Research Article

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Saponins extracted from by-product of *Asparagus officinalis* L. suppress tumour cell migration and invasion through targeting Rho GTPase signalling pathway

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Abstract

BACKGROUND: The inedible bottom part ($\sim$30–40%) of asparagus (*Asparagus officinalis* L.) spears is usually discarded as waste. However, since this by-product has been reported to be rich in many bioactive phytochemicals, it might be utilisable as a supplement in foods or natural drugs for its therapeutic effects. In this study it was identfed that saponins from old stems of asparagus (SSA) exerted potential inhibitory activity on tumour growth and metastasis.

RESULTS: SSA suppressed cell viability of breast, colon and pancreatic cancers in a concentration-dependent manner, with half-maximum inhibitory concentrations ranging from 809.42 to 1829.96 μg mL^{-1}. However, SSA was more functional in blocking cell migration and invasion as compared with its cytotoxic effect, with an effective inhibitory concentration of 400 μg mL^{-1}. A mechanistic study showed that SSA markedly increased the activities of Cdc42 and Rac1 and decreased the activity of RhoA in cancer cells.

CONCLUSION: SSA inhibits tumour cell motility through modulating the Rho GTPase signalling pathway, suggesting a promising use of SSA as a supplement in healthcare foods and natural drugs for cancer prevention and treatment.
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Keywords: *Asparagus officinalis* L.; saponin; cancer; Rho GTPase

INTRODUCTION

The global burden of cancer is still increasing and it is the leading cause of death in economically developed countries. About 12.7 million new cases and 7.6 million cancer deaths are estimated to have occurred in 2008 worldwide.[1] Most cancers can be cured surgically before cancer metastasis, and 5 year survival rates are often above 90%. However, once metastasis has taken place, the 5 year survival rate falls below 15%.[2] The process of cancer metastasis consists of a series of sequential and discrete steps. The malignant cells first escape from a primary tumour, then spread through the blood and lymph vessels to a secondary site and finally form secondary tumours.[3] Therefore early treatment to block cancer cell metastasis is paramount.

Ras homologue GTPases (Rho GTPases) are small molecules of the Ras superfamily. They share structural homology with a Rho-type GTPase-like domain located between the fifth β-strand and the fourth α-helix within the small GTPase domain.[4] A structural feature differentiates the Rho proteins from other small GTPases. The Rho family can be simply divided into six subfamilies, including the RhoA-related subfamily (RhoA, RhoB and RhoC), the Rac1-related subfamily (Rac1 (and Rac1b), Rac2, Rac3 and RhoG), the Cdc42-related subfamily (Cdc42 (and G25K), TC10, TCL, Chp/Wrch-2 and Wrch-1), the Rnd subfamily (Rnd1, Rnd2 and RhoE/Rnd3) and the RhoBTB subfamily.[5] Research has revealed that Rho GTPases play an acknowledged role in cell

motility, showing that they act to regulate cell adhesions and cytoskeleton networks.[6] Among them, RhoA, Cdc42 and Rac1 are three classical and well-studied members. RhoA promotes actin/myosin contractility and the formation of stress fibres and focal adhesions, Cdc42 causes the formation of filopodia, regulates cell polarity and thereby maintains the directionality of cell movement, while Rac1 promotes actin polymerisation and the formation of lamellipodia.[5,7]

Asparagus (*Asparagus officinalis* L.), a herbaceous plant with a perennial root, is a healthy and nutritious vegetable. Besides its edible value, this plant and its extracts have been reported to possess antifungal,[8] antioxidant,[9] hypolipidaemic,[10] hypoglycaemic[11] and hepatoprotective[12] activities. Regarding cancer treatment, Shao *et al.*[13] found that crude saponins from asparagus shoots (edible part) inhibited the growth of human leukaemia in a dose- and time-dependent manner. Saponins from asparagus have also been reported to repress the viability of liver and gastric cells by regulating the cell cycle and increasing intracellular Ca^{2+} and reactive oxygen species (ROS)

