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ORIGINAL ARTICLE

Isoliquiritigenin Induces Apoptosis through Caspases and Reactive Oxygen Species Signaling Pathways in Human Bladder Cancer Cells

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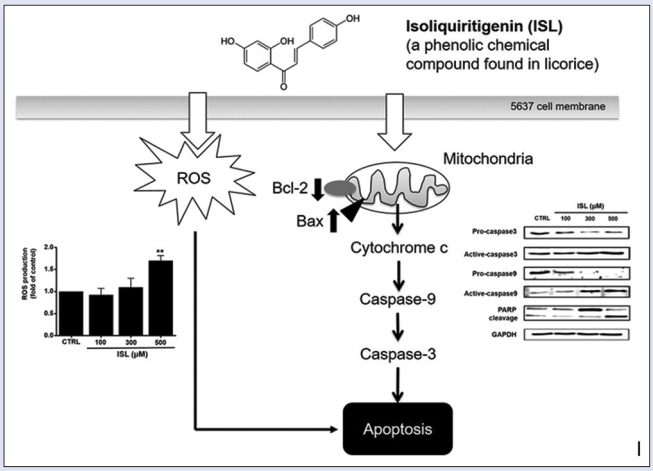
ABSTRACT

Background: Isoliquiritigenin (ISL) is a flavonoid isolated from the roots of various species of licorice plants. **Objectives:** Mechanisms underlying ISL-induced cell death were investigated in 5637 human bladder cancer cell line. **Materials and Methods:** Cell viabilities were measured with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide and cell counting kit-8 assay. Cell cycle analysis, caspase activity assay, western blotting, and reactive oxygen species (ROS) assay were also used to investigate the anticancer effects of ISL on 5637 cells. **Results:** ISL (100–500 μg/ml) inhibited cancer cell proliferation and increased sub-G1 cell cycle phase ratios. ISL-induced cell death resulted in reduced Bcl-2 and increased Bax. ISL also activated caspase-3 and -9 and increased the levels of intracellular ROS generated. In addition, TG100-115 (transient receptor potential [TRP] melastatin 7 inhibitor) and tranilast (TRP vanilloid 2 inhibitor) each exerted a synergistic effect with ISL on ISL-induced apoptosis. **Conclusion:** These findings suggest that ISL causes apoptosis in 5637 cancer cell line. Therefore, ISL may be a potential anticancer drug for treating bladder cancer and a good anticancer supplement.

Key words: 5637, anticancer, apoptosis, bladder cancer, isoliquiritigenin

SUMMARY

- Isoliquiritigenin (ISL) (100, 300, and 500 μM) inhibited 5637 cells [Figure 1a] and reduced cell viability [Figure 1b]. Sub-G1 phase ratios were increased by ISL [Figure 2]
- Bcl-2 was reduced by ISL, whereas Bax was increased [Figures 3a-c]
- ISL dose dependently increased caspase-3 and -9 activities, and zVAD-fmk (a broad-spectrum caspase inhibitor) pretreatment suppressed these activities [Figure 4a]. ISL upregulated the active forms of caspase-3 and -9, downregulated pro-caspase -3 and -9, and upregulated PARP protein levels [Figure 4b]
- ISL increased reactive oxygen species levels in 5637 cells [Figure 5]
- TG100-115, a transient receptor potential (TRP) melastatin 7 blocker, combined with ISL has a synergistic effect on ISL-induced apoptosis [Figure 6a]. Similarly, TRP vanilloid 2 blocker, tranilast, had the same effect on ISL-induced apoptosis [Figure 6b].



Abbreviations used: ISL: Isoliquiritigenin; ROS: Reactive oxygen species; TRP: Transient receptor potential.

