BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 31(1), 68-72 (2023)

MS-5, a Naphthalene Derivative, Induces Apoptosis in Human Pancreatic Cancer BxPC-3 Cells by Modulating Reactive Oxygen Species

Suman Giri[1,†], Gyu Hwan Park[2,†], Joon-Seok Choi[1], Eunsook Ma[1], Kyung-Soo Chun[3,*] and Sang Hoon Joo[1,*]

[1]*Department of Pharmacy, Daegu Catholic University, Gyeongsan 38430,*
[2]*College of Pharmacy and Research Institute of Pharmaceutical Sciences, Kyungpook National University, Daegu 41566,*
[3]*College of Pharmacy, Keimyung University, Daegu 42601, Republic of Korea*

Abstract

Pancreatic cancer is one of the most fatal cancers with a poor prognosis. Standard chemotherapies have proven largely ineffective because of their toxicity and the development of resistance. Therefore, there is an urgent need to develop novel therapies. In this study, we investigated the antitumor activity of MS-5, a naphthalene derivative, on BxPC-3, a human pancreatic cancer cell line. We observed that MS-5 was cytotoxic to BxPC-3 cells, as well as inhibited the growth of cells in a concentration- and time- dependent manner. Flow cytometry analysis revealed that the percentage of annexin V-positive cells increased after MS-5 treatment. We also observed cleavage of caspases and poly (ADP-ribose) polymerase, and downregulation of Bcl-xL protein. Flow cytometry analysis of intracellular levels of reactive oxygen species (ROS) and mitochondrial superoxide suggested that MS-5 induced the generation of mitochondrial superoxide while lowering the overall intracellular ROS levels. Thus, MS-5 may be potential candidate for pancreatic cancer treatment.

Key Words: Reactive oxygen species, Pancreatic cancer, Apoptosis

INTRODUCTION

Pancreatic cancer is one of the most lethal cancers with a poor prognosis. While the treatment of pancreatic cancer has not improved substantially, its incidence in the United States has increased by approximately more than ~2% annually from 2000 to 2017 (Ali *et al*., 2021). Estimates suggest that by 2030, pancreatic cancer will be the second leading cause of cancer mortality (Rahib *et al*., 2014). The use of chemotherapeutics, such as 5-fluorouracil, gemcitabine, and oxaliplatin, is limited for treating older patients and those with poor health status owing to their high toxicity (Orth *et al*., 2019). Moreover, the development of resistance reduces the effectiveness of these chemotherapeutics (Gillen *et al*., 2010). Thus, novel and promising therapeutic agents for treating pancreatic cancer must be developed urgently.

Reactive oxygen species (ROS) play versatile roles in cancer biology (Liou and Storz, 2010) and the carcinogenesis of pancreatic cancer (Durand and Storz, 2017). Cancer cells can proliferate as a consequence of the mitogenic function of ROS (Torres and Forman, 2003). ROS mediate various cellular functions, such as angiogenesis (Ushio-Fukai and Nakamura, 2008) and metastasis (Nishikawa, 2008), thereby aiding the survival of cancer cells. Therefore, utilizing ROS as a putative target for chemotherapy is a viable approach. Recently, we reported that MS-5 (Fig. 1A), a naphthalene derivative, induces apoptosis in Caov-3, a human ovarian cancer cell line, by modulating ROS generation (Ma *et al*., 2019). In this study, we analyzed the cytotoxic effects of MS-5 on the human pancreatic cancer cell line BxPC-3.

MATERIALS AND METHODS

Materials

Antibodies against cleaved caspases-3, -7, -8, -9, poly

Open Access **https://doi.org/10.4062/biomolther.2022.127**

Received Sep 30, 2022 **Revised** Oct 7, 2022 **Accepted** Oct 17, 2022
Published Online Nov 16, 2022

***Corresponding Authors**
E-mail: sjoo@cu.ac.kr (Joo SH), chunks@kmu.ac.kr (Chun KS)
Tel: +82-53-850-3614 (Joo SH), +82-53-580-6647 (Chun KS)
Fax: +82-53-359-6729 (Joo SH), +82-53-580-6645 (Chun KS)
[†]The first two authors contributed equally to this work.

