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Natural Compounds from Danshen Suppress the Activity of Hepatic Stellate Cells

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Danshen is an herbal medication frequently used in oriental medicine to treat liver or kidney malfunction. In the course of our studies, we observed that compounds purified from Danshen exhibit an inhibitory activity against Discoidin Domain Receptor 2 (DDR2) tyrosine kinase. Through this inhibition, these compounds also inhibited the growth of HSC T6 cells and suppressed the expression of alpha-smooth muscle actin and MMP2, as well as collagen synthesis, all of which are increased in activated liver stellate cells. Given that activation of liver stellate cells is the hallmark of liver fibrosis and that DDR2 plays a critical role in this activation, these results suggest that one of the pharmacological activities of Danshen extract that protects the liver is the inhibition of key cell-signaling kinases, such as DDR2, in liver stellate cells.

Key words: Danshen, Fibrosis, Liver stellate cell, Kinase inhibitor

INTRODUCTION

Continuous injury to the liver induces the transformation of liver stellate cells into proliferating myofibroblast-type cells that produce excessive collagen fiber in liver cirrhosis (Ramón *et al.*, 2005; Dan *et al.*, 1999; Friedman *et al.*, 1999). This is believed to be the main mechanism of cirrhotic liver induction, and the suppression of liver stellate cell proliferation is considered an important therapeutic strategy for this disease (Friedman, 1999; Dan *et al.*, 1999).

DDR2 belongs to the Discoidin Domain Receptor tyrosine kinase family, along with DDR1 (Vogel, 1999). This family of receptors is activated upon binding to its activating ligand, native collagen fiber, to become tyrosine phosphorylated in the c-terminal cytoplasmic domain (Vogel *et al.*, 1997; Shrivastava *et al.*, 1997). The activation kinetics of the DDR family are quite different from most other known receptor tyrosine kinase families, in that activation takes hours rather than minutes (Vogel *et al.*, 1997). In addition, once activated, its activated state seems to persist for a long time (Olaso *et al.*, 2001). A recent report suggested that the increased collagen synthesis and the prolonged activation of DDR2 are critical for the proliferation of activated liver stellate cells (Olaso *et al.*, 2001). This implies that inhibition of DDR2 activity could suppress the phenotype of activated stellate cells.

The water extract of Danshen, which is the root of *Salvia miltiorrhiza*, has been shown to have a pharmacological activity that protects organs, such as the liver and kidney, from damage (Zhou *et al.*, 2005; Hsu *et al.*, 2005). Various poly-phenolic natural products have been identified in its extracts (Zhou *et al.*, 2005; Li *et al.*, 2005) and have been shown to have antioxidant activity, a possible mechanism for its pharmacological effects (Zhu *et al.*, 2004). Among the identified components, magnesium lithospermate B and lithospermic acid A have been studied the most extensively and are regarded as the ingredients responsible for Danshen's pharmacological activity.

In this investigation, we studied the bioactivity of other ingredients purified from water-soluble Danshen extract, finding that some Danshen components specifically inhibit DDR2 tyrosine kinase and have properties that suppress activated liver stellate cells.

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MATERIALS AND METHODS

Cell culture and reagents

Purified natural compounds from water-soluble Danshen extracts were obtained from the Compound Bank of the Korea Institute of Science and Technology, Seoul, Korea. HSC T6 cells were kindly provided by Dr. Yang, Korea Institute of Science and Technology, Seoul, Korea. HSC T6 cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum, 1% glutamine (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C in a humidified incubator containing 5% CO_2.

Kinase inhibition assays

Purified activated DDR2 tyrosine kinase domain protein was obtained as previously described (Yang *et al.*, 2005). The DDR2 kinase inhibition assay was performed in a final volume of 20 μL; the reaction mixture contained 100 ng of DDR2 and 4 μg of Histone H2B (Sigma), the substrate, with various concentrations of test natural product compounds in a buffer of 20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.1 mM DTT, and 10 μM ATP with 0.1 μCi γ-[^{32}P]-ATP. The reaction was allowed to proceed for 15 minutes and then stopped by adding one-third the volume of 30% phosphoric acid. The terminated reaction mixture was spotted onto p81 paper (Millipore) and washed five times with a buffer containing 20 mM Tris-HCl (pH 8.0) and 0.1 M NaCl. The radioactivity of each spot was quantitated using a BAS image analyzer (Fuji).