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INTRODUCTION

Isoliquiritigenin (ISL) is a flavonoid isolated from the roots of various species of licorice plants.[1,2] It is available in common foods, beverages, and alternative medicines.[1,2] It reportedly has many biological properties, such as antioxidation and antiplatelet aggregation and antiviral, hepatoprotective, and cardioprotective effects.[3-5] ISL has also been considered to induce apoptotic effects in numerous cancers, including prostate, colon, lung, stomach, and breast cancer; melanoma; and osteosarcoma.[1,6-11]

Bladder cancer is a disease that, if not properly treated, indicates a high prevalence and mortality rate.[12] Men are generally more susceptible to the disease than women and more likely to develop it as they get older.[13] Most common symptoms are blood in the urine, and the most common form of bladder cancer is urothelial carcinomas.[14] Many new treatments for bladder cancer are being developed; however, it is

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still considered one of the most difficult cancers to treat.[15] However, research on anticancer properties of ISL and related mechanisms of action in bladder cancer is lacking. In this study, we studied the efficacy of ISL and related mechanisms of action in 5637 bladder cancer cell lines.

MATERIALS AND METHODS

Cell culture and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay

Human 5637 bladder carcinoma cells were cultured in RPMI-1640 medium (Gibco-BRL, St. Louis, MO, USA) supplemented with 10% heat inactivated fetal bovine serum and 1% antibiotic mixture (penicillin and streptomycin) at 37°C, cell viabilities were determined using an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, and absorbances were measured at 570 nm.[16] This cell line was established at the Cancer Research Center, College of Medicine, Seoul National University, Korea, and the cell passage number was 7. ISL (Sigma-Aldrich, St. Louis, MO, USA; Lot number: 78825) was used at concentrations of 100, 300, and 500 μM.

Cell counting kit-8 assay

Cells from the 5637 cell line were seeded at 1×10^4 cells/well, and 10 μl of cell counting kit-8 (CCK-8; Abbkine Co., Ltd., Hubei, China) was added. After incubation for 2 h, absorbances were measured at 450 nm.[17]

Measurement of cell cycle

The 5637 cells were treated with ethyl alcohol and treated at 4°C overnight before incubating them. Cells were stained with propidium iodine staining solution (5 mg/ml; 2 μl) containing RNase A, centrifuged at 20,000 ×*g* for 10 s, and incubated for 40 min. After that, they were measured with a fluorescence-activated cell sorter (FACS) at λ = 488 nm.[16]

Western blot analysis

Proteins isolated from 5637 cells were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and incubated with several antibodies. Antibodies against Bcl-2 (#sc-783), Bax (#sc-493), caspase-3 (#sc-7148), caspase-9 (#sc-7885), PARP (#sc-7150), β-actin (#sc-47778), and GAPDH (#sc-32233) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat antirabbit immunoglobulin (Ig) G and goat antimouse IgG (cat. no. SC-2004 and SC-2005, respectively; Santa Cruz Biotechnology, Dallas, TX, USA) were used as secondary antibodies. All procedures followed standard methods.[18]

Caspase assay

Assays were performed using caspase-3 and -9 assay kits. Cells were suspended with lysis buffer and cell pellet was incubated with caspase substrate (400lM AcDEVDpNA; 50 μl) at 37°C. Absorbances were measured at 405 nm.[19]

Measurement of reactive oxygen species levels

Using 20 μ DCF-DA: 2',7' dichlorofluorescin-diacetate (Molecular Probes, Eugene, OR, USA) at 37°C, reactive oxygen species (ROS) generation was measured with a FACS at 488 nm excitation/525 nm emission wavelengths.[19]

Statistical analysis

Using Origin 8.0 (OriginLab Corporation, Northampton, MA, USA) software, the analysis was performed. Results are suggested as mean ± standard error of the mean, and $P < 0.05$ was considered statistically significant.

RESULTS

Apoptotic effects of isoliquiritigenin in 5637 cells

MTT assays were used to check if 5637 cell growth was inhibited by ISL with 24 h. ISL (100, 300, or 500 μM) inhibited 5637 survival by 71.1% ± 4.4% ($P < 0.01$), 27.8% ± 1.1% ($P < 0.01$), or 20.8% ± 1.2% ($P < 0.01$), respectively [Figure 1a]. Furthermore, the survival of 5637 cells was investigated using CCK-8 assay method. ISL induced the reduction of cellular viability by 76.4% ± 4.7% ($P < 0.01$), 66.3% ± 5.4% ($P < 0.01$), or 53.1% ± 4.4% ($P < 0.01$), respectively [Figure 1b]. In addition, to determine whether ISL induces apoptosis, cell cycle analysis was conducted by flow cytometry. Sub-G1 phase ratios were increased by ISL by 15.7% ± 2.8% ($P < 0.01$) at 100 μM, 20.0% ± 2.4% ($P < 0.01$) at 300 μM, and 27.5% ± 3.5% ($P < 0.01$) at 500 μM as compared to untreated cells by flow cytometry [Figure 2]. These results indicate that ISL inhibits 5637 proliferation and that these effects are related to the induction of apoptosis.