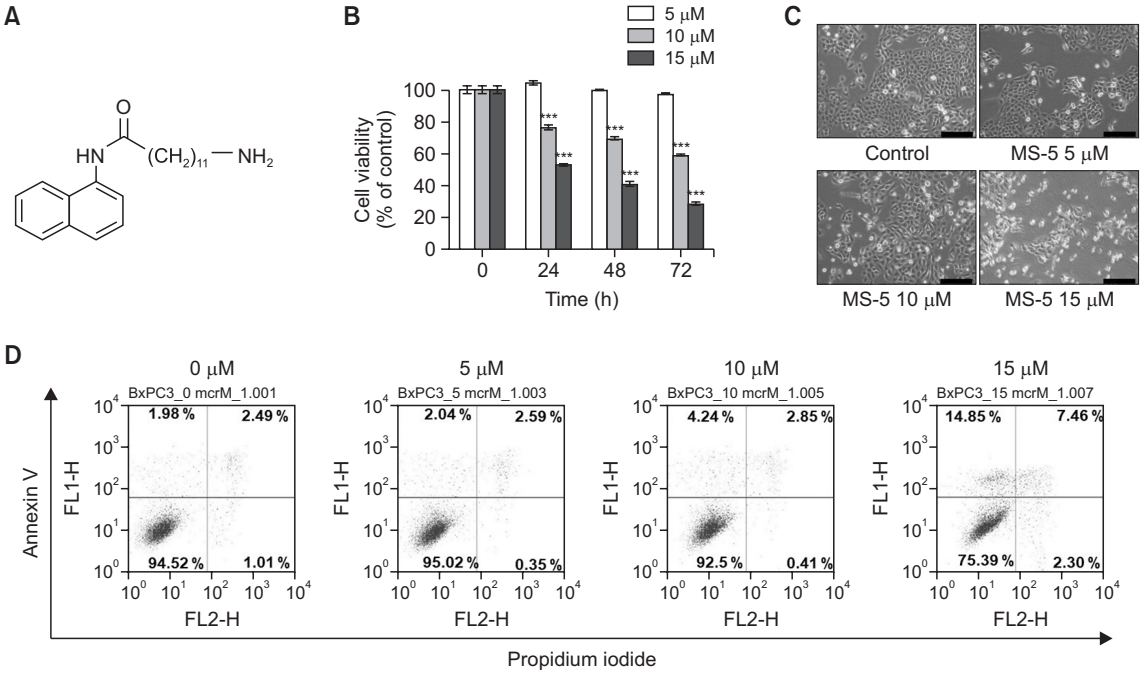

Fig. 1. Cytotoxicity of MS-5 in human pancreatic cancer BxPC-3 cells. (A) Chemical structure of MS-5. (B) BxPC-3 cell viability after MS-5 treatment (5, 10, or 15 μM) for 24, 48, and 72 h. ***p< 0.001 compared to control. (C) Microscopic morphology of BxPC-3 cells after MS-5 treatment of (0, 5, 10, or 15 μM) for 24 h (scale bar=200 μm). (D) Flow cytometry analysis of BxPC-3 cells after MS-5 treatment for 24 h after staining with annexin V and propidium iodide.

(ADP-ribose) polymerase (PARP), Bak, Bax, Bcl-xL, superoxide dismutase 1 (SOD1), heme oxygenase-1 (HO-1) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against Bcl-2, cyclin A, cyclin E, cyclin dependent kinase 2 (CDK2), p27, and SOD2 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibodies specific to mice and rabbits were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The antibody against β-actin was from Sigma-Aldrich (St. Louis, MO, USA). MS-5 was prepared as previously described (Ma *et al*., 2019).

Cell culture

Human pancreatic adenocarcinoma BxPC-3 cells were acquired from American Type Culture Collection (Manassas, VA, USA). Cultured cells were maintained in Hyclone RPMI-1640 medium (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin (GE Healthcare Life Sciences) at 37°C in a humidified incubator with 5% CO_2.