Cell growth inhibition assays

HSC T6 cell growth inhibition assays were performed by the MTT method. One-thousand cells were plated in each well of a 96-well plate and treated with increasing concentrations of the test compounds for 48 h. The number of viable cells after this treatment period was estimated with the MTT (Sigma) colorimetric dye reduction method; the absorbance of reduced MTT was read at 570 nm after its extraction with DMSO.

Measurement of matrix metalloprotease 2

Sub-confluent cultures of HSC-T6 cells were seeded in 10 cm dishes and, subsequently, changed into serum free media for treatment with increasing concentrations of isolated Danshen components for 48 h. Media were collected and concentrated five-fold using a Vivaspin concentrator (Vivascience). The total amount of MMP-2 protein in the concentrated media was measured using the enzyme-linked immunosorbent assay (ELISA) kit for MMP-2 according to the manufacturer's instructions (R&D systems).

Immunoblotting of alpha-smooth muscle actin

Cell lysate in 1×Laemmli buffer was boiled for 2 minutes and loaded onto 10% SDS-PAGE gels. Following electrophoresis, proteins were transferred to Immobilon-P membrane (Millipore) and blocked by 5% skim milk in 1 × TBS for an hour. The membrane was immunoblotted with alpha-smooth muscle actin monoclonal antibody (Sigma), followed by horseradish peroxidase-conjugated secondary antibody for an hour. Positive immunoreactivity for alpha-smooth muscle actin was detected by chemiluminescence (Amersham).

Measurement of collagen synthesis

Collagen synthesis was estimated by measuring the incorporation of proline in collagenous and non-collagenous proteins in the culture media after digestion with collagenase. 24-well plates were seeded with 5×10^4 HSC-T6 cells and grown for 24 h. The medium was then refreshed, and the cells were treated with 50 μg/mL ascorbic acid, 1μCi of [^{3}H]-proline per well and 80 μg/mL amino propionitrile, together with the test inhibitory compound for another 24 h. The media was harvested and adjusted to collagenase reaction conditions by the addition of 10 × collagenase reaction buffer containing 0.33 M Tris-HCl (pH 7.5), 0.5 M $CaCl_2$, and 50 mM NEM. The adjusted medium was divided into two equal volumes: to one, 2 U/μL collagenase (Sigma) was added, and to the other, an equal volume of water was added. Each was then incubated at 37°C for 3 h. After the addition of 10% BSA, the reaction mixtures were precipitated with 50% TCA and centrifuged to pellet the precipitated protein. The resulting protein pellets were washed with ice-cold 10% TCA and dissolved in 1N NaOH. The incorporation of [^{3}H] radioactivity was measured using a beta counter. Finally, the percent of collagen synthesis was calculated by the equation: [(cpm of medium sample without collagenase treatment – cpm of medium sample with collagenase treatment)/(cpm of medium sample without collagenase treatment)] × 100.

RESULTS

Screening natural compounds for inhibitors of DDR2 tyrosine kinase

Recent studies have shown that DDR2 tyrosine kinase activity is necessary for the proliferation and activity of activated liver stellate cells (Olaso *et al.* 2001). Furthermore, since Danshen extract has demonstrated pharmacological activities that prevent liver fibrosis (Zhou *et al.* 2005), we hypothesized that a natural compound(s) in the extract could inhibit the tyrosine kinase activity of DDR2, and that this action serves as one of the mechanisms for protecting the liver from fibrosis. To test this, we first performed *in vitro* DDR2 tyrosine kinase inhibition assays using purified natural compounds from Danshen extract. Through these assays, we have identified three compounds that are

capable of inhibiting DDR2 tyrosine kinase activity with IC_{50}s of approximately 5 μM (Fig. 1A and B). Moreover, the inhibition of DDR2 tyrosine kinase was relatively specific; DDR1 tyrosine kinase inhibition assays demonstrated no detectable inhibition at 100 μM concentrations, and other kinases, such as IRTK, EGFR. Akt1, and GSK3 beta, were likewise not inhibited by these compounds at the same high concentrations. All three of these compounds (Fig. 1A) share a similar structure to lithospermic acid A and are likely its derivatives (Li *et al.*, 2005). The three inhibitory compounds also have a dihydrobenzofuran structure in common, which suggests that the dihydrobenzofuran moiety might be a key pharmacophore in inhibiting DDR2 tyrosine kinase.

