Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Snail Mucus Induces Cytotoxicity and Chemosensitivity of Triple-Negative Breast Cancer Cells via Activation of Fas Signaling Pathway

Chien-Yi Ho
China Medical University Hsinchu Hospital
Dai-Wei Hu
China Medical University
Bo-Rong Chen
China Medical University
Chun-Chun Yang
National Taichung First Senior High School
Chun-Hsu Yao
China Medical University Hospital
Tien Ni
China Medical University Hospital
Yueh-Sheng Chen
China Medical University Hospital
Chih-Yen Tu
China Medical University Hospital
Wei-Chao Chang
China Medical University Hospital
Yang-Chang Wu
China Medical University
Chih-Hsin Tang
China Medical University
Hui-Chi Huang
China Medical University
Wei-Chien Huang (whuang@mail.cmu.edu.tw)
China Medical University
Tzong-Shiun Li
Show Chwan Memorial Hospital

Research Article

Keywords: snail mucus, anti-cancer, apoptosis, TNBC, chemotherapy

Posted Date: August 27th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-827784/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

Background

Triple-negative breast cancers (TNBCs) patients showed poor survival outcomes due to chemoresistance, and the development of new therapeutic strategies is urgently needed. The development of cancer is mainly related to chronic inflammation. Therefore, the pharmacological reduction of inflammation by natural extract components may promote anti-cancer activity and increase chemosensitivity. Snail mucus has been reported to possess the ability against inflammation, a process closely related to tumorigenesis, suggesting a potential anti-cancer function. The direct cytotoxic activity of snail mucus in cancer cells was investigated in this study.

Methods

The effect of snail mucus on cell viability in breast cancer cells and normal epithelial cells were measured by MTT and IncuCyte Live-cell analysis. The active fractions of snail mucus were isolation by performing MPLC and the anti-cancer ingredients were identified by NMR spectrometer analysis.

Results

Snail mucus significantly decreased the proliferation and viability of TNBC cells with relatively lower cytotoxicity to normal breast epithelial cells and enhanced their response to chemotherapy. Mechanistically, snail mucus induces an extrinsic apoptotic pathway through activation of Fas signaling by suppressing nucleolin. Two possible peptide fractions have also been identified as the anti-cancer ingredients of the snail mucus.

Conclusions

In summary, snail mucus can induce programmed cell death via the extrinsic apoptotic pathway and might have therapeutic potential with chemo-sensitizing effect for TNBCs.

Background

Breast cancer is one of the most common cancer types and is the second major cause of death in women. The symptoms of breast cancer include breast or nipple pain, skin irritation looking like an orange peel, breast skin inflammation, and redness. According to the expressions of molecular markers, breast cancers are divided into estrogen receptor (ER)/progesterone receptor (PR)-positive, HER2-positive, or triple-negative breast cancer (TNBC) (1). TNBC patients did not respond to hormonal and HER2-targeted therapies due to the lack of ER/PR and HER2 expressions and showed the worst clinical outcome. Thus, the main treatments for TNBC patients are surgery, radiation therapy, and chemotherapy (2).

The occurrence and progression of breast cancer are associated with long-term inflammation. In previous studies, TNBC cells have been reported to express critical inflammatory genes, such as cyclooxygenase 2 and other NF-κB-regulated target genes (3, 4). Targeting inflammation by inhibiting COX-2 with non-steroidal anti-inflammatory drugs (NSAIDs) has been found to repress cancer cell growth and sensitize cancer cells to chemotherapy (5, 6). More and more studies have proposed that natural extracts of anti-inflammatory ingredients have anti-cancer activity. Among animal-derived drugs, the snail has anti-inflammatory activity (7). In addition to the snail body, the mucus produced by the snail can also be used as an anti-inflammatory treatment. Snail slime is an externally secreted mucus produced by gastropod mollusks, including giant African snail *Achatina fulica*, and can protect snail skin from dryness, ultraviolet rays, and damage (8). As a traditional medicine, snail has been employed for the treatment of asthma (9). In recent years, snail slime was further widely used in cosmetics as an anti-aging compound to preserve human skin from wrinkle and calm skin due to its inducing effect on the production of collagen (10). According to the Greek physician Hippocrates, crushed snails and sour milk were mixed and used to treat inflammation as early as 2,000 years ago (11). Furthermore, snail mucus has been demonstrated to possess the functions of inhibiting inflammation and angiogenesis (12). Since chronic inflammation is one of the causes of many types of cancer and promotes tumor progression, snail mucus with anti-inflammatory activity may also possess anti-cancer functions. Snail mucus is a mixture and contains proteoglycan, glycosaminoglycan, and glycoprotein enzyme, etc.(13). Acharan sulfate, isolated from the giant African snail *Achatina fulica*, is a kind of glycosaminoglycan with a strong affinity to target cell membrane proteins nucleolin in lung A549 cancer cells (14, 15). Nucleolin has been shown as a nuclear matrix-binding protein interacting with telomerase and is involved in the regulation of apoptosis (16, 17). These clues suggest that snail mucus may affect apoptosis through regulating nucleolin functions.

Material And Methods

Source and preparation of snail mucus

Snail mucus was collected from *Achatina fulica* and supplied by Dr. Yao-Tern Li (Hsin-Ling Tang Chinese Medicine Clinic, Chia-Yi, Taiwan). 3% NaCl was used to stimulated mucus production, and mucus was collected from the surface of live snails. The stock of snail mucus is defined as 100% (6 mg/mL). The stock solution was diluted to 30–600 μg/mL as the working solution and was filtered out the impurities with 0.45mm pore size membrane disc filters.

Cell line and cell culture

The human breast cancer MDA-MB-231cell line was purchased from American Type Culture Collection (ATCC). All of the cell lines were cultured in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% fetal bovine serum, 100 unit/mL penicillin, and 100 μg/ mL streptomycin. Cells were incubated at 37°C, 95% air and 5% CO_2 (18).

MTT assay

Breast cancer cells were seeded in a 96-well plate at $0.5x10^4$ per well. Cells were treated with various concentrations of the snail mucus for 2 days followed by incubation with 20% of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, MO, USA) for 3 hours, and then dissolved with DMSO for 30 minutes (18). The absorbance was measured at the wavelength of 570 nm by using an ELISA reader.

IncuCyte Live-cell analysis

Breast cancer cells were seeded in a 96-well plate at $0.5x10^4$ per well. Cells were incubated in IncuCyte after treatment with snail mucus and/or doxorubicin. The growth rate was measured with IncuCyte Live-Cell Analysis System.

Cell cycle analysis

Breast cancer cells were seeded in a 6-well dish at the density of $0.1x10^6$/well. After treatment with snail mucus for 48 hours, the cells were washed with PBS and collected by trypsinization. Then cells were fixed in 75% ethanol and stored at -20°C for 2 hours. Cells were centrifuged at 1000 rpm for 5 minutes to remove the supernatant and stained with 0.1mg/ mL RNase (Cat no.12091-021), 1% 100X Triton, 20 µg/mL propidium iodide in 1X PBS at 37^{o}C for 30 minutes. The sample was detected for ten thousand cells and analyzed by using Flow Cytometry.

Apoptosis analysis

Breast cancer cells were seeded in a 6-well dish at the density of $0.1x10^6$/well. Following treatment with snail mucus for 24 hours, cells were collected by trypsinization and washed with PBS. Cells were stained with an Annexin V-FITC Apoptosis Detection Kit (Bio Vision). The sample was detected for ten thousand cells and analyzed by using Flow Cytometry (BD Biosciences).

DNA laddering assay

After the treatment for 24 and 48 hours, cells were collected and centrifugated at 1600g for 5 minutes. The cell lysate was prepared with lysis buffer (1% NP-40 in 20mM EDTA, 50mM Tris-HCl at PH 7.5) and was centrifuged at 10000g for 10 min. The supernatant was added with 1% SDS and was incubated for 2 hours at 37^{o}C. Then, the supernatant was added with 5 µg/µL proteinase K followed by incubation at 37^{o}C for 2 hours and was further added with 3 M sodium acetate to precipitate genomic DNA. DNA was mixed thoroughly with 100% ethanol then placed at -80^{o}C for 1 hour. The sample was centrifuged at

12000 rpm for 20 minutes to obtain the pellet. The pellet was washed with 75% cold ethanol and air-dried at room temperature. The dried pellet was dissolved with double-distilled H_2O. DNA laddering was observed in agarose gel electrophoresis.