Cell viability assay

Quantimax WST kit (BioMax, Seoul, Korea), based on the water-soluble tetrazolium-1 assay, was used to measure cell viability following the manufacturer's instructions. Cells were seeded in a 48-well plate (1.5×10^4/well), and incubated for 24 h before treatment with MS-5 in varying concentrations. Dimethylsulfoxide (0.1% final concentration) was used as the vehicle, and all experiments were performed in a quadruplicate. Cell viability was compared with that of the vehicle-only control.

Annexin V staining and cell cycle analysis

The FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) was used for Annexin V staining following the manufacturer's instruction. The fluorescence intensity was measured using a BD FACSCalibur flow cytometer (BD Biosciences). Cells were stained with propidium iodide solution (50 μg/mL propidium iodide and 100 μg/mL of RNase A) in filtered phosphate buffered solution to analyze the cell cycle. Subsequently, fluorescence was measured using a BD FACSCalibur flow cytometer.

Western blot analysis

Cell lysates were prepared by mixing the harvested cells with RIPA buffer containing sodium orthovanadate (1 mM), sodium fluoride (1 mM), phenylmethylsulfonyl fluoride (0.1 mM) and a protease inhibitor cocktail. Equal amounts of protein extract were denatured by boiling for 5 min for each sample. Proteins were resolved using 10-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The resolved proteins were transferred to polyvinylidene difluoride membranes. Then the membrane was blocked with 5% skim milk, or 3% bovine serum albumin, in Tris-buffered saline with Tween 20 (TBST; Tris-HCl 10 mM, 150 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h at room temperature. The membranes were washed three times and the respective primary antibody was added for overnight incubation at 4°C. The following day, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Then, the membranes were washed again proteins were detected using an ECL kit (GE Healthcare Life Sciences).

Measurement of intracellular ROS and mitochondrial ROS

BxPC-3 cells were stained after MS-5 treatment for the indicated time to measure ROS levels. Subsequently, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen, Carlsbad, CA, USA) 10 μM was used to measure intracellular ROS, and MitoSOX Red (Invitrogen) 5 μM was used to measure mitochondrial ROS. The fluorescence signals were detected using a BD FACSCalibur flow cytometer.

Statistical Analysis

Data are presented as mean ± standard deviation. Single comparisons were performed using the Student's t-test. *p* value less than 0.05 was considered significant statistically.

RESULTS

Cytotoxicity of MS-5 in BxPC-3 human pancreatic cancer cells

To determine whether MS-5 was toxic to BxPC-3 cells, we performed a cell viability assay using water-soluble tetrazolium salt (WST-1). The growth of BxPC-3 cells was inhibited by MS-5 in a concentration- and time-dependent manner (Fig. 1B). After a 24 h-incubation in the presence of MS-5 (15 μM), cell viability was approximately 53% compared with that of control. Observation of the cells using light microscopy revealed shrinkage and rounding of the cells (Fig. 1C).

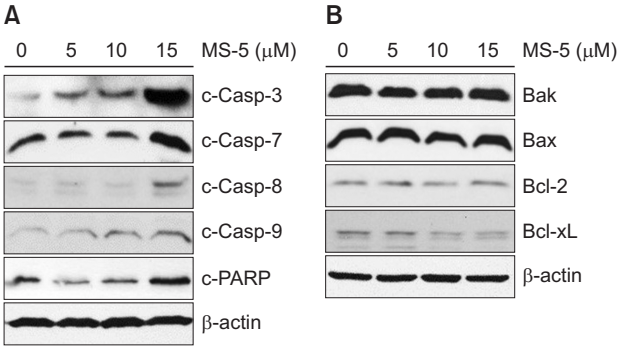

Fig. 2. Cellular markers of apoptosis in BxPC-3 cells after treatment of MS-5. Cell lysates were prepared for western blot analysis after treatment with MS-5 (0, 5, 10, and 15 μM) for 24 h. (A) Caspases-3, -7, -8, -9, and PARP cleavage. (B) Bak, Bax, Bcl-2, Bcl-xL. β-actin was used as the loading control.