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levels.[14] During industrial processing, around 30–40% of each asparagus spear is discarded as waste,[11] leading to significant environmental pollution. This throw-away part has been reported to be rich in many bioactive phytochemicals such as steroidal saponins,[15,16] flavonoids,[17] polysaccharides,[18] etc. Nevertheless, there have been few functional and mechanistic studies on the pharmacological effects of asparagus by-product to date. Our previous studies revealed that extracts from asparagus by-product exhibited strong hypolipidaemic and hepatoprotective actions (*n*-butanol extract) in high-fat-fed mice[10] and displayed hypoglycaemic and hypotriglyceridaemic functions (aqueous extract) in streptozotocin-induced diabetic rats.[11] In this study we further examined how saponins from old stems of asparagus (SSA) inhibited cancer cell migration and invasion by targeting the key cellular signalling pathway.

MATERIALS AND METHODS

Plant material
Asparagus by-product was kindly provided by Shanghai Green Asparagus Co. Ltd (Shanghai, China) and authenticated by Dr Hongqing Li (School of Life Science, East China Normal University, Shanghai, China). It comprised the bottom parts of asparagus spears (10–25 cm) remaining after the top (edible) parts (30–35 cm) had been taken for selling as food. A voucher specimen (No. zxl001) was deposited in the herbarium of East China Normal University. The asparagus by-product was cleaned, air dried and powdered for further use.

Preparation of SSA
SSA was prepared as described by Zhu *et al*.[10] with some modifications. Briefly, the asparagus by-product powder was first degreased and extracted to obtain butanolic fractions. These crude saponins were then applied to an AB-8 macroporous resin column (Chang Zhou Bao En Chemical Plant, Hebei, China) for futher purification to produce SSA. The total steroidal saponin content was determined as 51.8% by a spectrophotometric method.[19]

Reagents
Growth factor-reduced Matrigel was obtained from BD Biosciences (San Jose, CA, USA). Glutathione Sepharose 4B was purchased from GE Healthcare (Fairfield, CT, USA). Anti-Rac1 antibody was obtained from Millipore (Boston, MA, USA), anti-RhoA antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-Cdc42 antibody from Cell Signaling Technology (Danvers, MA, USA). Other analytical reagents were purchased from Sigma (St Louis, MO, USA). A 250 g L^{-1} stock solution of SSA was dissolved in dimethyl sulfoxide (DMSO) and stored in small aliquots at $-20\,^{\circ}$C until needed.

Cell lines and cell culture
Human breast cancer Bcap-37 cells were purchased from the China Center for Type Culture Collection (Shanghai, China). Other cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Human breast cancer MDA-MB-231 and colon cancer SW620 cells were cultured in L15 medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (Gibco) at 37 $^{\circ}$C without CO$_2$. Human breast cancer Bcap-37, colon cancer HCT116 and pancreatic cancer

PANC-1 and PANC-28 cells were cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% FBS at 37 $^{\circ}$C under a humidified 95:5 (v/v) mixture of air and CO$_2$.

Cell viability assay
Cell viability was determined by the sulforhodamine B (SRB) method.[20,21] Briefly, cancer cells (7×10^3 cells per 100 µL well) were seeded in 96-well plates and exposed to various concentrations of SSA for 48 h. Subsequently, 25 µL of TCA (trichloroacetic acid) (500 g L^{-1}) was added to each well and the cells were fixed at 4 $^{\circ}$C for 1 h. After being dried, treated cells were stained with 50 µL of 4 g L^{-1} SRB for 10 min and washed with 1% glacial acetic acid. The absorbance at 515 nm was recorded with a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Three independent experiments were performed in triplicate.

Annexin V/propidium iodide (PI) apoptosis assay
Human breast cancer MDA-MB-231 cells were treated with various concentrations of SSA for 48 h, collected and stained with annexin V-FITC according to the manual (Annexin V-FITC Apoptosis Detection Kit, Invitrogen). Stained cells were analysed by flow cytometry (FACSCalibur, BD Biosciences).

Cell migration assay
A cell migration assay (wound-healing assay) was performed as described by Pang *et al*.[22] Human breast cancer MDA-MB-231 cells were plated in six-well plates and grown until full confluence. Cell monolayers were wounded using a sterile pipette tip and washed with phosphate-buffered saline (PBS) to remove cell debris. The cells were then incubated with various concentrations of SSA for 24 h at 37 $^{\circ}$C. Digital images were obtained using an inverted microscope (magnification 16×). Cells that had migrated to wound closures were quantified by manual counting. All experiments were performed three times.