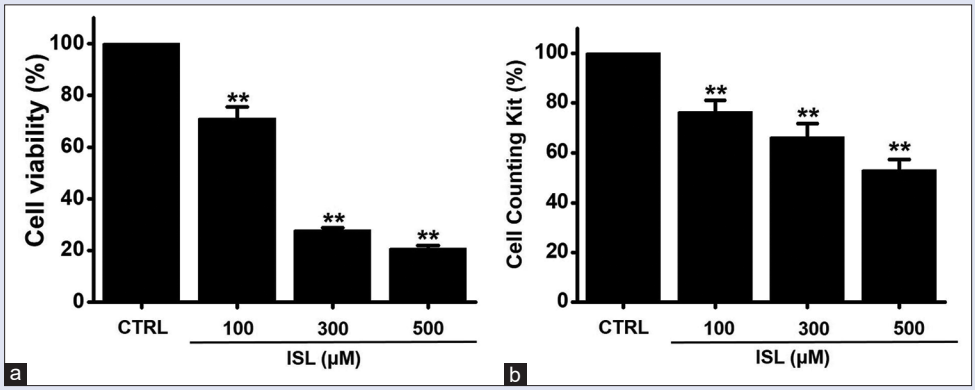

Figure 1: Effect of isoliquiritigenin on 5637 cell viability. Cell viabilities were investigated using (a) an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay and (b) a cell counting kit-8 assay. Isoliquiritigenin dose dependently reduced cell viabilities for 24 h. Results are presented as mean ± standard error of the mean. ISL: Isoliquiritigenin; CTRL: Control. **$P < 0.01$ versus untreated cells

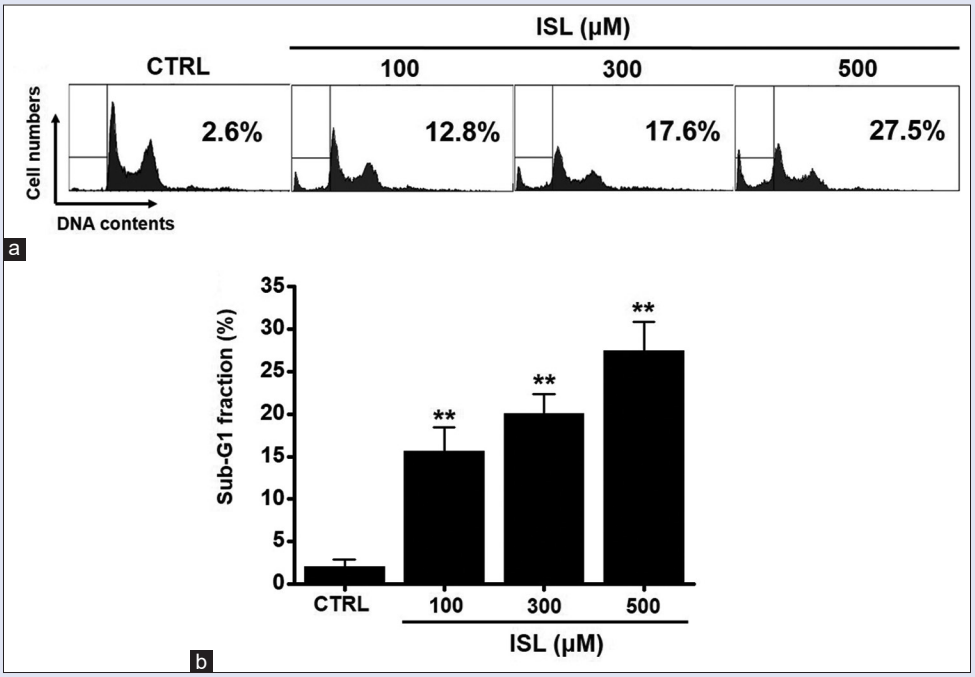

Figure 2: Effect of isoliquiritigenin on 5637 cell apoptosis. (a) Cell cycle analysis was conducted by flow cytometry. (b) Sub G1 fractions with isoliquiritigenin are expressed as percentages. Results are presented as mean ± standard error of the mean. ISL: Isoliquiritigenin; CTRL: Control. ***P* < 0.01 versus untreated cells