Apoptosis of BxPC-3 induced by MS-5

To determine whether BxPC-3 cells underwent apoptosis upon treatment of MS-5, we analyzed the cells by flow cytometry after staining with Annexin V/propidium iodide. The population of cells with Annexin V-positive staining was considered to have undergone apoptosis. The results revealed that BxPC-3 cells underwent apoptosis after treatment of MS-5. At 15 μM of MS-5, the cells undergoing apoptosis were approximately 22%, whereas less than 4% of the cells were undergoing apoptosis in untreated cells (Fig. 1D).

MS-5 induced modulation of caspases and Bcl-2 family proteins

The levels of caspases involved in apoptosis were analyzed by western blotting, as the initiation and progression of apoptosis are characterized caspase activation. BxPC-3 cells, treated with various concentrations of MS-5 for 24 h, were subjected to western blot analysis. The results revealed that treatment of MS-5 to BxPC-3 cells induced the activation of caspases-3, -7, -8, and -9, and cleavage of PARP (Fig. 2A). Encouraged by the results, we examined the levels of Bcl-2 family proteins. An increase in the levels of pro-apoptotic proteins, such as Bax and Bak, was not observed. However, the level of Bcl-xL, an anti-apoptotic protein, was downregulated by MS-5 treatment, shifting the balance of Bcl-2 family proteins. Bcl-2 protein level remained unaltered (Fig. 2B).

MS-5 induced cell cycle arrest in BxPC-3 cells

Flow cytometry analysis and western blotting were performed to monitor the cell cycle profile of BxPC-3 cells 24 h after treatment with MS-5. Flow cytometry analysis revealed that the cells in G1 and sub-G1 phases accumulated after treatment with MS-5, while the number of cells in the S and G_2/M phases decreased (Fig. 3A). Notably, western blot analysis showed the decrease in the levels of cyclins A and E at the highest concentration (15 μM) of MS-5 (Fig. 3B). Similarly, the level of CDK2 decreased as MS-5 concentration increased. Conversely, the levels of p27, an inhibitor of CDK, were elevated by the treatment with MS-5 (Fig. 3B).

MS-5 induced modulation of ROS generation

We then used flow cytometry to examine the generation of

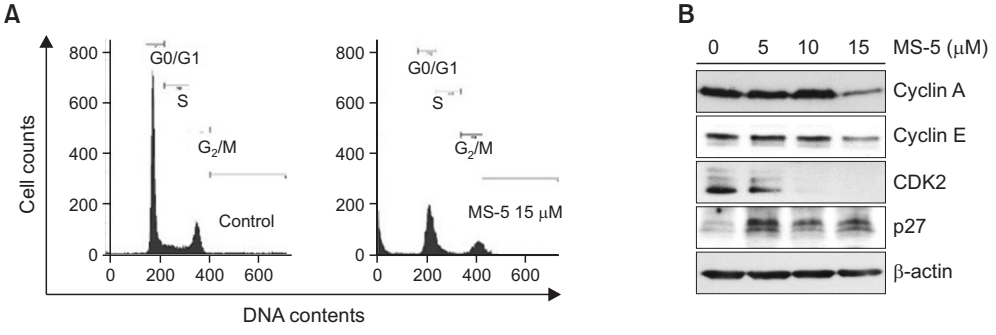

Fig. 3. Cell cycle arrest in BxPC-3 cells after treatment with MS-5. Cells were treated with the indicated concentrations of MS-5 for 24 h. (A) Cell cycle distribution was analyzed by flow cytometry after staining with propidium iodide. (B) Western blot analysis of cyclins A, and E, CDK2, and p27. β-actin was used as the loading control.

ROS in BxPC-3 cells 24 h after treatment with MS-5. Intracellular total ROS level was measured using H_2DCFDA, an oxidation-sensitive dye. After treating the cells with MS-5, we observed a marginal decrease in intracellular ROS levels (Fig. 4A). Conversely, an increase in ROS level was observed when MitoSOX Red was used to detect mitochondrial superoxide (Fig. 4B). Western blot analysis showed that the level of SOD1 was decreased after MS-5 treatment. A decrease in the level of SOD2 was observed at 15 μM MS-5, whereas the level of HO-1 remained relatively unchanged (Fig. 4C).