Transwell migration assay
A transwell migration assay was performed as described by Pang *et al*.[22] with some modifications. Briefly, MDA-MB-231 cells (5×10^4 cells per well) were suspended in 100 µL of serum-free L15 medium and pretreated with various concentrations of SSA for 30 min. Polycarbonate filters (6.5 mm diameter, 8 µm pore size) (Corning, Inc., NY, USA) were placed in a 24-well plate. Pretreated cells were seeded in the upper compartments, while the lower compartments were filled with 500 µL of L15 medium containing 10% FBS. The cells were then incubated at 37 $^{\circ}$C for 16–18 h. Cells on the upper surfaces of the filter membrane (non-migrated cells) were wiped away with cotton swabs. Cells that had migrated to the lower surface of the membrane were fixed with 40 g L^{-1} paraformaldehyde for 15–20 min and stained with 10 g L^{-1} crystal violet. Images were taken with an Olympus inverted microscope (magnification 16×), and migrated cells were quantified by manual counting. Percentage inhibition was expressed based on untreated wells at 100%.

Transwell invasion assay
A transwell invasion assay was carried out to examine tumour invasiveness as described previously.[23] The polycarbonate filters were coated with 100 µL of 10% Matrigel (diluted in L15 medium) to form a genuine reconstituted basement membrane. The other procedures were the same as described above for the transwell

migration assay. Images were taken with an Olympus inverted microscope (magnification 16×), and invasive cells were quantified by manual counting. Percentage inhibition was expressed based on untreated wells at 100%.

Immunofluorescence assay

Sterile coverslips were placed in a 24-well plate and coated with 10 g L^{-1} gelatin for 30 min. MDA-MB-231 cells (5×10^4 cells per well) were seeded on the coverslips and exposed to various concentrations of SSA. The cells were then fixed with 40 g L^{-1} paraformaldehyde, permeabilised with 0.1% Triton X-100/PBS and sequentially stained with FITC-phalloidin (1:500) and DAPI (1:1000). Stained slides with cells were air dried, mounted with mounting medium (Sigma-Aldrich, St. Louis, MO, USA) and analysed under a confocal laser scanning microscope (magnification 64×) (LSM 5, Pascal, Carl Zeiss, Jena, Germany).

Glutathione S-transferase (GST) pull-down assay

GTPase activation in the cells was evaluated by GST-PAK1 and GST-RBP pull-down assays as described previously.[24] Briefly, MDA-MB-231 cells were treated with various concentrations of SSA for 24 h and then lysed with corresponding lysis buffer (Rac1/Cdc42: 20 mmol L^{-1} N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 120 mmol L^{-1} NaCl, 10% glycerol, 2 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), 1 mmol L^{-1} phenylmethanesulfonyl fluoride (PMSF), 10 µg mL^{-1} aprotinin/leupeptin and 0.5% NP-40; RhoA: 50 mmol L^{-1} Tris, 50 mmol L^{-1} NaCl, 5 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} dithiothreitol (DTT), 1 mmol L^{-1} PMSF, 10 µg mL^{-1} aprotinin/leupeptin and 1% Triton X-100) supplemented with different kinds of proteinase inhibitor. About 500 µg of protein extract from each treatment was applied for pull-down assays. Protein concentration was determined by the bicinchoninic acid assay (BCA) (Thermo Fisher Scientific, Rockford, USA). GTP-bound Rac1 and Cdc42 were pulled down using the GST-PBD of PAK1 immobilised on glutathione beads, while GTP-bound RhoA was pulled down using the GST-RBP purified from *Escherichia coli*. The amounts of active Rac1, Cdc42 and RhoA (GTP-bound form) were determined by western blotting. Blotting bands were detected by electrochemiluminescence and then autoradiographed using X-ray film. The relative optical density of blotting bands was quantified by Image J software (NIH, Bethesda, MD, USA). All tests were performed independently in triplicate.