Western blot analysis

Cells were washed with ice-cold PBS, and lysates were collected in RIPA buffer (50 mM Tris, 150mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.1% SDS) containing protease inhibitors. Cell lysates were centrifuged at 15000 rpm for 15 minutes after sonication, and the supernatant was collected. The concentration of protein was measured by using Bradford protein assay (Bio-Rad). An equal amount of protein was separated by SDS-PAGE. After transfer to PVDF membrane (0.45 μM, GE Healthcare), the membrane was blotted with 5% milk in TBST buffer for 1 hour followed by hybridization with indicated primary antibodies and HRP-labeled secondary antibodies. The expression levels of target proteins were determined by detecting the chemoluminescence signal, which was catalyzed with ECL (18) (GE Healthcare or Millipore).

General of AFC components analysis

^{1}H-NMR spectra were recorded using a Avance Neo 400 NMR spectrometer (Bruker Instruments, Karlsruhe, Germany) with CD_3OD or D_2O as the solvent. MPLC was performed using a reverse-phase column (iLOK™ (12g, I.D. 21.4×H76 mm, C18, 460 mm × 36 mm i.d.) on a PuriFlash XS 420 series apparatus (Interchim Inc., Montlucon, France). The UV detector was set at 210, 254, and 280 nm, and the UV absorption spectra were recorded in the range of 210–400 nm.

Isolation of the active fractions

The *Achatina fulica* mucus (AFM, 45.3 mg) was separated with MPLC [C_{18} gel (460 mm × 36 mm i.d.), column (21.4×H76 mm i.d.)] using water (A) and acetonitrile (B) under gradient conditions (0–2 min, 2 % B; 2–3 min, linear gradient 2–5% B; 3–17: min, linear gradient 5–15% B; 17–32 min, linear gradient 15–100% B; 32–50 min, 100% B) as the mobile phase at a flow rate of 20.0 mL/min. The sample was dry-load onto the column and fractionated into seven fractions (AFM-1 ~ 7).

Statistical analysis

Data were presented as mean ± S.E.M. The difference between the experimental and control groups was assessed by Student's t-test and reached significance if the p-value is < 0.05.

Results

3.1 Sail mucus suppressed cell proliferation of TNBC cells

To test the inhibitory effects of snail mucus on cancer cell proliferation, MDA-MB-231 TNBC cells were treated with increasing doses of snail mucus followed by morphologic observation under a microscope. According to the toxicity of the venom of organisms to cancer cells, the fatal concentration of snake venom is about 6–12 μg (19), but the IC_{50} of cancer cell activity is about 1–5 μg (20, 21). In addition, the IC_{50} of honeybee venom on cancer cells is about 5 ng/μL (22). Therefore, based on the toxic concentration of these biological secretions to cells and the component concentration of the snail mucus masks (about 100 ppm), we adjusted the snail mucus as 60–600 μg/mL to treat breast cancer cells. We found that the cell shape was shrunken after treatment with snail mucus for three days (Fig. 1A). To further confirming the cytostatic activity of snail mucus, the proliferation of MDA-MB-231 cells in response to treatment with snail mucus was also measured by using IncuCyte live cell analysis. Our data showed that the proliferation of MDA-MB-231 cells was decreased by snail mucus, and 900 μg/mL of snail mucus almost abolished the cell growth (Fig. 1B). To further determine the effective concentration of snail mucus on cell viability inhibition, we performed an MTT assay with different doses of snail mucus (Fig. 2A). The IC_{50} of snail mucus for MDA-MB-231 cancer cell line was 210 μg/mL. In comparison with its effect on the cancer cell line, snail mucus showed less cytotoxicity in normal breast epithelial MCF-10A cells (IC_{50}: 446 μg/mL). These findings indicated a potential anti-cancer activity of snail mucus in breast cancer cells.

MDA-MB-231 breast cancer cells were treated with indicated concentrations of snail mucus for 3 days. (A) The cell morphology and number were observed under a microscope. (B) The cell numbers were counted every 12 hours in IncuCyte.

3.2 Snail mucus induced apoptosis of TNBC cells.

To elucidate the mechanism of the anti-proliferation activity of snail mucus, we first used FACS analysis with PI staining to examine its effect on cell cycle progression in MDA-MB-231 breast cancer cells. The data showed that the sub-G1 population of breast cancer cells was induced by snail mucus in a dose-dependent manner (Fig. 2B). Moreover, sub-G1 and G1 populations of breast cancer cells were also induced and inhibited, respectively, by increasing doses of snail mucus (Fig. 2B). To further assess the involvement of apoptosis in snail mucus-induced cell death, MDA-MB-231 cells were double-stained with PI and Annexin V following the treatments with 60, 300, 600 μg/mL of snail mucus for 48 hours. We found that the percentages of Annexin V/PI double-positive cells were induced in a dose-dependent manner, and 600 μg/mL snail mucus induced these populations at least 8 folds (Fig. 3A), indicating that snail mucus increased apoptosis in TNBC cells. Consistent with these results, 600 μg/mL snail mucus also induced DNA fragmentation in MDA-MB-231 cancer cells after treatment for 48 and 72 hours (Fig. 3B). These results revealed that snail mucus elicited apoptosis in cancer cells.

3.3 Snail mucus induced apoptosis via extrinsic pathway.

To further determine the mechanism of snail mucus-induced apoptosis, MDA-MB-231 cells were treated with different concentrations of snail mucus for 48 hours followed by detection of PARP and caspase 3 cleavages. Snail mucus induced PARP and caspase 3 cleavages in MDA-MB-231 cells (Fig. 4A) but did not activate p53 (Fig. 4B), suggesting the involvement of extrinsic pathway in the snail mucus-induced apoptosis. To corroborate that snail mucus induces extrinsic programmed cell death, we assessed if PARP cleavage would be overcome by inhibition of caspase 8. To this end, MDA-MB-231 cells were pretreated with caspase 8 inhibitor Ac-IETD-CHO for 2 hours followed by the treatment with 600 μg/mL snail mucus for 48 hours. The data showed that Ac-IETD-CHO suppressed snail mucus-induced PARP cleavage (Fig. 4B), suggesting that snail mucus induces cell death via an extrinsic apoptotic pathway. To further investigate the mechanism of snail mucus-induced extrinsic apoptosis, MDA-MB-231 cells were treated with snail mucus for 3 days followed by detection of Fas signaling in western blot. It was found that FADD, an adaptor for death receptor Fas, was induced by snail mucus in a dose-dependent manner (Fig. 5A). Furthermore, phosphorylation of p38, a kinase downstream of the Fas death receptor to induce cell apoptosis (23), was upregulated by snail mucus. It suggested that the Fas signaling pathway is involved in the snail mucus-induced extrinsic apoptotic pathway. We also found that snail mucus decreased the expression of nucleolin (Figs. 5A and 5B), which was found to bind and inhibit Fas (24). These results suggest downregulation of nucleolin may contribute to the snail mucus-induced Fas-dependent extrinsic apoptosis pathway.

3.4 Snail mucus sensitizes TNBC cells to doxorubicin

To further confirm whether snail mucus synergizes the anti-cancer activity of chemotherapy, MDA-MB-231 cells were co-treated with snail mucus and chemotherapeutic agent doxorubicin. By analyzing cell viability, we found that even low concentration (60 μg/mL) of snail mucus didn't affect cell viability (Fig. 1B) but increased sensitivity of breast cancer cells to doxorubicin (Fig. 6). The IC_{50} of doxorubicin for MDA-MB-231 cells was 0.1 μM. However, combination treatment with 30 μg/mL and 60 μg/mL snail mucus shifted IC_{50} of doxorubicin from 0.26 μM to 0.05 μM and 0.04 μM, respectively. It indicated that a low dose of snail mucus could function as a chemosensitizer in TNBC. This synergistic effect may decrease chemo drug-induced side effect and chemotherapy resistance.

