (PY), Src (Y416), Akt (S473), Akt, Fyn, Yes, and β-actin were purchased from Cell Signaling (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA).

3.2. Cell culture

Rat glioma C6, RAW264.7, and SYF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI1640 medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

3.3. Cell proliferation assay

Cell viability and the extent of proliferation were assessed by conventional MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum bromide] assays (Zund et al. 1999). Briefly, C6 cells (5×10^4 cells/well) were incubated with various concentrations of testing compounds for the indicated times and further incubated with MTT solution (0.5 mg/ml) for an additional 4 h at 37 °C. The absorbance of the samples was measured at 490 nm with a microplate reader manufactured by Molecular Devices Corp. (Menlo Park, CA).

3.4. Preparation of cell lysates, immunoprecipitation, and immunoblotting

C6 or SYF cells (5×10^6 cells/ml) were washed 3 times in cold PBS with 1 mM sodium orthovanadate and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM ethyleneglycotetraacetic acid, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM benzimide, and 2 mM PMSF) for 30 min with rotation at 4 °C. Lysates were clarified by centrifugation at 16,000 g for 10 min at 4 °C and stored at −20 °C until needed.

Cell lysates containing equal amounts of protein (500 μg) from C6 cells (1×10^7 cells/ml), treated with or without test compounds for the indicated times, were pre-cleared with 10 μl of protein A-coupled Sepharose Magnetic beads (50% v/v) (ELPIS BIOTECH, Daejeon, Korea) for 1 h at 4 °C. Pre-cleared samples were then incubated with 5 μl of anti-Src antibody overnight at 4 °C. Immune complexes were then mixed with 10 μl of protein A-coupled Sepharose Magnetic beads (50% v/v) and rotated for 3 h at 4 °C.

Whole cell lysates were analyzed by immunoblotting. Proteins were separated on 10% SDS-polyacrylamide gels and transferred by electroblotting to a polyvinylidenedifluoride (PVDF) membrane. Membranes were blocked for 60 min in Tris-buffered saline containing 3% bovine serum albumin, 20 mM NaF, 2 mM EDTA, and 0.2% Tween 20 at room temperature. The membrane was then incubated for 60 min with a specific primary antibody at 4 °C, washed 3 times with the same buffer, and incubated for an additional 60 min with an HRP-conjugated secondary antibody. Total and phosphorylated levels of Akt, Src and β-actin were visualized with an ECL system (Amersham, Little Chalfont, Buckinghamshire, UK).