DISCUSSION

In this study, we demonstrated that MS-5 induced apoptosis in the human pancreatic cancer cell line BxPC-3. The proliferation of cells decreased with increasing concentrations of MS-5 or exposure time, indicating that MS-5 was cytotoxic to BxPC-3 cells (Fig. 1B). Along with the reduced viability of BxPC-3 cells, we observed the typical morphological changes during apoptosis (Fig. 1C). In addition, the flow cytometry analysis of apoptotic subpopulations after staining with Annexin V and propidium iodide revealed that the reduction in proliferation correlated well with the increase in the ratio of apoptotic cells (Fig. 1D).

As we observed the apoptotic effect of MS-5 on BxPC-3 cells, we evaluated whether this effect participates in the mitochondria-dependent apoptotic pathway. We could see that caspases-3, -7, -8, -9, and PARP were cleaved (Fig. 2A). Moreover, we observed a shift in the balance of Bcl-2 family proteins during MS-5 treatment. The levels of Bak, Bax, and Bcl-2 remained relatively stable (Fig. 2B). However, the level of Bcl-xL was slightly decreased in MS-5-treated cells, possibly resulting in the release of cytochrome c (Fig. 2B). In addition to the activation of the mitochondrial apoptotic pathway, we noticed an accumulation of cells in the G1 and sub-G1 phases from the cell cycle analysis with flow cytometry (Fig. 3A). As cells progress through the cell cycle, the levels of several proteins, such as cyclins and CDKs, change. These proteins are critical cell cycle regulators (Dickson and Schwartz,

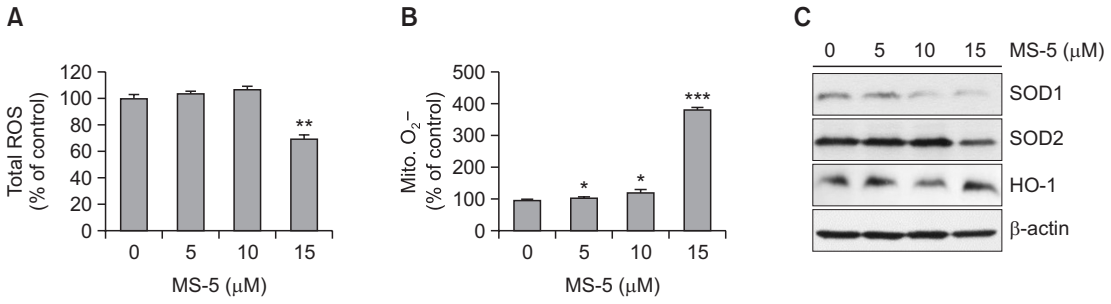

Fig. 4. Levels of total ROS and mitochondrial superoxide in BxPC-3 cells after MS-5 treatment. Cells treated with MS-5 (0, 5, 10, and 15 μM) for 24 h were subjected to flow cytometry analysis to measure the levels of total ROS (A) and mitochondrial superoxide (B). (C) Western blot analysis of cell lysates for SOD1, SOD2, and HO-1 expression. β-Actin was used as the loading control. *p<0.05, **p<0.01 and ***p<0.001 compared to control.