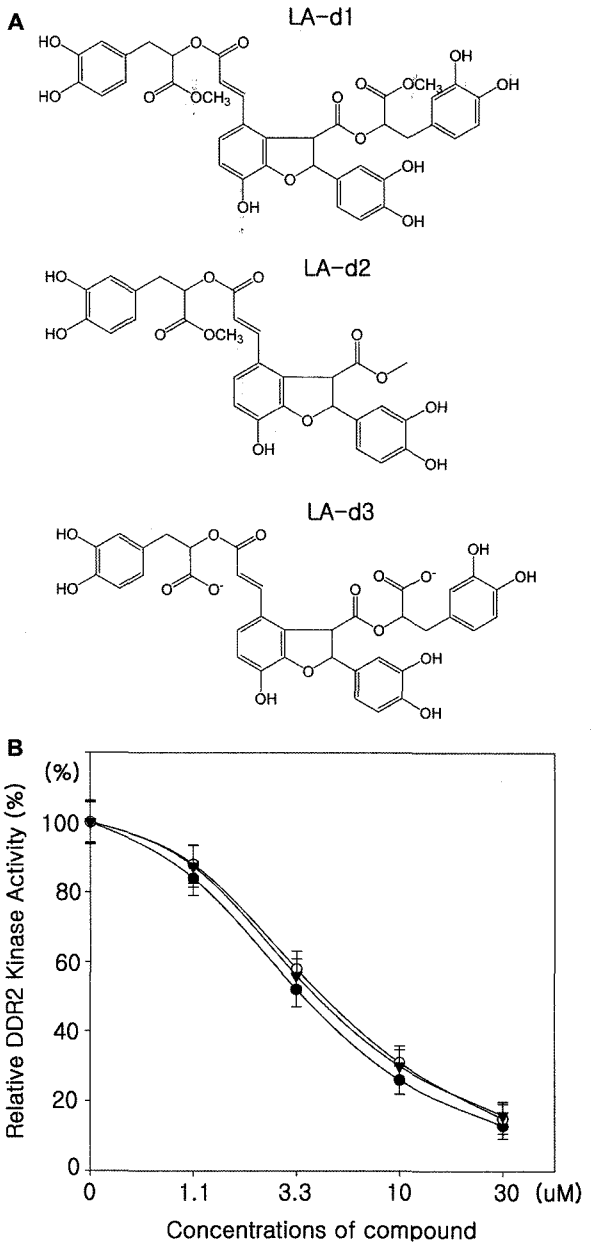

Fig. 1. Identification of three natural compounds from Danshen that inhibit DDR2 tyrosine kinase activity. (A) Chemical structures of the three compounds. These compounds have chemical structures similar to lithospermic acid A. Therefore, they were designated LA-d1 (lithospermic acid A derivative 1), LA-d2, and LA-d3, respectively. (B) DDR2 tyrosine kinase inhibition curves for LA-d1, LA-d2 and LA-d3. DDR2 activity without added test compound was assigned the value of 100%. The relative values are shown as mean±S.D. from three independent measurements. Filled circle: LA-d1, open circle: LA-d2, filled triangle: LA-d3.

DDR2 inhibitors from Danshen suppress HSC T6 cell growth

Having observed DDR2 tyrosine kinase inhibition by these compounds, we next tested whether these Danshen components could suppress cellular activities that are directly regulated by DDR2 tyrosine kinase. Because DDR2 tyrosine kinase is required for the growth of liver stellate cells (Olaso *et al.*, 2001), we measured the growth of HSC T6 cells, an established activated liver stellate cell line, following treatment with these compounds. Among the three natural compounds in Fig. 1A, two compounds, LA-d1 and LA-d2, effectively suppressed HSC T6 cell growth with EC_{50}s of 40.6 μM and 46.7 μM, respectively, whereas LA-d3 exhibited very little inhibition (Fig. 2). We think the two carboxylic acid groups in LA-d3 interfere with the cellular internalization of this compound through the cell membrane. Based on these results, all further experiments and characterizations at the cellular level were performed with the compound, LA-d1.