3.5 Isolation of the active fractions from the mucus of the snail of Achatina fulica powder

The *Achatina fulica* mucus (AFM) was introduced in MPLC with gradient elution by a dry loading and was fractionated into seven fractions (AFM-1 ~ 7) (Fig. 7). To identify the active fractions of AFM, MDA-MB-231 cells were treated with 50 μg/mL of each fractioned product, respectively. We found that only AFM-5, 6, and 7 significantly decreased the cell viability in MTT assay (Fig. 8). Thus, active fractions (AFM-5 ~ 7) were further analyzed using ^{1}H-NMR spectroscopy (Fig. 9). ^{1}H NMR spectra of peptide compounds in AFM-5 (Fig. 9A) and AFM-6 (Fig. 9B) are characterized by chemical shifts. In AFM-7 (Fig. 9C), ^{1}H-NMR

shifts of characteristic signals is a fatty acid. Among them, AFM-5 showed the highest inhibitory activity on cell viability; the IC_{50} of AFM-5 was 12.7 μg/mL (Fig. 10).

Discussion

Snail mucus has a moisturizing and anti-aging function, and it has been widely used in skin care products in recent years. According to our results, moreover, snail mucus effectively inhibits the viability of TNBC cells through induction of extrinsic apoptosis. The effective component of snail mucus may be developed as a potential therapeutic agent or chemosensitizer for TNBC patients.

Programmed cell death can be divided into extrinsic and intrinsic pathways. The apoptotic signaling transmitted via death receptors is called the extrinsic apoptosis pathway. Intrinsic apoptosis is also called mitochondrial apoptosis, relying on the cytochrome C release from the intermembrane space of mitochondria to activate caspase 9 in the cytoplasm. The activated caspase 9 further promotes the activation of downstream caspase and leads to apoptosis (25). In our study, not only death receptor Fas but also Fas-associated death domain (FADD) expressions were induced by snail mucus in a dose-dependent manner, indicating that snail mucus may promote the activation of Fas signaling to lead to apoptosis.

Nucleolin-Fas complexes were found in B-cell lymphoma cells but not in normal B-lymphocytes (24), suggesting Fas-mediated cell death in B lymphoma was inhibited due to the block of the Fas/Fas ligand interaction by nucleolin. In addition, nucleolin is phosphorylated by Casein kinase 2 (CK2) and interacts with Hdm2, which is an E3 ubiquitin ligase of p53, and therefore decreased p53 expression (26). These findings suggest that nucleolin may regulate both extrinsic and intrinsic apoptosis pathways. However, our study showed that snail mucus did not induce p53 activity or protein expression, implying the preferential effect of snail mucus on extrinsic apoptosis pathway. Indeed, our data also showed that Fas and FADD expressions were increased, but nucleolin expression was suppressed by snail mucus. Acharan sulfate, a glycosaminoglycan, is the major constituent of snail mucus (27). This glycosaminoglycan not only inhibits angiogenesis but also binds to plasma membrane nucleolin and suppresses the nucleolin-inhibited cell apoptosis (28), supporting our finding that snail mucus induces extrinsic apoptosis pathway through activation of Fas signaling via suppressing nucleolin (as illustrated in Fig. 11). Compared to normal cells, cancer cells abundantly express nucleolin, raising the possible reason that snail mucus showed more growth inhibition effect on cancer cells than on normal cells.

Recently, target therapies against HER2 or hormone receptors have been developed and used for breast cancers and showed a good prognosis by early treatment. However, there is no specific target for TNBC patients whose treatments still need to rely on chemotherapy. In colorectal cancer, Fas was considered to be induced by chemotherapy drug oxaliplatin and to be involved in oxaliplatin-induced cell apoptosis (29). However, MMP7 is related to the resistance of colorectal cancer to oxaliplatin, and inhibition of MMP7 was suggested to re-sensitize colorectal cancer cells to oxaliplatin by increasing the expression of Fas. In addition to colorectal cancer, it was already known that activation of Fas determines

chemosensitivity in many solid tumor types, such as breast cancer, small cell lung cancer, and renal cell carcinoma (30). Our data also showed that snail mucus could increase the expression and activity of Fas. Therefore, induction of Fas activity by snail mucus may be a strategy to sensitize breast cancer cells to chemotherapeutic drugs.

In our research, we found that snail mucus has the effect of cytotoxic for cancer cells. Although, it is known that acharan sulfate, which is extracted from the large African snail, induces lung cancer cell apoptosis (14). Although we provided the possible peptide fractions (AFM-5 and AFM-6), which have the potential to be used as therapeutic agents, the exact composition and compound structures of the active fractions will be further analyzed to facilitate the development of anti-cancer drugs.

Conclusion

In this study, we demonstrated the inducing effect of snail mucus on the extrinsic apoptosis pathway and chemosensitivity in the MDA-MB-231 TNBC line. Mechanistically, treatment with snail mucus induced Fas signaling via suppressing nucleolin expression (Fig. 11). Our data suggest that snail mucus may have the potential to be developed as an anti-cancer agent or chemosensitizer for TNBC.

Abbreviations

American Type Culture Collection: ATCC

Achatina fulica mucus: AFM

Estrogen receptor: ER

Non-steroidal anti-inflammatory drugs: NSAIDs

Progesterone receptor: PR

Triple-negative breast cancers: TNBCs

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The data in this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

Funding

Ministry of Science Technology, Taiwan (MOST-109-2314-B-039-015); China Medical University Hospital (CMU106-ASIA-18); China Medical University Hsin-Chu Hospital (CMUHCH DMR-110-003, CMUHCH DMR-110-006).

Author contributions

C.Y.H proposed the study concept and designed the study experiments. D.W.H acquired and interpreted data and prepared the manuscript; B.R.C acquired and analyzed the data. C.C.Y acquired and analyzed the data. C.H.Y supervised the quality of data and algorithms. T.N supervised the quality of data and algorithms. Y.S.C supervised the quality of data and algorithms. C.Y.T supervised the quality of data and algorithms. W.C.C performed the HPLC analysis. Y.C.W edited the manuscript. C.H.T edited the manuscript. H.C.H performed the HPLC analysis and edited the manuscript. W.C.H: proposed the study concept, designed the study experiments, and reviewed the manuscript. T.S.L proposed the study concept and edited the manuscript.

Acknowledgement

This work was supported by grants from the Ministry of Science Technology, Taiwan (MOST-109-2314-B-039-015), China Medical University Hospital (CMU106-ASIA-18) and China Medical University Hsin-Chu Hospital (CMUHCH DMR-110-003, CMUHCH DMR-110-006). This work was also financially supported by the “Drug Development Center, China Medical University” from The Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) in Taiwan. The grantee had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

References

1. Szajnik M, Szczepanski MJ, Czystowska M, Elishaev E, Mandapathil M, Nowak-Markwitz E, et al. TLR4 signaling induced by lipopolysaccharide or paclitaxel regulates tumor survival and chemoresistance in ovarian cancer. Oncogene. 2009;28(49):4353-63.
2. Yagata H, Kajiura Y, Yamauchi H. Current strategy for triple-negative breast cancer: appropriate combination of surgery, radiation, and chemotherapy. Breast Cancer. 2011;18(3):165-73.
3. Denkert C, Kobel M, Berger S, Siegert A, Leclere A, Trefzer U, et al. Expression of cyclooxygenase 2 in human malignant melanoma. Cancer Res. 2001;61(1):303-8.

