Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Anticancer Effect of Benzyl Isothiocyanate on the Apoptosis of Human Gemcitabine-Resistant Pancreatic Cancer MIA PaCa-2/GemR Cells (MIA RG100)

Chao-Wen Hsueh, Chun Chen[1], Po-Yen Chiu[2], Ni-Na Chiang[3], Chi-Cheng Lu[4], Jai-Sing Yang[5], Fu-An Chen[2]

Department of Internal Medicine, Division of Gastroenterology, Kaohsiung Armed Forces General Hospital, Kaohsiung 802, [1]School of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, [2]Department of Pharmacy and Master Program, Tajen University, Pingtung 907, [3]Kaohsiung Veterans General Hospital, Kaohsiung 813, [4]Department of Sport Performance, National Taiwan University of Sport, Taichung 404, [5]Department of Medical Research, China Medical University Hospital, China Medical University, Taichung 404, Taiwan

Submitted: 25-Feb-2022 Revised: 30-May-2022 Accepted: 12-Jul-2022 Published: 19-Sep-2022

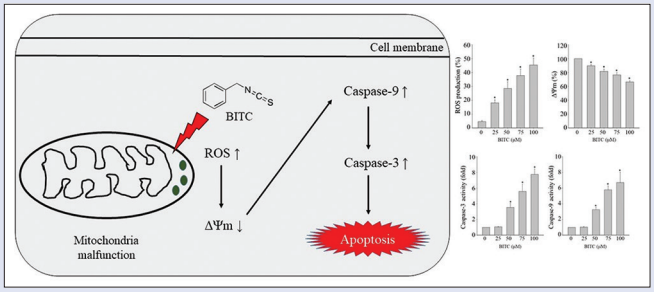
ABSTRACT

Background: Benzyl isothiocyanate (BITC) is a natural compound found in numerous cruciferous vegetables, and research has indicated that it has diverse biological activities. Isothiocyanate and its derivatives are the major anticancer natural compounds in cruciferous vegetables; these compounds help inhibit tumor cell proliferation through various mechanisms such as promoting tumor cell apoptosis, prompting cycle arrest, and increasing the generation of reactive oxygen species (ROS). **Objectives:** In human pancreatic cancer, gemcitabine is the first-line treatment; however, pancreatic cancer cells readily develop resistance to gemcitabine. Studies have demonstrated that natural products can promote the effect of gemcitabine and enhance the apoptosis process; however, the relevant mechanism and potential of BITC in human pancreatic cancer cells with gemcitabine resistance, namely, MIA PaCa-2/GemR cells (MIA RG100), are unclear. **Materials and Methods:** To elucidate the extent to which BITC induces apoptosis, we investigated the time and dose-dependent cell viability of PaCa-2/GemR cells under treatment with BITC. **Results:** Following BITC treatment, the PaCa-2/GemR cells exhibited DNA condensation, as indicated by transferase-mediated d-UTP nick end labeling (TUNEL) stain, with a corresponding increase in ROS production in mitochondria. Moreover, colorimetric assay analyses revealed that BITC increased caspase-9 and caspase-3 activities in PaCa-2/GemR cells. Our results indicate that BITC induces apoptotic cell death in PaCa-2/GemR cells through a mitochondrial-dependent signaling pathway.

Key words: Apoptosis, benzyl isothiocyanate (BITC), gemcitabine-resistant pancreatic cancer cells (PaCa-2/GemR)

SUMMARY

- BITC increased ROS production in PaCa-2/GemR cells and regulated ΔΨm.
- BITC increased caspase-3/9 activity in PaCa-2/GemR cells.
- BITC induced cell death via mitochondria-dependent signaling pathways.



Abbreviations used: BITC: Benzyl isothiocyanate; ROS: Reactive oxygen species; PaCa-2/GemR cells: Human pancreatic cancer cells with gemcitabine resistance; TUNEL: Transferase-mediated dUTP nick-end labeling; ITCs: Isothiocyanates; PEITC: Phenylethyl isothiocyanate; MTT: Thiazolyl blue tetrazolium bromide; Ig: Immunoglobulin; $DiOC_6$ (3): 3′-dihexyloxacarbocyanine iodide; H_2DCFDA: 2′, 7′-dichlorodihydrofluorescein diacetate; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; Akt: Protein kinase B; NF: Nuclear factor; SHH: Sonic hedgehog; dCK: Deoxycytidine kinase; ΔΨm: mitochondrial membrane potential. Fas: Apoptosis antigen 1; FasL: Fas-ligand; DR4: Death receptor 4; DR5: Death receptor 5; TNFR: Tumor necrosis factor receptor; p53: Tumor protein p53.