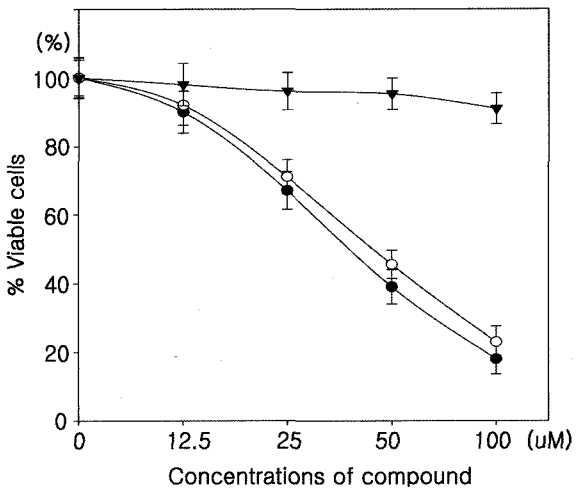

Fig. 2. Proliferation of HSC T6 liver stellate cells is inhibited by the DDR2 inhibitors from Danshen in a dose-dependent manner. HSC T6 cells were treated with DDR2 inhibitors from Danshen for 48 h. The relative number of viable cells following treatment was estimated using the MTT assay. A 100% value was assigned to the absorbance value without test compound treatment. The values are shown as mean±S.D. from three independent experiments. Filled circle: LA-d1, open circle: LA-d2, filled triangle: LA-d3.

DDR2 inhibitor from Danshen suppresses the expression of SMA and MMPs, as well as the invasive activity of HSC T6 cells

Increased expression of matrix metalloprotease 2 (MMP-2) and alpha-smooth muscle actin (SMA) are observed when stellate cells become activated in liver fibrosis, and these increases are considered characteristic features of activated stellate cells (Friedman, 1999). According to a recent study, MMP-2 is downstream target of activated DDR2 signaling (Olaso *et al.*, 2001). We therefore tested the ability of LA-d1 to suppress the expression of alpha-smooth muscle actin and matrix metalloprotease 2 in HSC T6 cells.

Treatment with LA-d1 significantly reduced the expression of smooth muscle actin in HSC T6 cells with an EC_{50} approximately equal to 50 μM (Fig. 3A). LA-d1 also inhibited the total expression of MMP-2, as assayed by ELISA, with an EC_{50} approximately equal to 60 μM (Fig. 3B). The suppression of MMP2 and alpha-smooth muscle actin by LA-d1 was also associated with the inhibition of HSC T6 cell invasion through matrigel, as shown in Fig. 4A and B. In the presence of 80 mM LA-d1, the invasion of HSC T6 cells through matrigel was virtually abolished.

DDR2 inhibitor from Danshen reduces collagen synthesis in HSC T6 cells

Finally, we tested the ability of LA-d1, one of the DDR2 kinase inhibitors from Danshen extract, to suppress collagen synthesis in HSC T6 cells. Activated stellate cells are responsible for producing excessive amounts of collagen fiber in the liver, and the over-accumulation of collagen is the main pathological event in liver cirrhosis. Furthermore, it has previously been shown that DDR2 signaling induces collagen synthesis in liver stellate cells (Olaso *et al.*, 2001). We therefore examined LA-d1's