Statistical analysis

All data were analysed and presented as mean ± standard deviation (SD) using Microsoft Excel. Statistical comparisons between groups were made by one-way analysis of variance (ANOVA) followed by Student's *t* test, with $P \leq 0.05$ indicating statistical significance.

RESULTS

SSA inhibits viability of various cancer cells

In this study the cytotoxic effects of SSA were examined on three kinds of cancer cell, namely breast, colon and pancreatic cancer cells. Our SRB results showed that SSA inhibited cell viability in a dose-dependent manner, with half-maximal inhibitory concentrations (IC_{50}) of 809.42, 1829.96, 921.14, 902.68, 1629.34 and 906.45 µg mL^{-1} in MDA-MB-231, Bcap-37, SW620, HCT116, PANC-1 and PANC-28 cancer cells respectively (Fig. 1A).

To clearly show the ability of SSA to inhibit tumour cell growth, we directly recorded the cell images after treatment for 48 h. As shown in Fig. 1B, the cell numbers of MDA-MB-231 and SW620 were decreased by SSA in a concentration-dependent manner. These results indicated that SSA had the ability to block tumour growth of various cancers.

SSA induces cancer cell apoptosis

To examine whether SSA induced cancer cell apoptosis, we further performed an annexin V staining assay in human breast cancer MDA-MB-231 cells. The results showed that the population of apoptotic cells was increased after 48 h of incubation with SSA, by 12.59 and 14.54% at concentrations of 600 and 1200 µg mL^{-1} respectively (Fig. 2). This was consistent with previous findings that SSA could inhibit cell viability[13,16] and induce cell apoptosis to some extent.

SSA inhibits cancer cell migration

Migration activity (motility) is an essential feature of most malignant tumours. Various biological assays have been developed to assess the metastatic potential of cancer cells *in vitro*. In this study we chose human breast cancer MDA-MB-231 cells and applied *in vitro* wound-healing (Fig. 3A) and transwell migration (Fig. 3B) assays to evaluate the inhibitory action of SSA on the chemotactic motility of tumour cells. Our representative images and quantitative analysis revealed that SSA dramatically reduced the migratory ability of MDA-MB-231 cells, with an effective inhibitory concentration of 400 µg mL^{-1}, which is much lower than the IC_{50} of cytotoxicity (809.42 µg mL^{-1} in Fig. 1A). These findings suggested that SSA was more effective in suppressing cancer cell migration than in blocking cancer cell growth.

SSA inhibits cancer cell invasion

Tumour cell invasion occurs by cell-secreted proteolytic degradation of the cellular basement membrane and spreads to distant organs.[23] This is the leading cause of cancer mortality. Our *in vitro* transwell invasion assay (Fig. 4A) showed that SSA significantly inhibited invasion of MDA-MB-231 cells at a concentration of 400 µg mL^{-1} compared with untreated cells. Together, these results indicated that SSA could suppress cancer cell migration and invasion.

Furthermore, to clearly show the changes in cellular morphology after treatment, we next performed an FITC-phalloidin immunofluorescence assay. It has been reported that some highly motile cells exhibit amoeboid movement with a rounded morphology.[25] As shown in Fig. 4B, after 24 h of incubation with various concentrations of SSA, the shape of MDA-MB-231 cells appeared to be altered. Untreated (control) cells remained tightly in contact, whereas treated cells were elongated without strong cell–cell adhesions but still attached to the substrate, with marked protrusions. The cytoskeletal networks of treated cells were seriously disorganised. Instead of forming the roundish aspect required for amoeboid motility, the cells took on a sword-like shape, resulting in a reduction in cell motility.