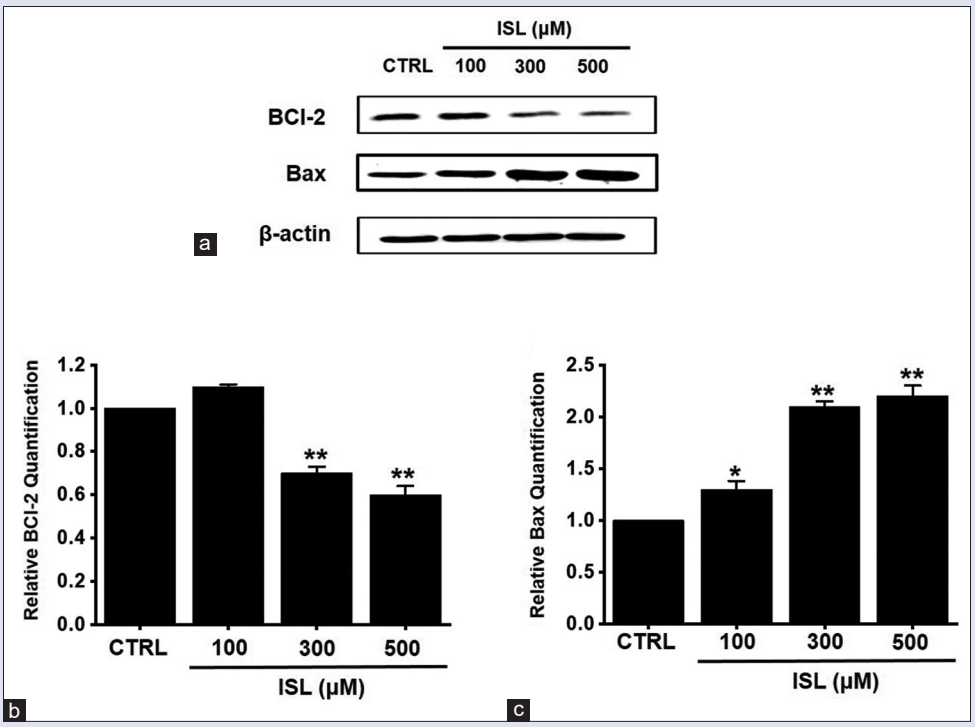

Figure 3: Effects of isoliquiritigenin on Bcl-2 and Bax in 5637 cells. (a) Bcl-2 expression was reduced gradually by isoliquiritigenin, whereas Bax expression was increased gradually using western blot. (b) Bcl-2 and (c) Bax protein expressions were normalized versus β-actin. Results are presented as mean ± standard error of the mean β-Actin was used as the loading control. ISL: Isoliquiritigenin; CTRL, control. **P* < 0.05. ***P* < 0.01 versus untreated controls