4. Eckert LB, Repasky GA, Ulku AS, McFall A, Zhou H, Sartor CI, et al. Involvement of Ras activation in human breast cancer cell signaling, invasion, and anoikis. Cancer Res. 2004;64(13):4585-92.
5. Basu GD, Pathangey LB, Tinder TL, Gendler SJ, Mukherjee P. Mechanisms underlying the growth inhibitory effects of the cyclo-oxygenase-2 inhibitor celecoxib in human breast cancer cells. Breast Cancer Res. 2005;7(4):R422-35.
6. Hilovska L, Jendzelovsky R, Fedorocko P. Potency of non-steroidal anti-inflammatory drugs in chemotherapy. Mol Clin Oncol. 2015;3(1):3-12.
7. Yue GG, Lee JK, Chan BC, Kwok HF, Hoi SW, Sze DM, et al. An innovative anti-cancer Chinese herbal formula exhibited multi-targeted efficacies in metastatic breast cancer mouse model. Chin Med. 2018;13:64.
8. Kuanpradit C, Jaisin Y, Jungudomjaroen S, Akter Mitu S, Puttikamonkul S, Sobhon P, et al. Attenuation of UV-B exposure-induced inflammation by abalone hypobranchial gland and gill extracts. Int J Mol Med. 2017;39(5):1083-90.
9. van Ree R, Antonicelli L, Akkerdaas JH, Pajno GB, Barberio G, Corbetta L, et al. Asthma after consumption of snails in house-dust-mite-allergic patients: a case of IgE cross-reactivity. Allergy. 1996;51(6):387-93.
10. Quay ER, Chang YC, Graber E. Evidence for Anti-Aging South Korean Cosmeceuticals. J Drugs Dermatol. 2017;16(4):358-63.
11. Bonnemain B. Helix and Drugs: Snails for Western Health Care From Antiquity to the Present. Evid Based Complement Alternat Med. 2005;2(1):25-8.
12. Ghosh AK, Hirasawa N, Lee YS, Kim YS, Shin KH, Ryu N, et al. Inhibition by acharan sulphate of angiogenesis in experimental inflammation models. Br J Pharmacol. 2002;137(4):441-8.
13. Cilia G, Fratini F. Antimicrobial properties of terrestrial snail and slug mucus. J Complement Integr Med. 2018;15(3):2017-0168.
14. Joo EJ, Yang H, Park Y, Park NY, Toida T, Linhardt RJ, et al. Induction of nucleolin translocation by acharan sulfate in A549 human lung adenocarcinoma. J Cell Biochem. 2010;110(5):1272-8.
15. Ginisty H, Sicard H, Roger B, Bouvet P. Structure and functions of nucleolin. J Cell Sci. 1999;112 (Pt 6):761-72.
16. Gotzmann J, Eger A, Meissner M, Grimm R, Gerner C, Sauermann G, et al. Two-dimensional electrophoresis reveals a nuclear matrix-associated nucleolin complex of basic isoelectric point. Electrophoresis. 1997;18(14):2645-53.
17. Khurts S, Masutomi K, Delgermaa L, Arai K, Oishi N, Mizuno H, et al. Nucleolin interacts with telomerase. J Biol Chem. 2004;279(49):51508-15.
18. Lee SC, Tsai CC, Yao CH, Hsu YM, Chen YS, Wu MC. Effect of arecoline on regeneration of injured peripheral nerves. Am J Chin Med. 2013;41(4):865-85.
19. Ghazala Parveen1 FKM, Hussain Ali, Tanveer Ibrahim and Rahim Shah. Determination of Lethal Dose (LD50) of Venom of four Different Poisonous Snakes found in Pakistan. Biochemistry &

Molecular Biology Journal 2017;3:No.3:18.

20. Kollipara PS, Won do H, Hwang CJ, Jung YY, Yoon HS, Park MH, et al. Enhanced Anti-Cancer Effect of Snake Venom Activated NK Cells on Lung Cancer Cells by Inactivation of NF-kappaB. Biomol Ther (Seoul). 2014;22(2):106-13.
21. Park MH, Jo M, Won D, Song HS, Han SB, Song MJ, et al. Snake venom toxin from Vipera lebetina turanica induces apoptosis of colon cancer cells via upregulation of ROS- and JNK-mediated death receptor expression. BMC Cancer. 2012;12:228.
22. Duffy C, Sorolla A, Wang E, Golden E, Woodward E, Davern K, et al. Honeybee venom and melittin suppress growth factor receptor activation in HER2-enriched and triple-negative breast cancer. NPJ Precis Oncol. 2020;4:24.
23. Farley N, Pedraza-Alva G, Serrano-Gomez D, Nagaleekar V, Aronshtam A, Krahl T, et al. p38 mitogen-activated protein kinase mediates the Fas-induced mitochondrial death pathway in CD8+ T cells. Mol Cell Biol. 2006;26(6):2118-29.
24. Wise JF, Berkova Z, Mathur R, Zhu H, Braun FK, Tao RH, et al. Nucleolin inhibits Fas ligand binding and suppresses Fas-mediated apoptosis in vivo via a surface nucleolin-Fas complex. Blood. 2013;121(23):4729-39.
25. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007;35(4):495-516.
26. Xiao S, Caglar E, Maldonado P, Das D, Nadeem Z, Chi A, et al. Induced expression of nucleolin phosphorylation-deficient mutant confers dominant-negative effect on cell proliferation. PLoS One. 2014;9(10):e109858.
27. Vieira TC, Costa-Filho A, Salgado NC, Allodi S, Valente AP, Nasciutti LE, et al. Acharan sulfate, the new glycosaminoglycan from Achatina fulica Bowdich 1822. Structural heterogeneity, metabolic labeling and localization in the body, mucus and the organic shell matrix. Eur J Biochem. 2004;271(4):845-54.
28. Joo EJ, ten Dam GB, van Kuppevelt TH, Toida T, Linhardt RJ, Kim YS. Nucleolin: acharan sulfate-binding protein on the surface of cancer cells. Glycobiology. 2005;15(1):1-9.
29. Almendro V, Ametller E, Garcia-Recio S, Collazo O, Casas I, Auge JM, et al. The role of MMP7 and its cross-talk with the FAS/FASL system during the acquisition of chemoresistance to oxaliplatin. PLoS One. 2009;4(3):e4728.
30. Fulda S, Los M, Friesen C, Debatin KM. Chemosensitivity of solid tumor cells in vitro is related to activation of the CD95 system. Int J Cancer. 1998;76(1):105-14.

Figures

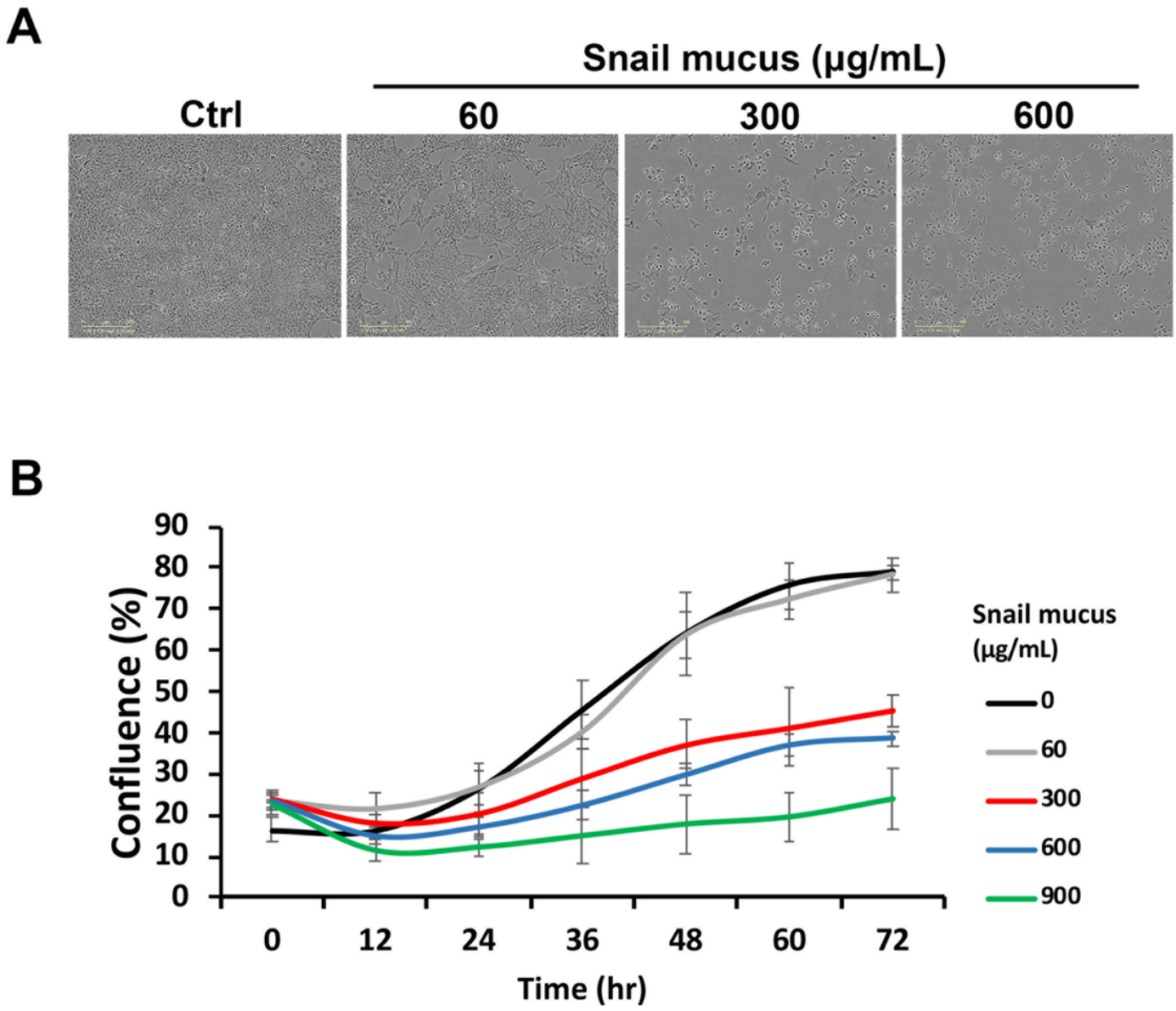

Figure 1

Snail mucus suppressed the cell proliferation of breast cancer cells. MDA-MB-231 breast cancer cells were treated with indicated concentrations of snail mucus for 3 days. (A) The cell morphology and number were observed under a microscope. (B) The cell numbers were counted every 12 hours in IncuCyte.