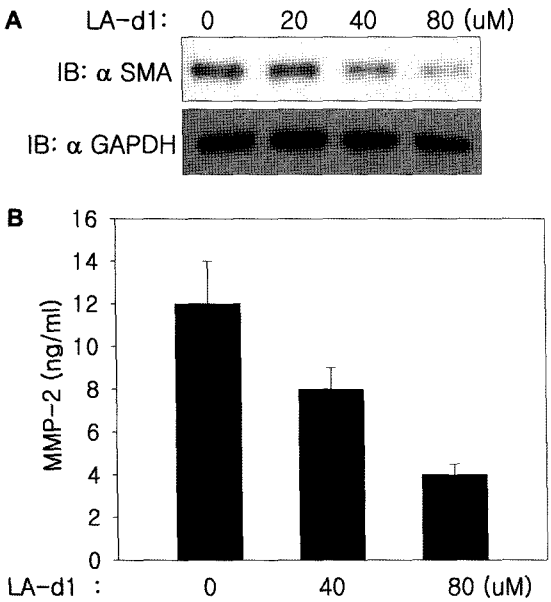

Fig. 3. DDR2 inhibitor LA-d1 from Danshen inhibits characteristic activities of activated liver stellate cells. (A) Expression of alpha-smooth muscle actin was estimated by immunoblotting after treating HSC T6 cells with LA-d1 for 24 h. GAPDH immunoblotting served as an internal control for equal loading. (B) The amount of MMP-2 in HSC T6 culture medium was estimated by ELISA after treatment with LA-d1 for 24 h. The values are shown as mean±S.D. for n=3.

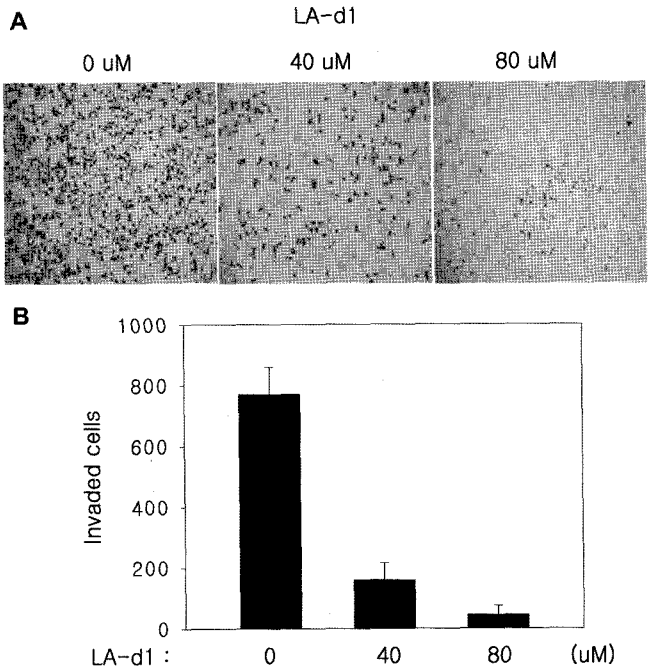

Fig. 4. DDR2 inhibitor LA-d1 from Danshen inhibits invasiveness of HSC T6 cells. Matrigel invasion assays of HSC T6 cells were performed in the presence of LA-d1. (A) The invading cells were stained with crystal violet and pictured under light microscope (×100). (B) Invading cells were counted, and the average values with standard deviations from three independent experiments are shown in the bar graph.

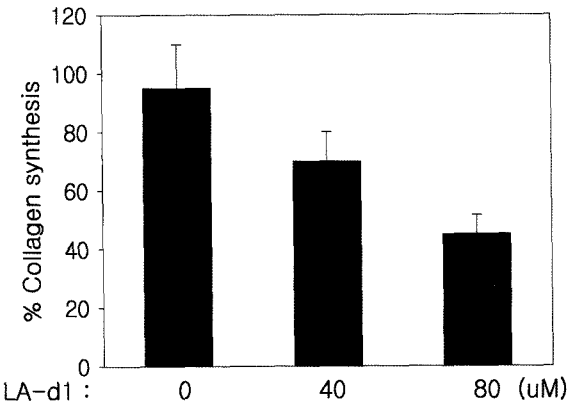

Fig. 5. DDR2 inhibitor LA-d1 from Danshen inhibits collagen synthesis. The amount of total collagen protein secreted by HSC T6 cells following treatment with LA-d1 for 24 h were estimated and shown as a bar graph with average values±S.D for n=3.

ability to suppress the synthesis of collagen in HSC T6 cells. Fig. 5 shows that treatment with 80 μM LA-d1 reduced collagen synthesis by more than 50% in HSC T6 cell cultures.