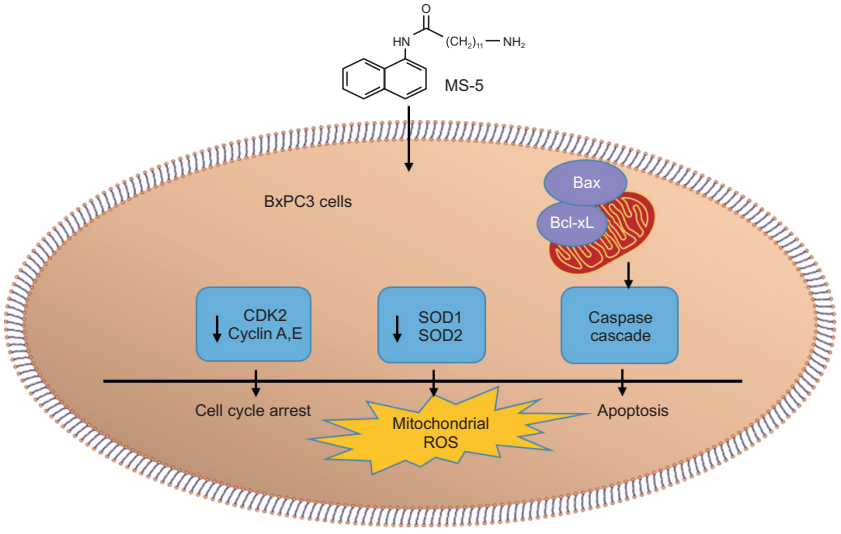

Fig. 5. Schematic diagram showing the mechanism of action underlying MS-5-induced cytotoxicity in human pancreatic cancer BxPC-3 cells. MS-5 induces the apoptosis and G1 phase arrest of BxPC-3 cells through modulating the ROS-regulating enzymes and by modulating reactive oxygen species.

2009). Immunoblot analysis revealed a decrease in the levels of cyclins A, and E, and CDK2 (Fig. 3B). We also observed increased p27 levels upon MS-5 treatment (Fig. 3B). The cyclin E/CDK2 complex could be inhibited by p27 binding to the complex (Polyak *et al*., 1994; Chu *et al*., 2007), possibly increasing p27 levels, leading to CDK2 inhibition and halting cell cycle progression.

Oxidative stress caused by excessive ROS generation is harmful; oxidative stress caused by various chemicals can induce apoptosis (Bilinski *et al*., 1989; Cabiscol *et al*., 2000). Intracellular ROS levels, as analyzed by flow cytometry with H_2DCFDA staining, were slightly decreased upon MS-5 treatment. The level remained relatively the same as that of the control when the MS-5 concentration was 10 μM. At 15 μM MS-5, total ROS decreased by approximately 25% compared with that in the control (Fig. 4A). In contrast, the mitochondrial ROS in BxPC-3 cells increased significantly after MS-5 treatment (Fig. 4B). We observed a four-fold increase in mitochondrial ROS levels at 15 μM MS-5. The expression of HO-1 is regulated by several transcription factors, including nuclear factor erythroid-2-related factor 2 and nuclear factor-κB. HO-1 is considered a marker of oxidative stress (Dennery, 2000). MS-5 did not induce a significant change in HO-1 levels (Fig. 4C). Instead, we noticed a decrease in the levels of SOD1 and SOD2. SODs are antioxidant enzymes that catalyze the dismutation of superoxide ($O_2^{\bullet-}$) and play a vital role in antioxidant protection (Fridovich, 1995). The decreased levels of SOD1 and SOD2 likely contribute to the increase in mitochondrial ROS. SOD1 inhibition reportedly promotes cancer cells apoptosis by modulating ROS signaling (Che *et al*., 2016). Conversely, SODs promotes the epithelial-mesenchymal transition of pancreatic cancer cells (Li *et al*., 2015). Our data indicate that MS-5 induces apoptosis in pancreatic cancer BxPC-3 cells via a mechanism mediated by perturbation of ROS signaling (Fig. 5). Further studies are required to elucidate the detailed mechanism underlying the MS-5-induced alterations in the levels of intracellular and mitochondrial ROS. Our preliminary data indicate that MS-5 decreased the phosphorylation of JAK2 and STAT3 (Supplementary Fig. 1). The suppression of JAK2/STAT3 signaling pathway contributes cancer cell apoptosis (Park *et al*., 2022), and MS-5 may suppress the JAK2/STAT3 signaling pathway to induce apoptosis.

In conclusion, our results indicate that MS-5 induces apoptosis and G_1 phase arrest in human pancreatic cancer BxPC-3 cells by modulating ROS-regulating enzymes. Further studies may lead to a novel target for treating pancreatic cancer cells.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (2017R1D1A1A02018536; 2021R1I1A3058531 to SJ and 2020R1I1A3066367 to KC).

REFERENCES

Ali, H., Pamarthy, R., Vallabhaneni, M., Sarfraz, S., Ali, H. and Rafique, H. (2021) Pancreatic cancer incidence trends in the United States from 2000-2017: analysis of Surveillance, Epidemiology and End Results (SEER) database. *F1000Res.* **10**, 529.