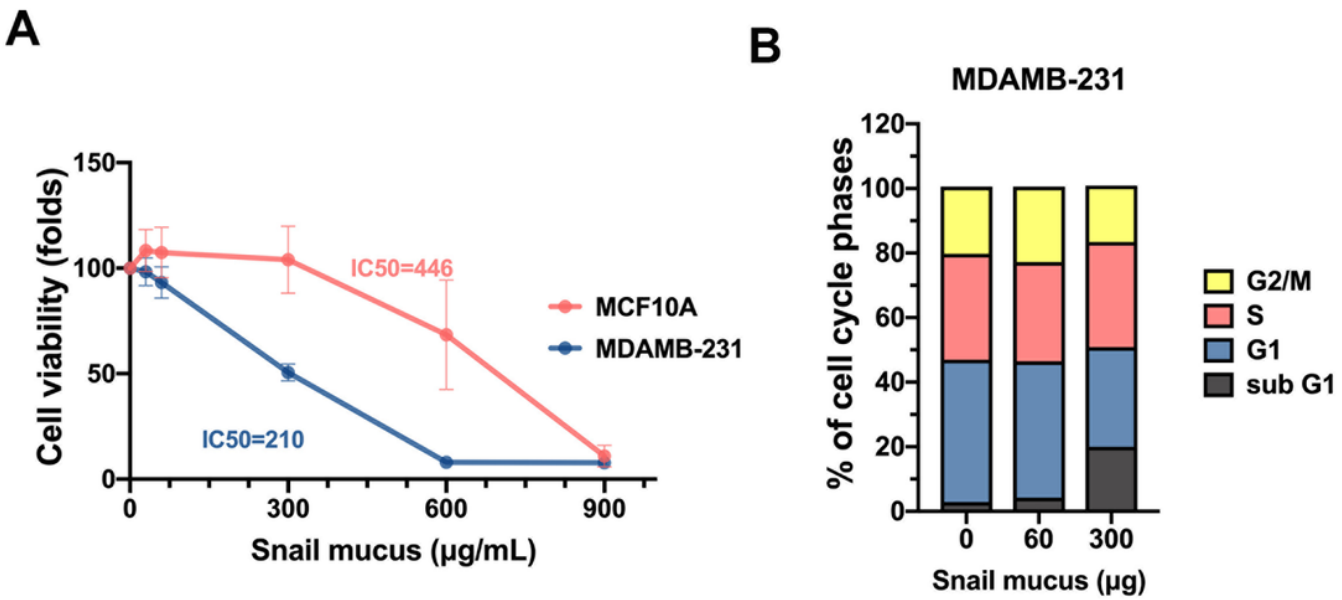

Figure 2

Snail mucus induced sub-G1 population of breast cancer cells. (A) The indicated cell lines were treated with different concentrations of snail mucus (0, 30, 60, 300, 600, or 900 μg/mL), and their viabilities were measured by using MTT assays. (B) MDA-MB-231 cell line was treated with 0, 60, and 300 μg/mL of snail mucus for 48 hours followed by PI staining. The distribution of the cell cycle was determined by using flow cytometry.

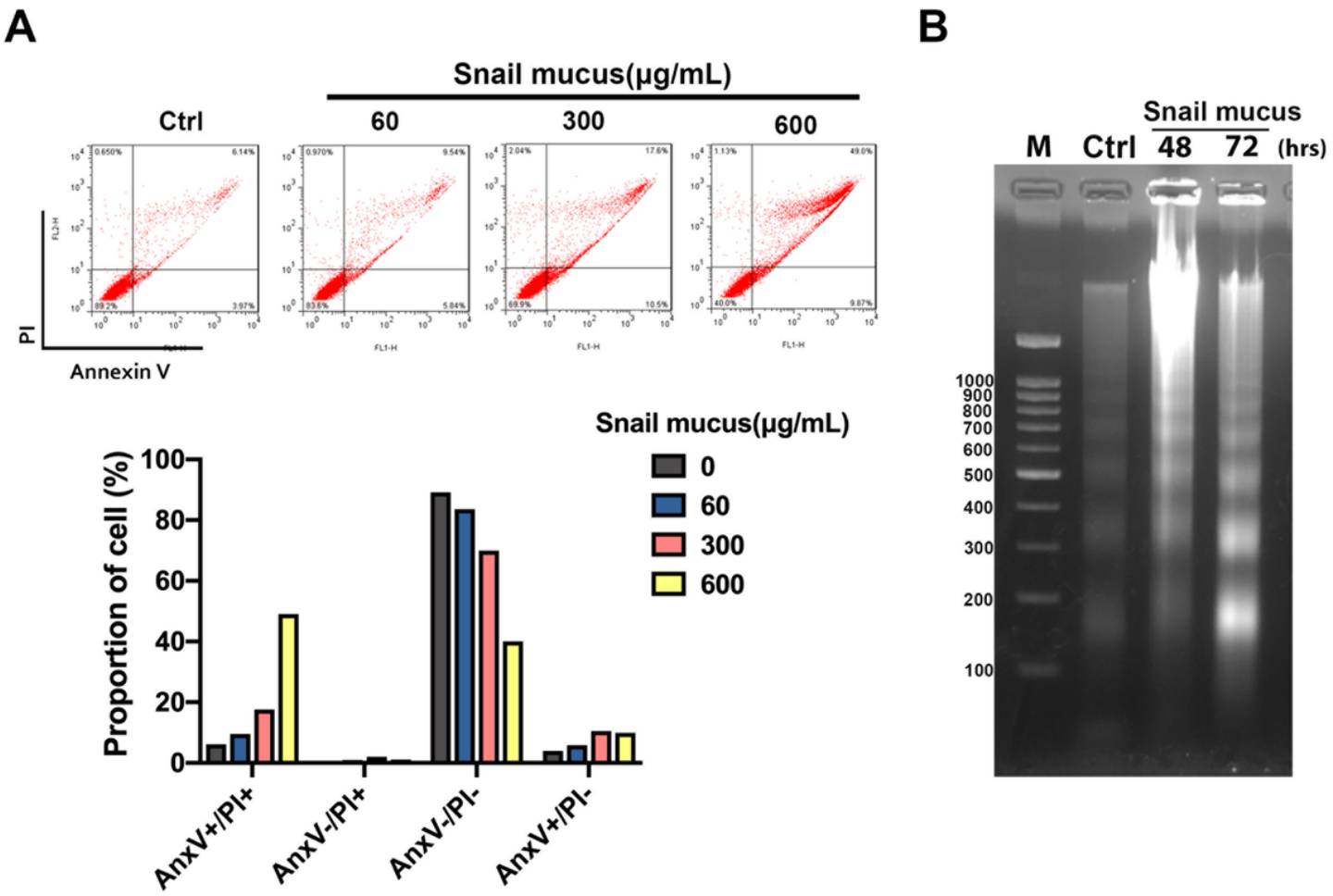

Figure 3

Snail mucus induced cell apoptosis of breast cancer cells. (A) MDA-MB-231 cells treated with 60, 300, and 600 μg/mL of snail mucus for 48 hours were stained with Annexin-V and propidium iodide. Ten thousand cells were collected and analyzed by flow cytometry. (B) DNA fragments were harvested from cancer cells treated with 600 μg/mL snail mucus for 48 and 72 hours and were analyzed in a 1.5% agarose gel and visualized with ethidium bromide staining under ultraviolet light. M: marker