Correspondence:

Dr. Fu-An Chen, Ph.D.
Department of Pharmacy and Master Program,
Tajen University, 20 Weixin Road,
Yanpu, Pingtung 907, Taiwan.
E-mail: fachen@tajen.edu.tw

DOI: 10.4103/pm.pm_101_22

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Pancreatic cancer is a very malignant cancer with an extremely fast course of development. Pancreatic cancer is typically accompanied by upper abdominal pain, and by the time it is detected, few treatment options may be available. Therefore, symptom relief is the chief concern in end-stage pancreatic cancer. Symptoms of abdominal pain, depression, insomnia, jaundice, thrombosis, and loss of appetite are common in end-stage pancreatic cancer. In developed countries, pancreatic cancer has an extraordinarily high mortality rate, thus producing considerable patient suffering.[1,2] Pancreatic cancer is among the 10 most prevalent cancers among both men and women in Taiwan. Because of the challenging management of pancreatic cancer, patients with this cancer exhibit a 5-year survival rate of >10%.[2-4] Moreover, because of the difficulty involved in diagnosing this cancer at a stage when potentially curative surgery is feasible, 80% of patients with pancreatic cancer rely solely on

Cite this article as: Hsueh CW, Chen C, Chiu PY, Chiang NN, Lu CC, Yang JS, *et al*. Anticancer effect of benzyl isothiocyanate on the apoptosis of human gemcitabine-resistant pancreatic cancer MIA PaCa-2/GemR cells (MIA RG100). Phcog Mag 2022;18:675-8.

chemotherapy.[5] The United States registered 57,600 pancreatic cancer cases in 2020, with 47,050 deaths being caused by this disease.[6] Similar to the trends observed in countries worldwide, in Taiwan, approximately 2000 new pancreatic cancer cases are reported annually, with only 20%–25% of these patients being eligible for surgery.[2,7]

Gemcitabine is the first choice of human pancreatic cancer treatment; however, pancreatic cancer cells readily develop gemcitabine resistance.[8,9] Studies have demonstrated that natural products can promote the effect of gemcitabine and enhance the apoptosis process.[10,11] Isothiocyanates are the hydrolyzates of a type of glucosinolate, and they are commonly found in cruciferous vegetables; examples include sulfur-containing cyanide isothiocyanates (ITCs), such as phenylethyl isothiocyanate (PEITC), benzyl isothiocyanate (BITC), and indole-like compounds.[12] In many animal model experiments examining carcinogen-induced cancer, ITC has demonstrated the ability to prevent cancer from forming in tissues and organs, such as the lungs, liver, pancreas, bladder, chest line, esophagus, small intestine, and large intestine.[13] Studies have also revealed that ITCs can cause cancer cell apoptosis, and the path and concentration of apoptosis caused by different ITC derivatives are distinct. BITC, allyl isothiocyanate, PEITC, and sulforaphane are common ITC derivatives. Studies have reported that these ITCs can resist cancer cell growth and induce apoptosis.[12] However, how these ITCs affect human gemcitabine-resistant MIA RG100 pancreatic cancer cells remains to be elucidated.

This study detected and evaluated the impact of BITC on the apoptosis of human gemcitabine-resistant MIA RG100 pancreatic cancer cells and explored the underlying mechanism driving apoptosis; the aim was to clarify the mechanism of BITC's inhibition of drug-resistant pancreatic cancer as well as its transfer and message transmission path.

MATERIALS AND METHODS

Materials

Thiazolyl blue tetrazolium bromide (MTT), BITC, and the *in situ* Cell Death Detection Kit (fluorescein; Roche Diagnostics GmbH) were purchased from Sigma–Aldrich (Merck KGaA, Darmstadt, Germany); other reagents and chemicals were purchased from the same supplier unless otherwise stated. We purchased all primary antibodies and antimouse and antirabbit immunoglobulin (Ig) G horseradish peroxidase (HRP)-linked secondary antibodies from GeneTex (Hsinchu, Taiwan). We obtained Muse Caspase-3/9 assay kits from Millipore (Merck KGaA, Darmstadt, Germany). We purchased 3, 3′-dihexyloxacarbocyanine iodide [DiOC6 (3)] and 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCFDA) from Molecular Probes (Thermo Fisher Scientific; Waltham, MA, USA). We obtained Dulbecco's modified Eagle's medium (DMEM) from Sigma–Aldrich. Finally, we sourced L-glutamine, penicillin/streptomycin, fetal bovine serum (FBS), and trypsin-ethylenediaminetetraacetic acid (EDTA) from HyClone (GE Healthcare Life Sciences; Logan, UT, USA).