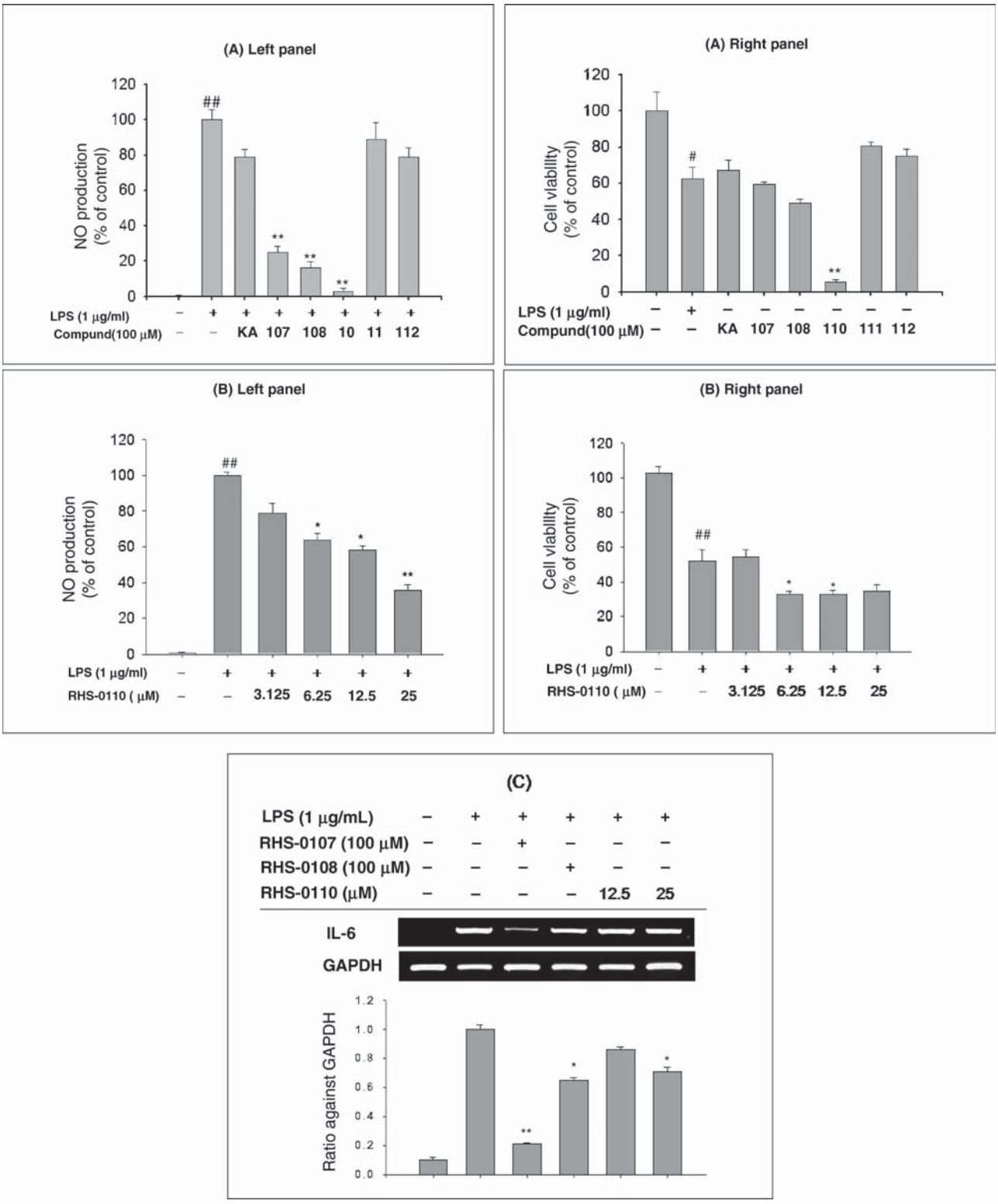

Fig. 4: The effect of RHS-0110 on the activation of macrophages induced by LPS. (A left panel and B left panel) RAW264.7 cells (1×10^6 cells/ml) were incubated with kojic acid derivatives for 24 h. NO levels from culture supernatants were determined by a Griess assay. (A right panel and B right panel) RAW264.7 cells (1×10^6 cells/ml) were incubated with kojic acid derivatives in the presence or absence of LPS (1 μg/ml) for 24 h. Cell viability was determined by conventional MTT assay, as described in the Materials and Methods section. (C) RAW264.7 cells (5×10^6 cells/ml) were incubated with kojic acid derivatives for 6 h, after which the mRNA level of IL-6 was determined by RT-PCR. $^{\#}$: $p < 0.05$ and $^{\#\#}$: $p < 0.01$, as compared to normal group, and *: $p < 0.05$ and **: $p < 0.01$, as compared to the control

3.5. DNA fragmentation

After discarding cellular media, RAW264.7 cells incubated with kojic acid derivatives were treated with TRIzol (1 ml/each plate) and incubated for 10 min on ice. After incubation, TRIzol-cell lysates were used for the extraction of DNA fragments, as described previously (Cho et al. 2004; Kang et al. 2009).

3.6. Nucleus staining

Cells were washed twice with PBS, fixed with 10% formalin for 5 min, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. After two more washes with PBS, nuclei were stained with 10 μg/ml Hoechst33342 for 15 min. Cells were visualized using an IX51 fluorescent microscope, and images were captured using a DP Controller (Olympus Optical, Japan).

3.7. Evaluation of NO production

The inhibitory effects of the test compounds on NO production were determined as previously described (Lee et al. 2009). Briefly, RAW264.7 cells (2×10^6 cells/ml) were incubated with LPS (1 μg/ml) in the presence or absence of compounds for 24 h, and supernatants were assayed for NO content using Griess reagent.

3.8. Determination of IL-6 expression

For the evaluation of IL-6 mRNA expression levels, total RNA from LPS treated-RAW264.7 cells (5×10^6 cells/ml) was prepared by adding TRIzol Reagent (Gibco BRL), according to the manufacturer-suggested protocol, as reported previously (Lee et al. 2008). The primers (Bioneer, Seoul, Korea) used in these experiments were as follows: IL-6 (F-5′-GTACTCCAGAAGACCAGAGG-3′ and 5′-TGCTGGTGACAACCACGGCC-3′); and GAPDH (F-5′-CACTCACGGCAAATTCAACGGCAC-3′ and 5′-GACTCCACGACATACTCAGCAC-3′).

3.9. Statistical analysis

The Student's *t*-test and one-way ANOVA were used to determine the statistical significance of differences between values for the various experimental and control groups. Data were expressed as the mean ± standard error (SEM). Results were obtained from at least three independent experiments performed in triplicate. P values of 0.05 or less were considered statistically significant.

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