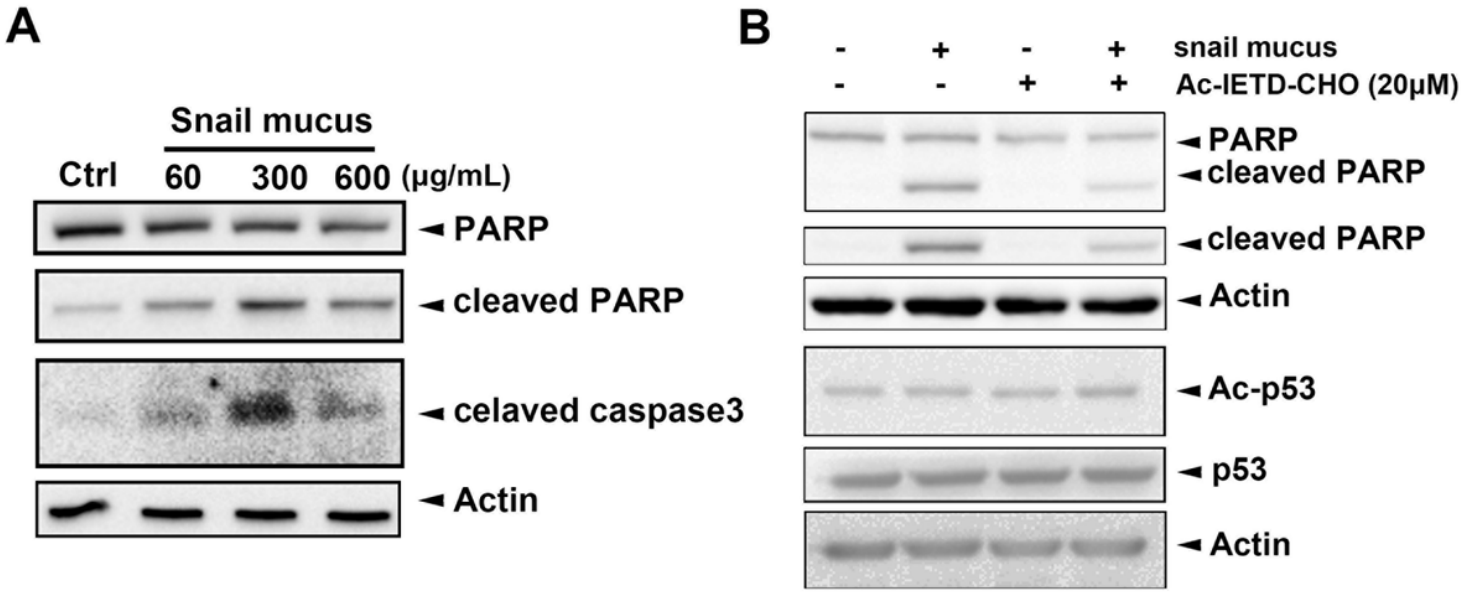

Figure 4

The expressions of the apoptosis-related gene were induced by snail mucus via an extrinsic pathway. (A) MDA-MB-231 cells were treated with different concentrations of snail mucus for 48hours. (B) MDA-MB-231 cells were pretreated with 20 μM Ac-IETD-CHO (a caspase 8 inhibitor) for 2 hours followed by the treatment with 600 μg/mL snail mucus for 48hours. Total protein was harvested and subjected to Western blot analysis with indicated antibodies. Actin served as a loading control.

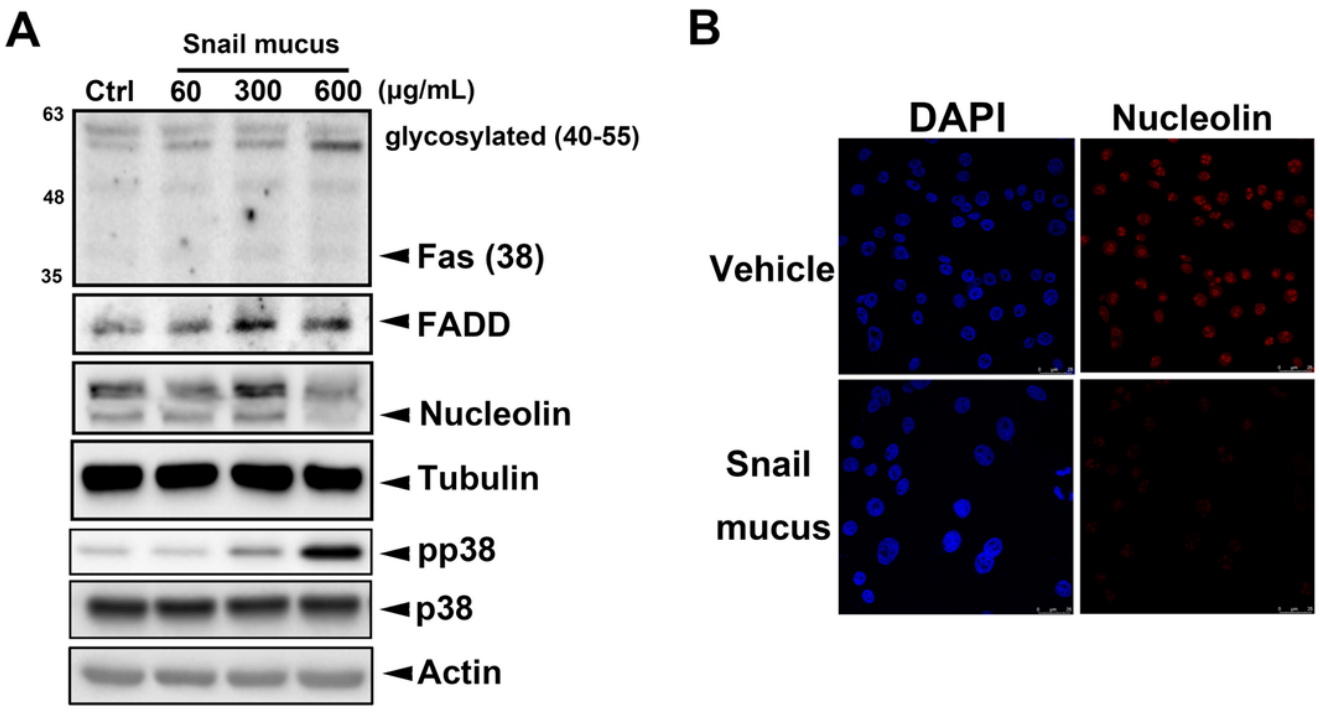

Figure 5

Activation of Fas death receptor was induced by snail mucus in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with snail mucus at indicated concentrations, and total protein was harvested for Western blot analysis with indicated antibodies. Actin served as a loading control. (B) MDA-MB-231 cells were treated with snail mucus and stained with an anti-nucleolin antibody. The images were shown by confocal microscopy.