Cell culture

We obtained MIA PaCa-2 cells from the Bioresource Collection and Research Center (Hsinchu, Taiwan, BCRC Number: 60139). Human gemcitabine-resistant pancreatic cells MIA RG100 cells derived to MIA PaCa-2 after MIA PaCa-2 were subjected to treatment with gemcitabine at concentrations of 5 nM to 100 nM for approximately 9 months; drug resistance to 100-nM gemcitabine was confirmed, and follow-up experiments were performed. The details can be found in the literature.[14] DMEM containing 100 U/mL penicillin, 100 ng/mL streptomycin, 10% FBS, and 1% L-glutamine was used to culture the PaCa-2/GemR cells until 100% confluence; this was performed in a humidified 5% CO_2 atmosphere at 37°C, with culture medium renewal every 2–3 days. After the cells were stained with trypan blue, the viable cells were counted and the survival rate was calculated with a hemocytometer; the number of cells in the passage was controlled at $2–5 \times 10^5$ cells/mL. The cells were treated for 24 h with 0, 25, 50, 75, and 100 μM BITC. Harvested cells subsequently underwent the cell viability test, transferase-mediated d-UTP nick end labeling (TUNEL) assay, reactive oxygen species (ROS) production assay, caspase-3/9 assay, and western blot analysis.

Cell viability assay–MTT assay

The PaCa-2/GemR cells were seeded in 96-well plates, after which exposure to various concentrations (0, 25, 50, 75, and 100 μM) of BITC was performed for 24 or 48 h. Subsequently, the cells were incubated with MTT solution for a further 4 h. After the removal of the medium, we added dimethyl sulfoxide (DMSO) to dissolve the formazan crystals and replace the culture medium. A spectrophotometer was used to measure the optical density at 570-nm absorbance, as previously described.[15]

TUNEL staining

Following 48 h BITC treatment at 0, 5, 10, or 20 μM, the PaCa-2/GemR cells were harvested. At room temperature, we fixed them for 10 min in methanol, and DNA break was detected through probing with the *in situ* Cell Death Detection Kit in accordance with the manufacturer's instructions. A fluorescence microscope was used to observe and photograph apoptotic cells.

Caspase-3/9 activity assay

PaCa-2/GemR cells in 6-well plates were exposed to BITC at the following levels for 48 h: 0, 25, 50, 75, or 100 μM. After harvesting the cell lysates, we incubated the supernatants with the relevant reaction buffer by following the manufacturer's protocols. Subsequently, a phase-contrast microscope was used to visualize and photograph the cells.

Determination of mitochondrial electrical potential and ROS through flow cytometry

PaCa-2/GemR cells were exposed to various BITC concentrations (0, 25, 50, 75, or 100 μM) for 48 h. After being harvested, the cells were probed with 500 nM DiOC6 (3) or H2DCFDA; this was achieved through flow cytometry performed for 30 min at 37°C, as previously described.

Statistical analysis

All experiments were performed in triplicate, and relevant data are presented as the mean ± standard deviation. All statistical analyses involved a one-way analysis of variance; $P < 0.05$ was considered significant.

RESULTS

BITC lowers PaCa-2/GemR cell viability

The survival rates (according to MTT detection) for gemcitabine-resistant human pancreatic cancer PaCa-2/GemR cells treated with BITC were significantly higher ($p < 0.05$) than for untreated cells, highlighting the high growth-inhibitory activity of BITC.

TUNEL analysis

TUNEL analysis was employed to observe BITC's effect on the cell morphology of human gemcitabine-resistant pancreatic cancer PaCa-2/GemR cells; the results indicated that BITC could prompt PaCa-2/GemR cell apoptosis.

Effect of BITC on caspase-dependent apoptosis in PaCa-2/GemR cells

Analysis of the influence of BITC treatment on the caspase activity of the cells of interest indicated that BITC activated caspase-3/9.

Effects of BITC on mitochondrial electrical potential and ROS in PaCa-2/GemR cells

We used H2DCFDA to detect the increase in ROS in drug-resistant pancreatic cancer PaCa-2/GemR cells following BITC treatment; the results indicated that BITC induced a dose-dependent increase in ROS.