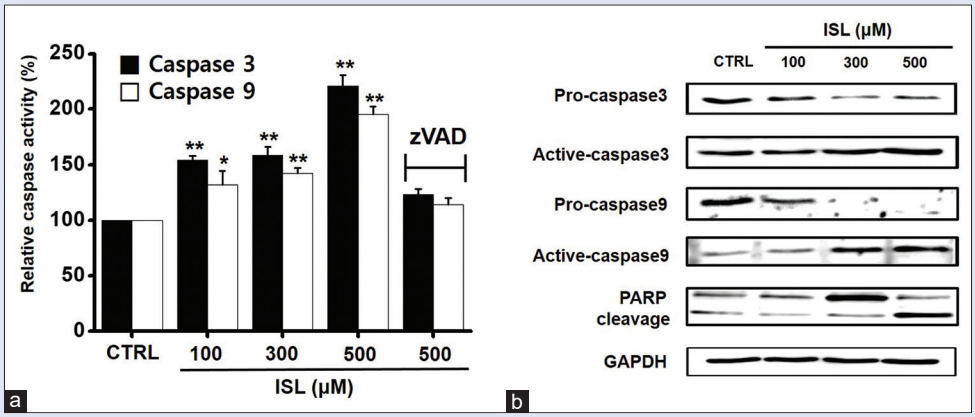

Figure 4: Caspases activation by isoliquiritigenin in 5637 cells. (a) 5637 cells were lysed and lysates were assayed for caspase-3 and -9 activities. (b) The changes of caspase-3, -9, and PARP cleavage activity were investigated by western blot. Results are presented as mean ± standard error of the mean GAPDH was the loading control. ISL: Isoliquiritigenin; CTRL: Control; PARP: Poly (adenosine diphosphate-ribose) polymerase. *$P < 0.05$, **$P < 0.01$ versus untreated controls

Effect of isoliquiritigenin on the mitochondria-dependent apoptotic pathway in 5637 cells

To check whether ISL-induced apoptosis is regulated by Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic), we performed western blotting on the proteins isolated from 5637 cells. Bcl-2 was reduced by ISL, whereas Bax was increased [Figure 3a-c]. These results indicate that ISL-induced apoptosis is related to activation of mitochondria in 5637 cells.

Caspase activation by isoliquiritigenin in 5637 cells

Caspases are important mediators of apoptosis through intrinsic and extrinsic pathways.[20] ISL dose-dependently increased caspase-3 and -9 activity, and zVAD-fmk (a broad-spectrum caspase inhibitor) pretreatment suppressed their activity [Figure 4a]. Western blotting also indicated that ISL treatment upregulated the active forms of caspase-3 and -9 and downregulated pro-caspase-3 and -9. In addition, ISL upregulated PARP protein levels [Figure 4b]. These results indicate that ISL-induced apoptosis is related to activation of caspase in 5637 cells.

Effect of isoliquiritigenin on intracellular reactive oxygen species generation in 5637 cells

As ROS is one of the key factors in cell apoptosis, we investigated whether ISL generates ROS in 5637 cells. To determine whether ROS generation was related to apoptosis by ISL, we used DCF-DA (a fluorescent dye) in the cells to measure ROS levels with flow cytometry. As indicated in Figure 5, flow cytometry showed that ISL increased ROS levels in 5637 cells.

Synergistic effects of transient receptor potential melastatin 7 channel or transient receptor potential vanilloid 2 channel blockers on isoliquiritigenin-induced apoptosis in 5637 cells

Regulation of transient receptor potential (TRP) melastatin 7 channel is known to be important for apoptosis of bladder cancer cells.[21,22] To check the role of TRP melastatin 7 channel in ISL-induced apoptosis in 5637 cells, we used the TRP melastatin 7 blocker, TG100-115.[23] TG100-115 and ISL exerted a synergistic effect on ISL-induced apoptosis [Figure 6a]. Similarly, for TRP vanilloid 2 channels,[24] TRP vanilloid 2 blocker, tranilast,[25] combined with ISL exerted a synergistic effect on ISL-induced apoptosis [Figure 6b].

DISCUSSION

Common cancer treatments, such as surgery and chemotherapy, do not always produce good clinical results.[26,27] These treatments also have serious side effects.[28] Therefore, herbal medicine, which is known to have fewer side effects, has been widely used to treat cancer in recent times.[27] Herbal drugs, made from a single plant or a combination of several plants, were used to treat diseases long before modern medicines were developed and are still in use.[29] ISL is a flavonoid extracted from licorice root and is known to possess anticancer properties effective on many types of cancer cells.[1,6-11] Apoptosis is a controlled mechanism for removing old or damaged cells, and inducing apoptosis is the most important role of anticancer drugs.[30] It is well known that several natural substances, including plants, are used as anticancer drugs, which can induce apoptosis and kill cancer cells.[31] In the present study, we investigated the efficacy of ISL and the mechanisms underlying ISL-induced apoptosis in bladder cancer cells. ISL (100–500 μg/ml) inhibited 5637 cell proliferation [Figure 1] and increased sub-G1 cell cycle phase ratios [Figure 2]. ISLinduced cell death was related to reduce Bcl-2 and increase Bax [Figure 3]. In addition, ISL activated caspase-3 and -9 [Figure 4] and increased intracellular ROS generation [Figure 5]. These findings suggest that ISL causes apoptosis in 5637 cells, and therefore, ISL may be a novel anticancer drug for treating bladder cancer and an effective anticancer supplement.