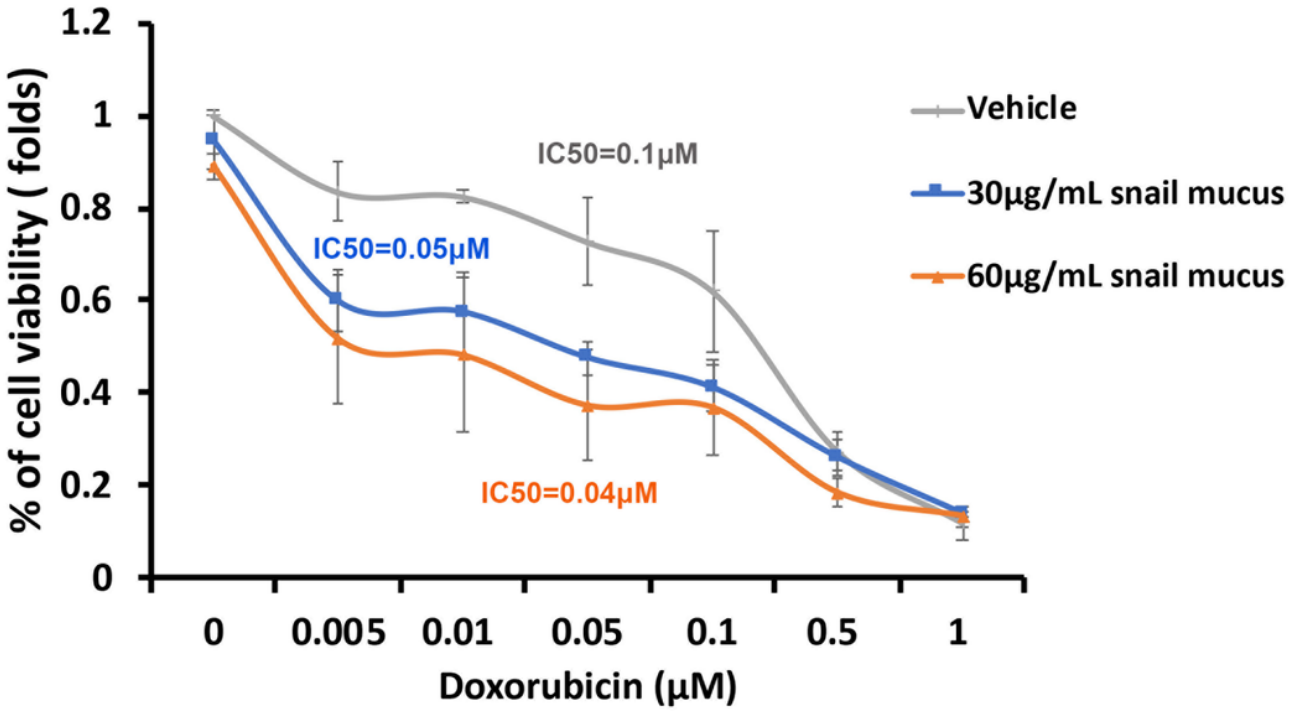

Figure 6

Snail mucus synergizes the anti-cancer activity of chemotherapy in MDA-MB-231 cells. MDA-MB-231 cells were treated with 0.005, 0.01, 0.05, 0.1, 0.5, and 1 μM of doxorubicin or the combination treatment with 30 and 60 μg/mL of snail mucus plus doxorubicin dose for 3 days respectively. The cell viability was measured by using an MTT assay.

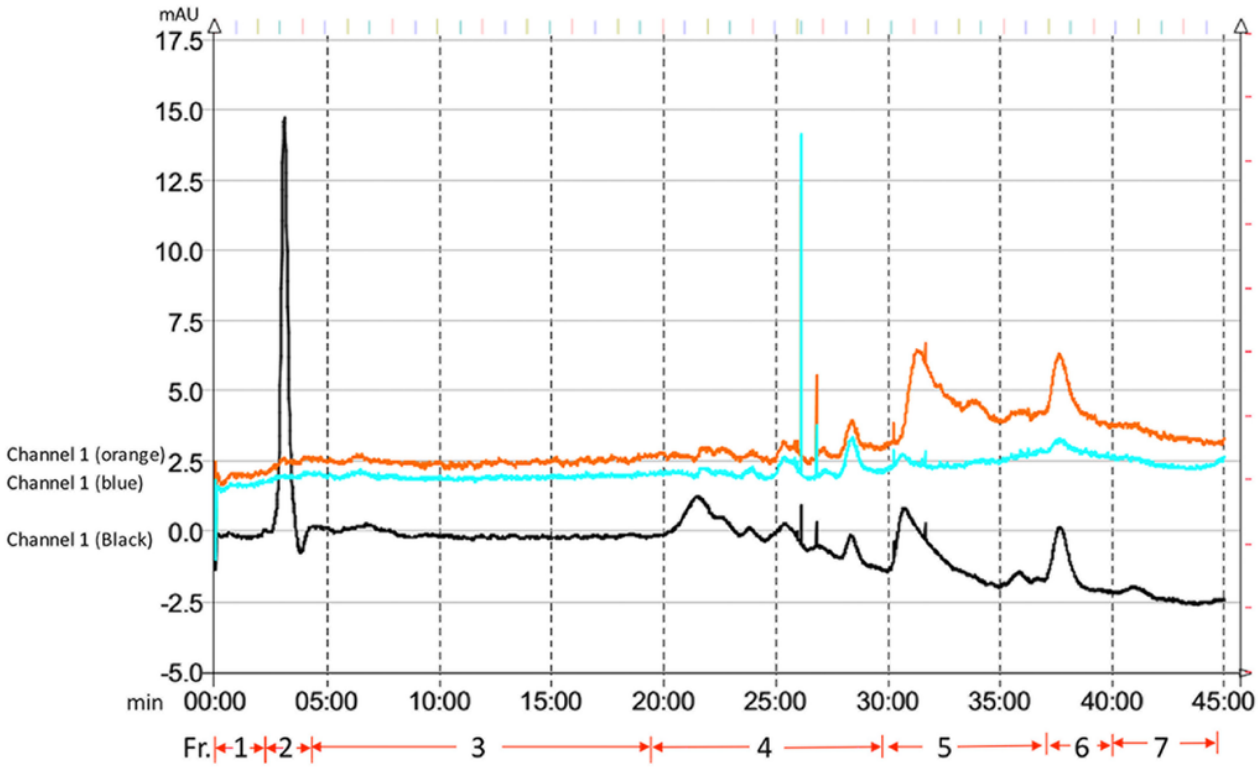

Figure 7

MPLC-UV chromatogram of AFM. Wavelengths: channel 1: 210 nm, channel 2: 280 nm, and channel 3: 254 nm.

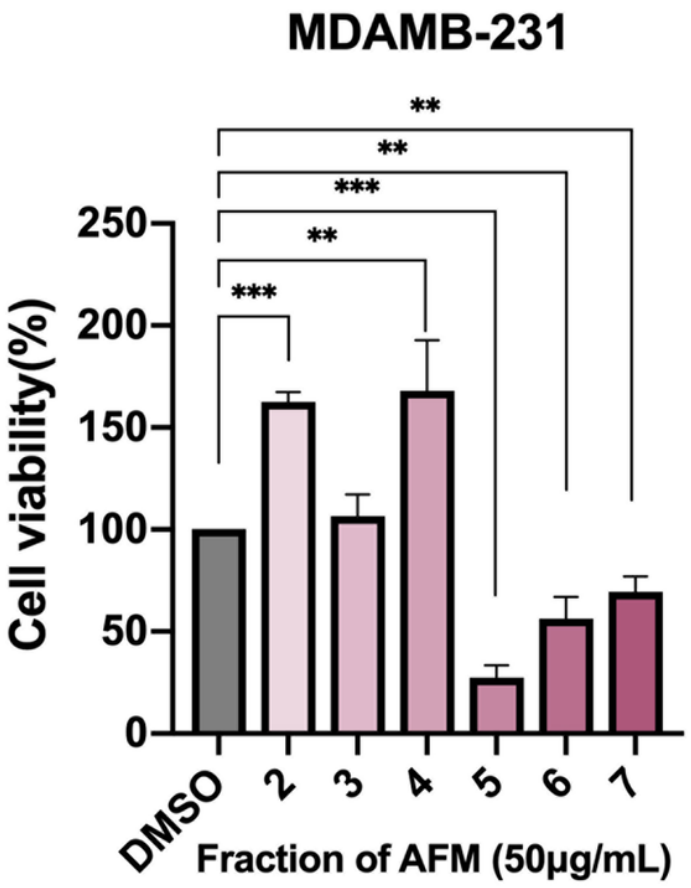

Figure 8

The cytotoxic effects of the different fractions on the viability of MDA-MB-231 cells. MDA-MB-231 cells were treated with 50 μg/mL of the different fractioned products of AFM for 48 h, and their viabilities were measured by using MTT assays.