DISCUSSION

Cancer has long been the leading cause of death in Taiwan, and the incidence rate has been increasing. In 2016, cancer resulted in the death of 47,760 people in Taiwan.[16] For pancreatic adenocarcinoma and other rare exocrine cancers, determining the prognosis is challenging, and these cancers have a high mortality rate.[17] Unresectable pancreatic cancer has a dismal prognosis as an untreated disease; moreover, the advanced-stage disease is common at diagnosis, and chemotherapy or radiotherapy has limited effectiveness in halting pancreatic cancer development.[18] Gemcitabine remains a major treatment compound in all disease stages.[19-22] However, gemcitabine resistance readily develops in pancreatic cancer. Regarding pancreatic cancer, studies have revealed several signaling pathways that act as control factors for both intrinsic and acquired resistance; numerous examples include epidermal growth factor receptor, mitogen-activated protein kinases, Akt, Notch, sonic hedgehog (SHH), and nuclear factor (NF)-κB pathways as well as expression of dCK.[18-25]

Numerous studies have indicated that natural medicines with fewer side effects than conventional treatments can serve as anticancer drugs. According to epidemiological studies, increased intake of cruciferous vegetables can not only reduce the incidence of cardiovascular disease but also reduce the incidence of cancer. Cruciferous vegetables have various anticancer effects because glucosinolates and their derivatives are closely related to the anticancer mechanism.[10-12]

In this study, we found that BITC results in a significant dose-dependent reduction in the viability of PaCa-2/GemR cells [Figures 1 and 2]. When BITC treatment is administered, numerous calcium ions are released into the cytoplasm under the pressure of the endoplasmic reticulum, which causes the mitochondria to release cytochrome c, leading to apoptosis. The external pathway is mainly affected by death signaling molecules, such as FasL, tumor necrosis factor [TNF]-related apoptosis-inducing ligand (TRAIL) combined with Fas/DR4, DR5, and TNFR; these molecules activate the downstream FAS-associating protein with death domain (FADD) protein, activate caspase-8, and regulate caspase-3 to cause the cell apoptosis. As a result, PaCa-2/GemR cells treated with BITC exhibited significant elevations of caspase-3 and caspase-9 activity [Figure 3]. Increased caspase-3/9 activity results in mitochondria depending on the apoptosis pathway and caspase-3 and caspase-9 for morphological changes; the subsequent increase in ROS production suggests a mitochondrial malfunction, resulting in inadequate energy reserves and intracellular signaling pathway activation. Moreover, endoplasmic reticulum stress can cause cells to undergo apoptosis through unfolded protein response and calcium ion transmission. Protein synthesis, folding, and positioning in the cell can occur at the endoplasmic reticulum. Furthermore, the endoplasmic reticulum is the principal site for calcium ion storage in the cell and participates in the transmission of calcium ions and the constant regulation of cellular calcium ions. Mitochondria are affected by apoptosis pathways involving DNA damage, ROS production, ΔΨm imbalance, and signal transporter

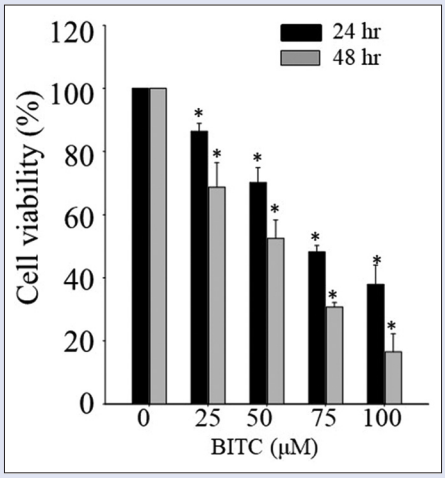


Figure 1: Viability of PaCa-2/GemR cells treated with BITC (0, 25, 50, 75, and 100 μM) for 24 h or 48 h. The values represent means ± standard errors (n = 3). *p < 0.05 versus untreated cells in cell viability % (Dunnett's test)

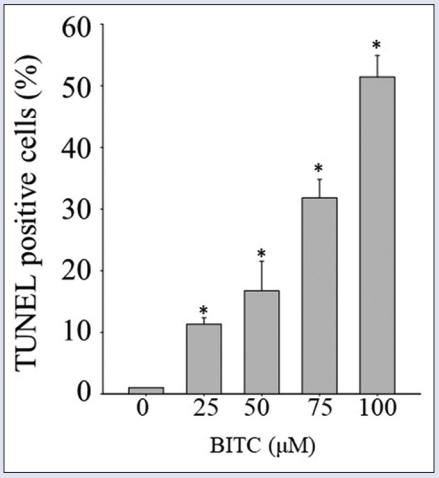


Figure 2: TUNEL positive cells of BITC-treated PaCa-2/GemR cells under BITC levels of 0, 25, 50, 75, and 100 μM. The values represent means ± standard errors (n = 3). *p < 0.05 versus untreated cells (Dunnett's test)