SSA regulates activation of Rho GTPases in cancer cells

Rho GTPases play a pivotal role in coordinating cellular motility, affecting the adhesion of cells and the organisation of cytoskeleton networks.[6] To test whether SSA exerted inhibitory action on cell motility by regulating the Rho GTPase signalling pathway, we
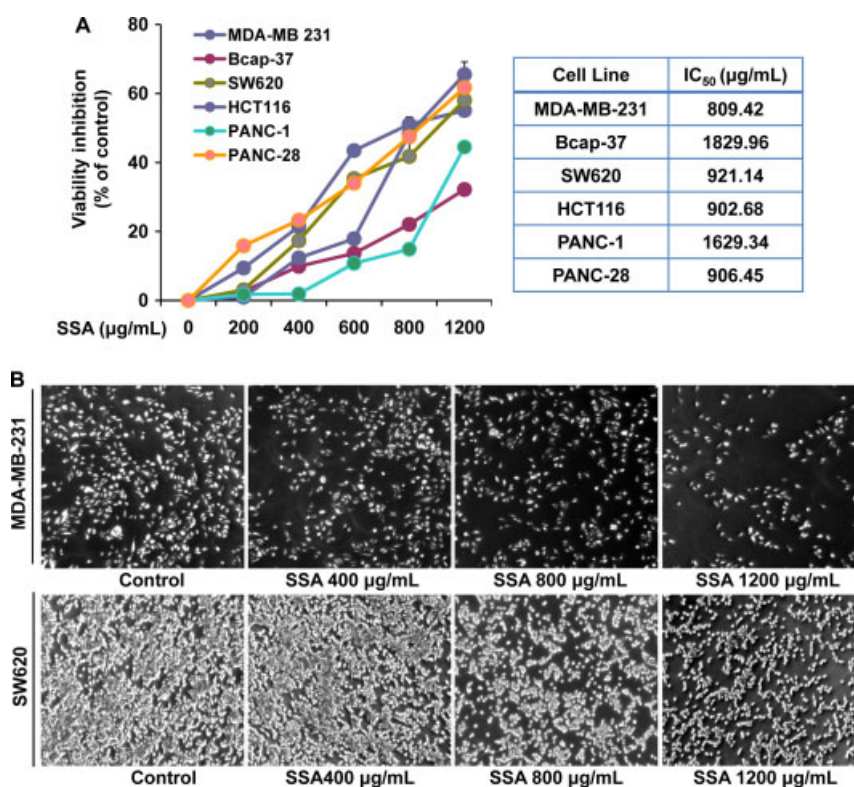
Figure 1. SSA inhibits cell viability in cancer cell lines. (A) Left: SSA inhibited cancer cell viability in a dose-dependent manner. MDA-MB-231, Bcap-37, SW620, HCT116, PANC-1 and PANC-28 cells (7×10^3 cells) were directly treated with or without various concentrations of SSA for 48 h. Cell viability was then analysed by the SRB assay. Symbols, mean; bars, standard error. Right: the IC$_{50}$ value of each cancer cell line is shown. (B) MDA-MB-231 and SW620 cells were seeded in 24-well plates and exposed to various concentrations of SSA for 48 h. The images shown give visual evidence of the ability of SSA to inhibit cell viability and growth. Three independent experiments were performed.
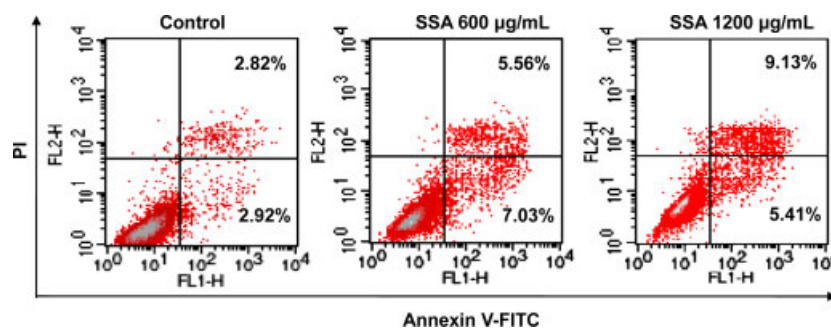

Figure 2. SSA induces cancer cell apoptosis. SSA induced apoptosis in MDA-MB-231 cells. Cells were seeded in 6 cm dishes and incubated with 600 and 1200 µg mL^{-1} SSA for 48 h. Cells were then stained with annexin V/PI and analysed by flow cytometry for the apoptotic effect of SSA. Three independent experiments were performed.

further performed a GST pull-down assay to examine the activities of Rac1, Cdc42 and RhoA in cancer cells. The results in Fig. 5A show that activation of Rac1 and Cdc42 was significantly elevated by SSA in a concentration-dependent manner, whereas activation of RhoA was markedly blocked. The relative optical density of blotting bands is correspondingly shown in Fig. 5B. These results indicated that the effects of SSA on the activation of Rho GTPases might contribute to its suppression of cancer cell migration and invasion.