DISCUSSION

Several studies have reported the therapeutic efficacy of Danshen extract for hepatitis, heart diseases, blood circulation diseases and kidney malfunction (Ling *et al.,* 2005; Zhou *et al.,* 2005; Hsu *et al.,* 2005). However, its pharmacological mechanism of action still remains unclear. Although in recent years water-soluble compounds isolated from Danshen were shown to have antioxidant activities that allow removal of free radicals (Zhu *et al.,* 2004; Zhou *et al.,* 2005), further studies are still needed to explain the wide spectrum of Danshen's pharmacological activities at the molecular level. In this paper, we suggest that inhibition of DDR2 tyrosine kinase by Danshen extract might be associated with one aspect of its pharmacological activity. Recent studies have established that DDR2 is a key signaling molecule in various activated fibrotic cells in human diseases (Wang *et al.,* 2002; Olaso *et al.,* 2001; Ferri *et al.,* 2004). Fibrotic cells are responsible for wound healing in organs following organ injury by trauma, burning, toxic materials, or excessive inflammation (Diegelmann *et al.,* 2004). The wound healing process includes the proliferation of fibrotic cells and the production of collagen fibers by these cell types. However, continuous injury, such as chronic inflammation in the liver, induces an excessive accumulation of collagen, which, in the liver, ultimately results in cirrhosis (Ramon *et al.,* 2005). Given that activated fibrotic cells are involved in various human diseases and the role of DDR2 in their activity, and that water-soluble Danshen extract inhibits DDR2 activity, it is reasonable to speculate that activated DDR2 is one of the key molecular targets for the therapeutic efficacy of Danshen extract.

We find it interesting that compounds in Danshen extract can suppress collagen synthesis, possibly through the inhibition of DDR2 kinase. Deregulated collagen synthesis is involved in the pathogenesis of several human diseases, such as atherosclerosis, as well as organ fibrosis (Hou *et al.,* 2002; Katsuda *et al.,* 2003; Friedman 1999). In atherosclerosis, collagen deposition on an injured blood vessel wall activates monocytes, inducing foam cell deposition and activating smooth muscle cell proliferation, and eventually leading to irreversible clot formation. In this disease, the suppression of collagen synthesis by components in Danshen extract could be the most important activity for its therapeutic efficacy.

Recently, protein kinases have been exploited extensively as targets for new drug discovery (Cohen, 2002; Fan *et al.,* 2006). The most common approach for developing kinase inhibitors has relied on the search for compounds that interfere with ATP binding to the active pocket of the target kinases (Cohen, 2002). In this regard, it is interesting that the common chemical moiety in the three natural DDR2 inhibitors isolated from Danshen extract is the dihydrobenzofuran ring. We believe that this chemical structure could be a useful pharmacophore for other kinase inhibitors as well. The IC_{50} values of these natural products from Danshen against DDR2 kinase were in the range of single-digit micromolar concentrations. Further derivatizations based on the dihydrobenzofuran structure could lead to even more potent DDR2 inhibitors.

REFERENCES

Cohen, P., Protein kinases - the major drug targets of the twenty-first century? *Nat. Rev. Drug Discov.*, 1, 309-315 (2002).

Dan, L. and Friedman, S. L., Liver fibrogenesis and the role of hepatic stellate cells : New insights and prospects for therapy. *Journal of gastroenterology and hepatology,* 14, 618-633 (1999).

Diegelmann, R. F. and Evans, M. C., Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci.,* 9, 283-289 (2004).

Fan, Q. W. and Weiss, W. A., Chemical genetic approaches to the development of cancer therapeutics. *Curr. Opin. Genet. Dev.,* 16, 85-91 (2006).

Ferri, N., Carragher, N. O., and Raines, E. W., Role of discoidin domain receptors 1 and 2 in human smooth muscle cell-mediated collagen remodeling: potential implications in atherosclerosis and lymphangioleiomyomatosis. *Am. J. Pathol.,* 164, 1575-1585 (2004).

Friedman, S L., Stellate cell activation in alcoholic fibrosis. *Alcohol Clin. Exp. Res.,* 23, 904-910 (1999).