Several ion channels are involved in the apoptosis of cancer cells, especially TRP channels.[32] The TRP channel family is a cell membrane protein and serves to control various physiological and pathological processes by controlling the transmission of signals inside/outside the cell.[33] TRP melastatin 7 channels are common in bladder cancer and promote the growth and migration of bladder cancer cells.[21] Therefore, TRP melastatin 7 plays a role in controlling the prognosis of bladder cancer. In addition, a decrease in the expression of TRP melastatin 7 channel is associated with an increase in ROS and eventually with increased apoptosis of the bladder cancer cells.[22] Temperature-sensitive TRP vanilloid channels are important for sensing pain and temperature.[34] Among them, the TRP

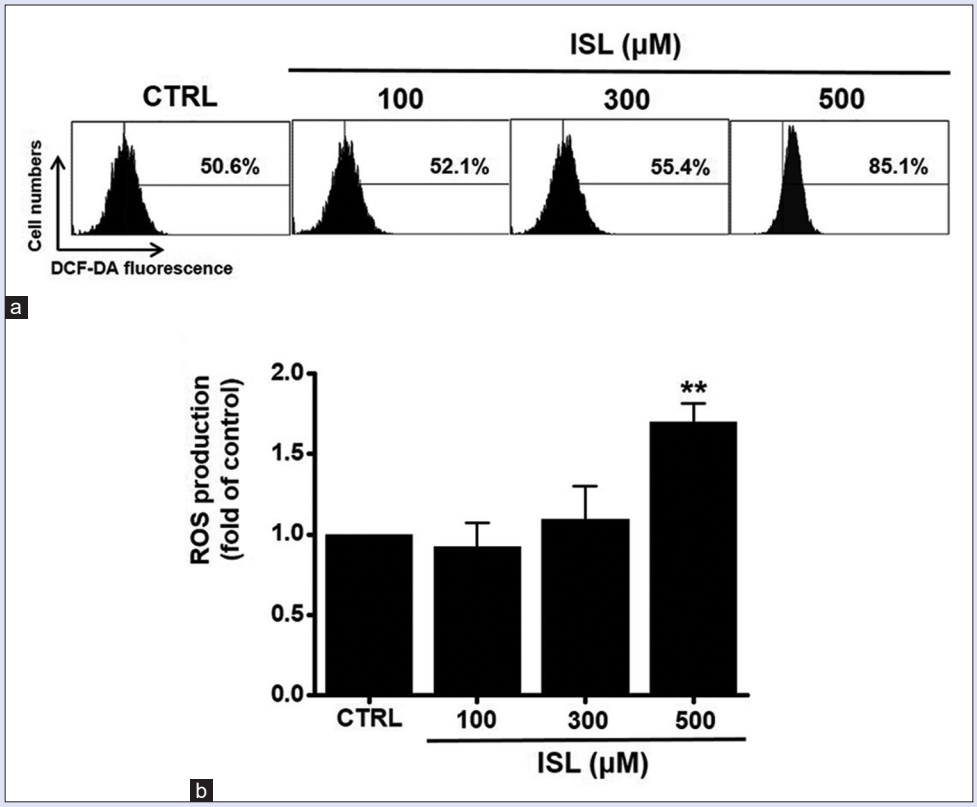

Figure 5: Isoliquiritigenin increased reactive oxygen species levels in 5637 cells. (a and b) Intracellular reactive oxygen species was detected with isoliquiritigenin. Reactive oxygen species levels are expressed as percentages of untreated controls. Results are presented as mean ± standard error of the mean ISL: Isoliquiritigenin; CTRL: Control. $^{**}P < 0.001$ versus untreated cells