DISCUSSION

Despite significant improvements in diagnostic and medical techniques, the primary cause of cancer deaths is still the resistance of metastases to conventional therapies.[26] Therefore novel remedies for suppressing metastasis are desperately needed. Natural products are widely applied in cancer chemotherapy and regarded as leads to anticancer drugs.[27] As a matter of fact, over 70% of anticancer compounds are obtained either from natural products or from natural product-derived substances designed following a natural compound as a model.[28] Anticancer agents from edible phytochemicals are now considered to be inexpensive and readily acceptable, with greater pharmacological functions and fewer side effects for cancer control and management. Asparagus (*A. officinalis* L.), a popular and healthy vegetable worldwide, has been reported to possess various biological activities, especially effects on metabolic syndrome[10,11] and cancer.[29] Besides, saponins exert the main bioactivity among
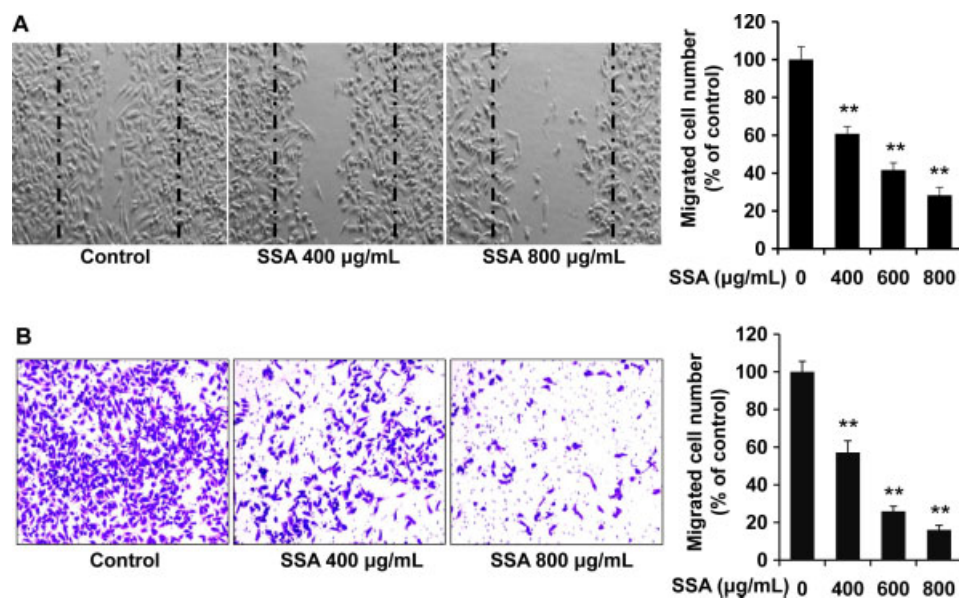
Figure 3. SSA inhibits cancer cell migration. (A) SSA inhibited wound-healing migration of MDA-MB-231 cells. Cells were grown to full confluence in six-well plates, wounded and treated with various concentrations of SSA for 24 h. Migrated cells were quantified by manual counting. (B) SSA inhibited transwell migration of MDA-MB-231 cells. Cells (5×10^4 per well) were seeded in the upper chamber of transwells and treated with various concentrations of SSA as described in 'Materials and methods'. Cells that migrated to the lower surface of the membrane were fixed, stained and photographed (magnification 16×). Migrated cells were quantified by manual counting. Columns, mean; bars, standard error. **$P < 0.01$ *versus* control group. The experiments were conducted at least three times.
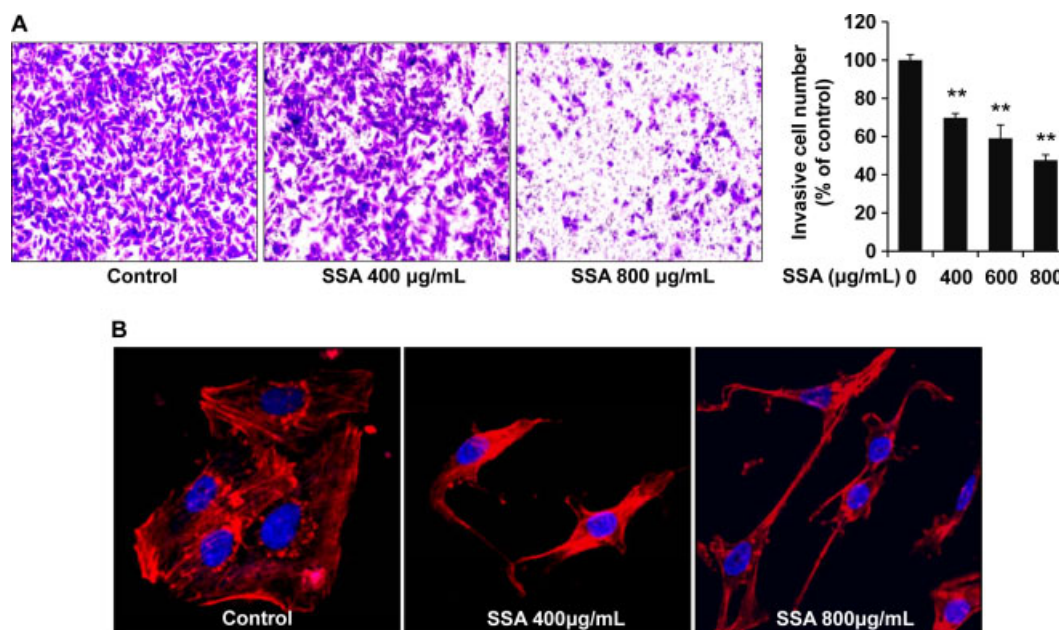