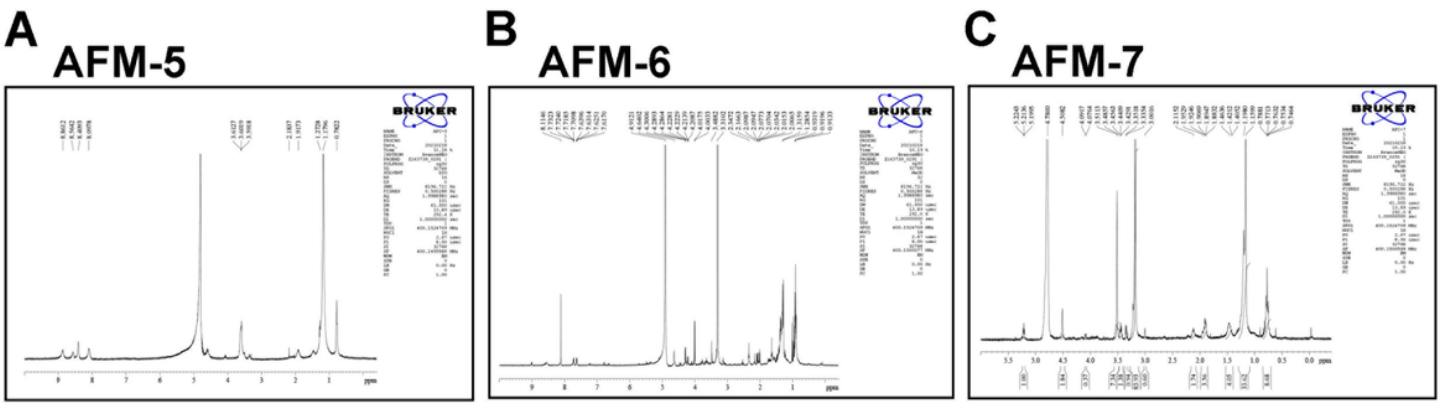

Figure 9

Nuclear magnetic resonance (NMR) analysis of the AFM fraction 5, 6, and 7. (A) 1H-NMR spectrum of AFM-5 (D2O, 400 MHz). (B) 1H-NMR spectrum of AMF-6 (CD3OD, 400 MHz). (C) 1H-NMR spectrum of AMF-7 (CD3OD, 400 MHz).

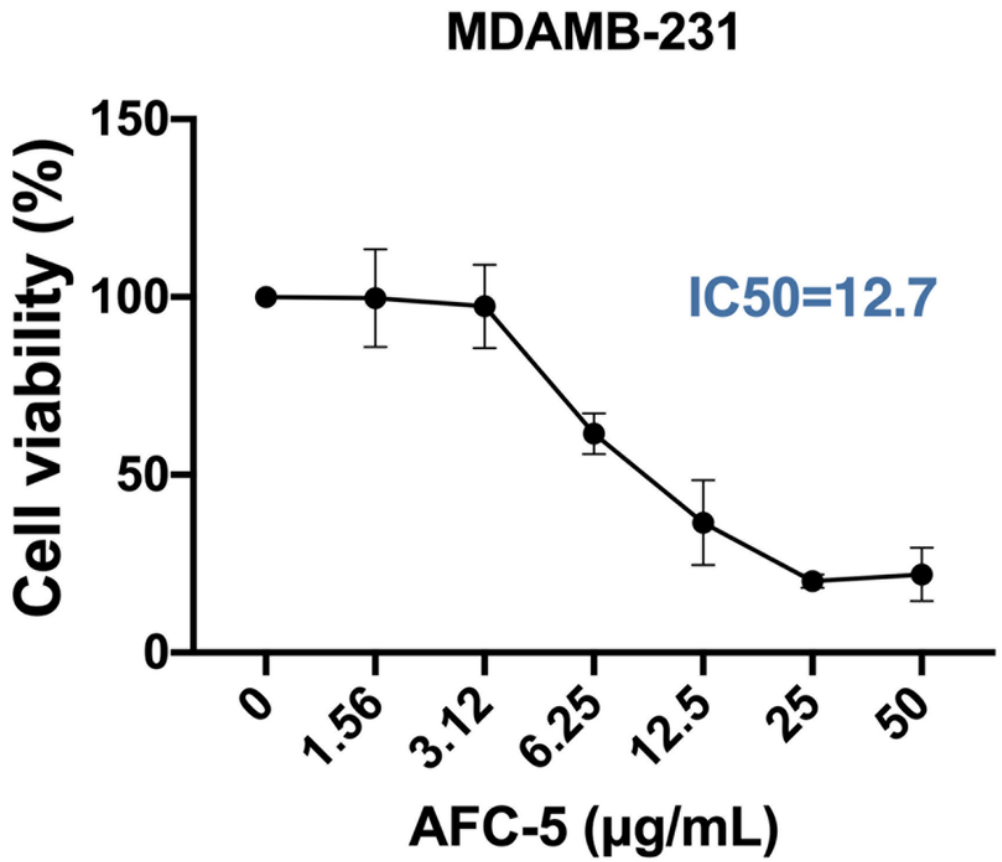

Figure 10

The IC50 of breast cancer cells in response to AFM-5. MDA-MB-231cells were treated with different concentrations of snail mucus (0, 1.56, 3.12, 6.25, 12.5, 25 and 50 μg/mL) for 48 h, and their viabilities were measured by using MTT assays.

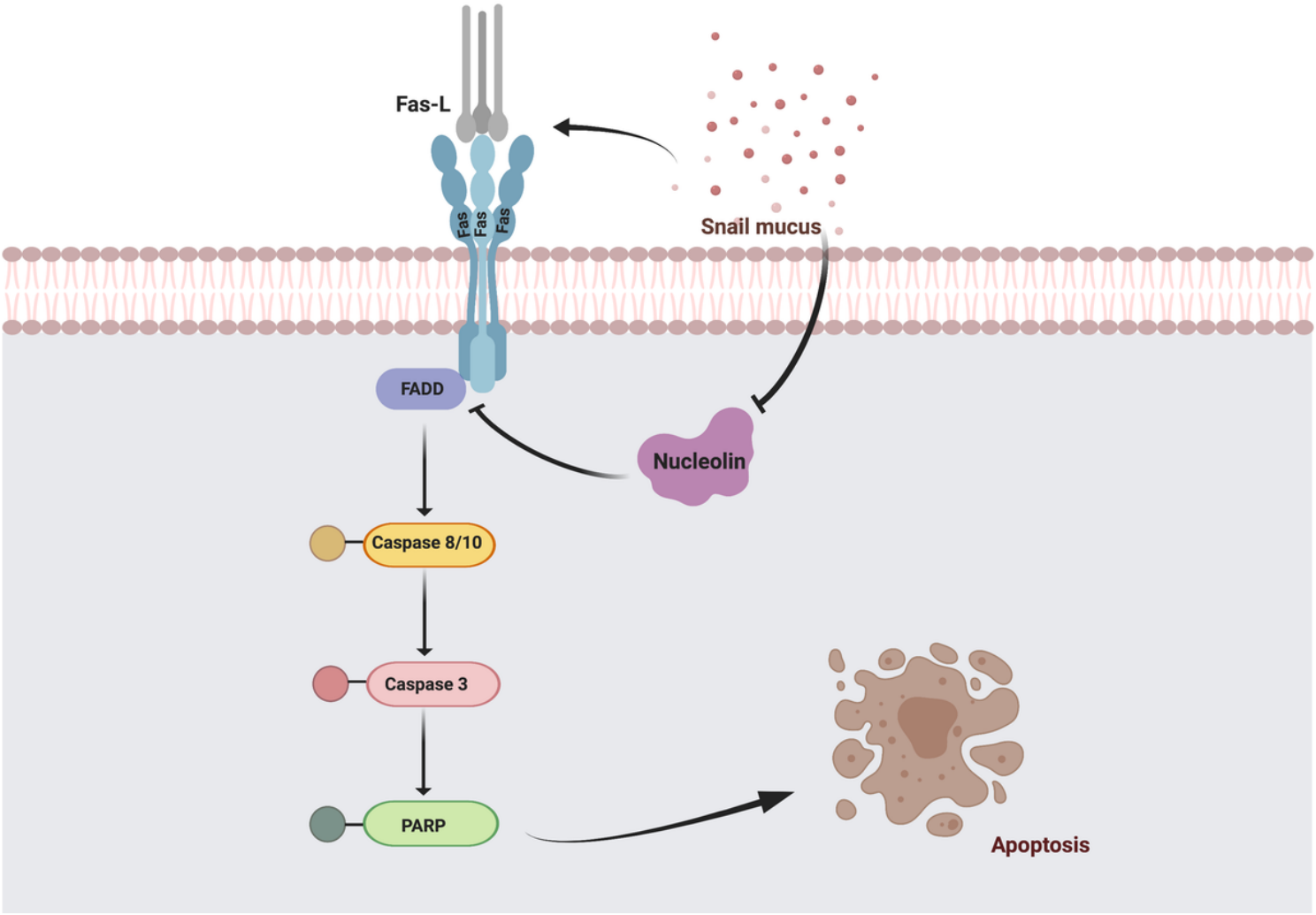

Figure 11

The hypothetic model of this study Snail mucus inhibits triple-negative breast cancer cell growth by reducing nucleolin expression and increasing Fas-mediated apoptosis.