Bilinski, T., Litwinska, J., Blaszczynski, M. and Bajus, A. (1989) Superoxide dismutase deficiency and the toxicity of the products of autooxidation of polyunsaturated fatty acids in yeast. *Biochim. Biophys. Acta* **1001**, 102-106.

Cabiscol, E., Piulats, E., Echave, P., Herrero, E. and Ros, J. (2000) Oxidative stress promotes specific protein damage in Saccharomyces cerevisiae. *J. Biol. Chem.* **275**, 27393-27398.

Che, M., Wang, R., Li, X., Wang, H. Y. and Zheng, X. F. S. (2016) Expanding roles of superoxide dismutases in cell regulation and cancer. *Drug Discov. Today* **21**, 143-149.

Chu, I., Sun, J., Arnaout, A., Kahn, H., Hanna, W., Narod, S., Sun, P., Tan, C. K., Hengst, L. and Slingerland, J. (2007) p27 phosphorylation by Src regulates inhibition of cyclin E-Cdk2. *Cell* **128**, 281-294.

Dennery, P. A. (2000) Regulation and role of heme oxygenase in oxidative injury. *Curr. Top. Cell. Regul.* **36**, 181-199.

Dickson, M. A. and Schwartz, G. K. (2009) Development of cell-cycle inhibitors for cancer therapy. *Curr. Oncol.* **16**, 36-43.

Durand, N. and Storz, P. (2017) Targeting reactive oxygen species in development and progression of pancreatic cancer. *Expert Rev. Anticancer Ther.* **17**, 19-31.

Fridovich, I. (1995) Superoxide radical and superoxide dismutases. *Annu. Rev. Biochem.* **64**, 97-112.

Gillen, S., Schuster, T., Meyer Zum Buschenfelde, C., Friess, H. and Kleeff, J. (2010) Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of response and resection percentages. *PLoS Med.* **7**, e1000267.

Li, W., Cao, L., Han, L., Xu, Q. and Ma, Q. (2015) Superoxide dismutase promotes the epithelial-mesenchymal transition of pancreatic cancer cells via activation of the H2O2/ERK/NF-kappaB axis. *Int. J. Oncol.* **46**, 2613-2620.

Liou, G. Y. and Storz, P. (2010) Reactive oxygen species in cancer. *Free Radic. Res.* **44**, 479-496.

Ma, E., Jeong, S. J., Choi, J. S., Nguyen, T. H., Jeong, C. H. and Joo, S. H. (2019) MS-5, a naphthalene derivative, induces the apoptosis of an ovarian cancer cell CAOV-3 by interfering with the reactive oxygen species generation. *Biomol. Ther. (Seoul)* **27**, 48-53.

Nishikawa, M. (2008) Reactive oxygen species in tumor metastasis. *Cancer Lett.* **266**, 53-59.

Orth, M., Metzger, P., Gerum, S., Mayerle, J., Schneider, G., Belka, C., Schnurr, M. and Lauber, K. (2019) Pancreatic ductal adenocarcinoma: biological hallmarks, current status, and future perspectives of combined modality treatment approaches. *Radiat. Oncol.* **14**, 141.

Park, K. H., Joo, S. H., Seo, J. H., Kim, J., Yoon, G., Jeon, Y. J., Lee, M. H., Chae, J. I., Kim, W. K. and Shim, J. H. (2022) Licochalcone H induces cell cycle arrest and apoptosis in human skin cancer cells by modulating JAK2/STAT3 signaling. *Biomol. Ther. (Seoul)* **30**, 72-79.

Polyak, K., Kato, J. Y., Solomon, M. J., Sherr, C. J., Massague, J., Roberts, J. M. and Koff, A. (1994) p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev.* **8**, 9-22.

Rahib, L., Smith, B. D., Aizenberg, R., Rosenzweig, A. B., Fleshman, J. M. and Matrisian, L. M. (2014) Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res.* **74**, 2913-2921.

Torres, M. and Forman, H. J. (2003) Redox signaling and the MAP kinase pathways. *Biofactors* **17**, 287-296.

Ushio-Fukai, M. and Nakamura, Y. (2008) Reactive oxygen species and angiogenesis: NADPH oxidase as target for cancer therapy. *Cancer Lett.* **266**, 37-52.