Figure 4. SSA inhibits cancer cell invasion. (A) SSA inhibited invasion of MDA-MB-231 cells. The Matrigel invasion assay was performed as described in 'Materials and methods'. The invasive capability of MDA-MB-231 cells was evaluated by counting the number of cells that passed through the transwell filter coated with Matrigel. Invading cells were stained, photographed (magnification 16×) and counted in at least five independent fields. Columns, mean; bars, standard error. *$P < 0.05$, **$P < 0.01$ *versus* control group. (B) Confocal images of phalloidin-labelled F-actin in MDA-MB-231 cells. Cells (5×10^4 per well) were seeded on coverslips, treated with SSA and stained with FITC-phalloidin (1:500)/DAPI (1:1000). Images were taken with a confocal laser scanning microscope. Experiments were performed three times in triplicate.

phytochemicals from asparagus.[30] However, little is known about their effects in prevention and treatment of tumour metastasis.

In this study we evaluated the anticancer property of saponins extracted from asparagus by-product (SSA) and revealed the novel biological function of SSA as an inhibitor of tumour cell migration and invasion. It is worth mentioning that breast cancer is the most diagnosed cancer and the leading cause of cancer death in females.[1] Cancer cells with high motility are more likely to emigrate from the solid tumour, which is the first step in cancer metastasis.[31] In the present study, MDA-MB-231 cells with highly metastatic property were applied to appraise the effects of SSA on cancer cell motility in *in vitro* wound-healing migration and transwell
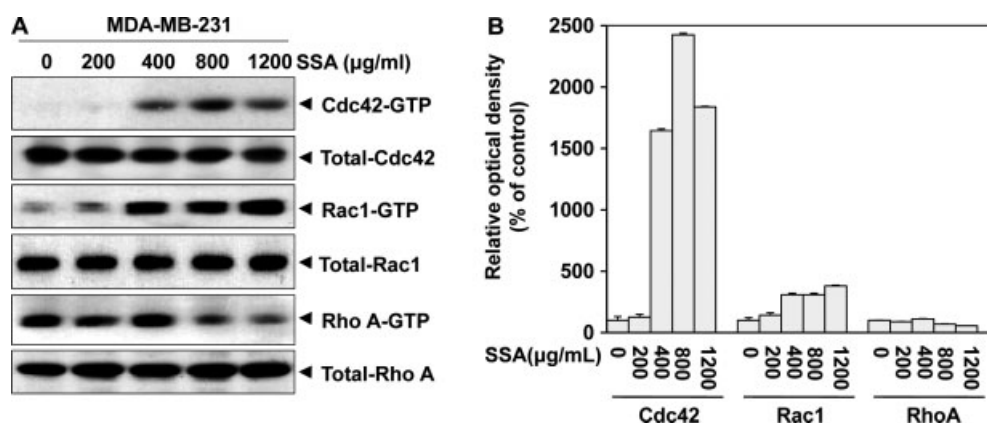
Figure 5. SSA regulates Rho GTPase signalling pathway. (A) SSA blocked RhoA activation but promoted Rac1 and Cdc42 activation. MDA-MB-231 cells were treated with various concentrations of SSA for 24 h and whole-cell extracts were prepared. GTP-bound Rac1 and Cdc42 were pulled down using GST-PBD of PAK1 immobilised on glutathione beads, while GTP-bound RhoA was pulled down using GST-RBP. The amounts of active GTPases were processed by 120 g L^{-1} SDS-PAGE and subjected to western blotting analysis using specific antibodies. The results shown are representative of three independent experiments. (B) The relative optical density of blotting bands is shown. Quantitative results for Rho-GTPase bands were analysed by Image J software (NIH). Columns, mean; bars, standard deviation.

invasion assays. Unlike widely used anticancer agents that have side effects[32] or severe cytotoxicity to induce cell apoptosis, we found that SSA presented low cytotoxicity but with admirable motility inhibition. The functional concentration of SSA to block cell migration and invasion (400 µg mL^{-1}) was much lower than the IC$_{50}$ (809.42 µg mL^{-1}) in breast cancer MDA-MB-231 cells. This indicates that the effect of SSA on inhibition of tumour metastasis might be much earlier than its cytotoxicity effects.