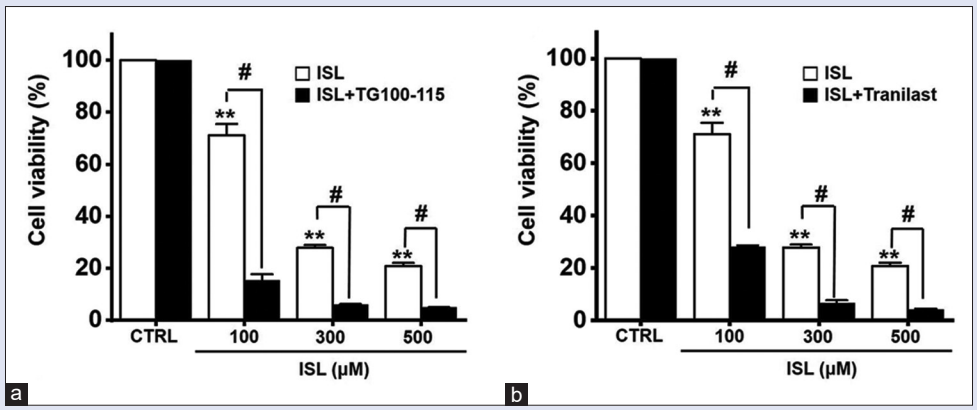

Figure 6: Effect of transient receptor potential channel inhibitor on the effects of isoliquiritigenin in 5637 cells. Cell viability was checked after co-treating 5637 cells with isoliquiritigenin plus (a) TG100-115 (transient receptor potential melastatin 7 inhibitor) or (b) tranilast (transient receptor potential vanilloid 2 inhibitor) for 24 h. Results are presented as mean ± standard error of the mean. ISL: Isoliquiritigenin; CTRL: Control. $^{**}P < 0.01$ versus untreated controls. $^{\#}P < 0.05$ for comparisons between treatments

vanilloid 2 channel increases the migration of bladder cancer cells but does not affect cell proliferation in 5637 cells.[24] Moreover, activation of TRP vanilloid 2 by matrix metalloproteinase 2 regulation is important for the development of bladder cancer.[24] However, in T24 bladder cancer cells, intracellular calcium influx through the TRP vanilloid 2 channel causes apoptosis.[35] In the present study, TG100-115, a TRP melastatin 7 blocker, combined with ILS had a synergistic effect on ISL-induced apoptosis [Figure 6a]. Similarly, tranilast, a TRP vanilloid 2 blocker, had the same effect on ISL-induced apoptosis [Figure 6b]. Therefore, it is thought that the TRP melastatin 7 or TRP vanilloid 2 channel has a significant effect on activating the apoptotic reaction by ISL. It is necessary to further investigate the effects of ISL on the TRP melastatin 7 and TRP vanilloid 2 channels themselves and to understand their respective mechanisms of action.

ISL inhibits proliferation and metastasis of MKN28 gastric cancer cells, osteosarcoma, and A549 lung cancer cells.[1,8,9] ISL induces apoptosis

of T24 human bladder cancer cells through CDK2 activities and mitochondrial signaling pathways[2] and of human prostate cancer cells via cell cycle arrest.[6] In addition, ISL induces apoptosis in A375 human melanoma cells through mitochondrial dysfunction[7] and in breast and colon HT29 cancer cells.[10,11] In addition to showing the efficacy of ISL on the various cancer cells described above, this study shows that ISL causes apoptosis in bladder cancer cells *in vitro*, mediated by caspase and ROS activities. Moreover, apoptosis is controlled by the regulation of TRP melastatin 7 and TRP vanilloid 2 channels.

CONCLUSION

The present study showed that ISL inhibited cell proliferation and increased the sub-G1 phase in 5637 cells. In addition, ISL-induced apoptosis was associated with the downregulation of Bcl-2 and the upregulation of Bax. ISL increased caspase-3 and -9 activities and intracellular ROS levels. Furthermore, ISL-induced apoptosis was controlled by the activation of TRP melastatin 7 and TRP vanilloid 2 channels. We hope these results contribute to increasing the efficiency of treatment of bladder cancer.

Financial support and sponsorship

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Conflicts of interest

There are no conflicts of interest.

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