Hou, G., Vogel, W. F., and Bendeck, M. P., Tyrosine kinase activity of discoidin domain receptor 1 is necessary for smooth muscle cell migration and matrix metalloproteinase expression. *Circ. Res.,* 90, 1147-1149 (2002).

Hsu, Y. C., Lin, Y. L., Chiu, Y. T., Shiao, M S., Lee, C. Y., and Huang, Y. T., Antifibrotic effects of Salvia miltiorrhiza on dimethylnitrosamine-intoxicated rats. *J. Biomed. Sci.,* 12, 185-195 (2005).

Kang, D. G., Oh, H., Sohn, E. J., Hur, T. Y., Lee, K. C., Kim, K. J., Kim, T. Y., and Lee, H S., Lithospermic acid B isolated from Salvia miltiorrhiza ameliorates ischemia/reperfusion-induced renal injury in rats. *Life Sci.,* 75, 1801-1816 (2004).

Katsuda, S. and Kaji T., Atherosclerosis and extracellular matrix. *J. Atheroscler. Thromb.,* 10, 267-74 (2003).

Li, X., Yu, C., Cai, Y., Liu, G., Jia, J., and Wang, Y., Simultaneous determination of six phenolic constituents of danshen in human serum using liquid chromatography/

tandem mass spectrometry. *J. Chromatography B.,* 820, 41-47 (2005)

Ling, S., Dai, A., Guo, Z., Yan, X., and Komesaroff, P. A., Effects of a Chinese herbal preparation on vascular cells in culture: mechanisms of cardiovascular protection. *Clin. Exp. Pharmacol. Physiol.,* 32, 571-578 (2005).

Olaso, E., Ikeda, K., Eng, F. J., Xu, L., Wang, L. H., Lin, H. C., and Friedman, S. L., DDR2 receptor promotes MMP-2-mediated proliferation and invasion by hepatic stellate cells. *J. Clin. Invest.,* 108, 1369-1378 (2001).

Olaso, E., Labrador, J. P., Wang, L., Ikeda, K., Eng, F. J., Klein, R., Lovett, D. H., Lin, H. C., and Friedman, S. L., Discoidin domain receptor 2 regulates fibroblast proliferation and migration through the extracellular matrix in association with transcriptional activation of matrix metalloproteinase-2. *J. Biol. Chem.,* 277, 3606-3613 (2002).

Ramón, B. and David, A. B., Liver fibrosis. *J. Clin. Invest.,* 115, 209-218 (2005).

Shrivastava, A., Radziejewski, C., Campbell, E., Kovac, L., McGlynn, M., Ryan, T. E., Davis, S., Goldfarb, M. P., Glass, D. J., Lemke, G., and Yancopoulos, G. D., An orphan receptor 14. tyrosine kinase family whose members serve as nonintegrin collagen receptors. *Mol. Cell,* 1, 25-34 (1997).

Vogel, W., Gish, G. D., Alves, F., and Pawson, T., The discoidin domain receptor tyrosine kinases are activated by collagen. *Mol Cell.,* 1, 13-23 (1997).

Vogel, W., Discoidin domain receptors : structural relations and functional implications. *FASEB J.,* Suppl, S77-82 (1999).

Wang, J., Lu, H., Liu, X., Deng, Y., Sun, T., Li, F., Ji, S., Nie, X., and Yao, L., Functional analysis of discoidin domain receptor 2 in synovial fibroblasts in rheumatoid arthritis. *J. Autoimmun.,* 19, 161-168 (2002).

Yang, K., Kim, J. H., Kim, H. J., Park, I. S., Kim, I. Y., and Yang, B. S., Tyrosine 740 phosphorylation of discoidin domain receptor 2 by Src stimulates intramolecular autophosphorylation and Shc signaling complex formation. *J. Biol. Chem.,* 280, 39058-39066 (2005).

Zhou, L., Zuo, Z., and Chow, M S., Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. *J. Clin. Pharmacol.,* 45, 1345-1359 (2005).

Zhu, Y. Z., Huang, S. H., Tan, B. K., Sun, J., Whiteman, M., and Zhu, Y. C., Antioxidants in Chinese herbal medicines: a biochemical perspective. *Nat. Prod. Rep.,* 21, 478-489 (2004).