Cell migration, a key step in cancer onset, is a series of discrete multistage processes involving a number of proteins in different 'metastatic cascades'. The Rho GTPases of the Ras family have come to the notice of researchers. They work as sensitive molecular switches regulating a wide range of cellular processes in tumour progression and tumour angiogenesis.[33,34] The Rho GTPases account for as many as 23 members. The archetypes RhoA, Rac1 and Cdc42 have been most characterised and studied. Like other small GTPase superfamilies, these proteins cycle between an inactive GDP-bound form and an active GTP-bound form. The active Rho GTPase is generally believed to be an important regulator of cell migration, and various evidence suggests that the contribution of each member is restricted to a specific condition and cell type.[35] Researchers have found that some carcinoma cells move at very high speed (up to 4 µm min^{-1}) with an amoeboid morphology *in vivo*. These cells show a rounded morphology and require Rho activation.[25] Also, overexpression of Cdc42 inhibits breast cancer migration and invasion,[35] and Cdc42 in turn cross-activates Rac1,[7] implying a coincident relevance. These two proteins assemble as a focal complex and facilitate extension of the lamellipodium of the cell to attach the extracelluar matrix at the leading edge, thus maintaining a forward direction for cell movements. Otherwise, RhoA cleaves the adhesion of the focal complex to promote tail detachment.[25] In the present study we found that SSA suppressed cancer cell motility by activating Cdc42 and Rac1 but deactivating RhoA. The activation of Cdc42 and Rac1 provided prolonged movement in the leading edge of the cell, but the reduction in RhoA activity inhibited tail detachment. As a result, the cell was further elongated, not presenting a rounded morphology, which was in agreement with our finding in the immunofluorescence assay (Fig. 4B).

In conclusion, our results indicated that SSA could significantly inhibit cell motility and cell growth in a dose-dependent manner by targeting the Rho GTPase signalling pathway. We identified a novel function and a potential use of SSA for cancer therapy as a supplement in healthcare foods and natural drugs. Additionally, our study also suggests an economically beneficial avenue to extend the asparagus industry chain and thereby reduce pollution.

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