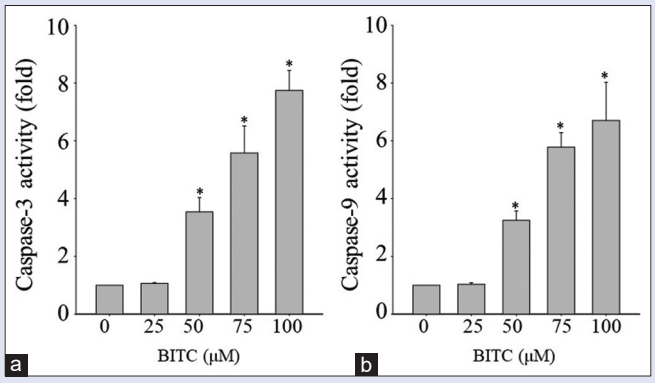


Figure 3: Influence of BITC on PaCa-2/GemR cell caspase activity. Fold changes in (a) caspase-3 and (b) caspase-9 activity in PaCa-2/GemR cells after 24 h of incubation with 0, 25, 50, 75, and 100 μM BITC (vs. 0 μM). The values represent means ± standard errors (n = 3). *p < 0.05 versus untreated cells (Dunnett's test)

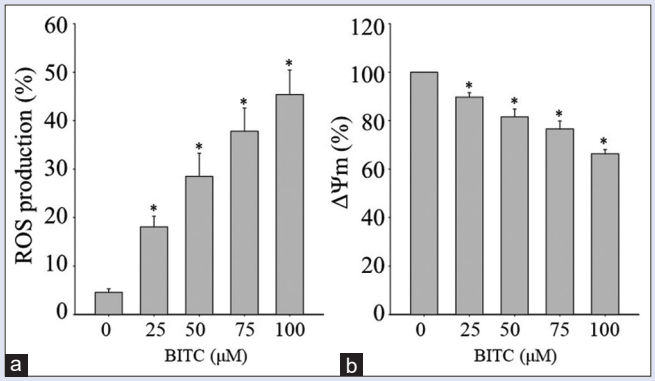


Figure 4: (a) ROS production results for PaCa-2/GemR cells that were subjected to 0, 25, 50, 75, or 100 μM BITC treatment. (b) ΔΨm in PaCa-2/GemR cells incubated for 24 h with 0, 25, 50, 75, or 100 μM BITC. BITC treatment was confirmed and ROS levels were assessed using DiOC6 (3) and H2DCFDA fluorescent dye, respectively, in flow cytometry. The values represent means ± standard errors (n = 3). *p < 0.05 versus untreated cells (Dunnett's test)

release.[15,26] ROS-induced injury to DNA results in the kinase acting upstream of p53 autophosphorylation in ataxia-telangiectasia mutating and triggering apoptotic signal phosphorylation, thus inducing apoptosis.[15,27,28] We found that BITC can induce PaCa-2/GemR cell apoptosis through mitochondrial-dependent signaling pathways, which along with BITC raise ROS production to reduce the mitochondrial membrane potential [Figure 4]. We detected and evaluated the effect of BITC on the apoptosis of PaCa-2/GemR cells. This study provides evidence that BITC has the potential as a natural supplement for enhancing gemcitabine treatment in PaCa-2/GemR cells.

CONCLUSION

Promoting PaCa-2/GemR cell apoptosis, BITC is associated with mitochondrial malfunction via increasing ROS production and caspase-3/9. In addition, caspase-3/9 plays a major trigger-inducing role in autophagy or apoptosis.

Financial support and sponsorship

This study was supported by the Department of Medicine, Kaohsiung Armed Forces General Hospital. Funding for this study was provided by plans 108-10.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Raimondi S, Maisonneuve P, Lowenfels AB. Epidemiology of pancreatic cancer: An overview. Nat Rev Gastroenterol Hepatol 2009;6:699-708.
2. Lu TP, Wu CH, Chang CC, Chan HC, Chattopadhyay A, Lee WC, *et al.* Distinct survival outcomes in subgroups of stage III pancreatic cancer patients: Taiwan cancer registry and surveillance, epidemiology and end results registry. Ann Surg Oncol 2022;29:1608-15.
3. Luo J, Xiao L, Wu C, Zheng Y, Zhao N. The incidence and survival rate of population-based pancreatic cancer patients: Shanghai Cancer Registry 2004-2009. PLoS One 2013;8:e76052. doi: 10.1371/journal.pone. 0076052.
4. Ilic M, Ilic I. Epidemiology of pancreatic cancer. World J Gastroenterol 2016;22:9694-705.
5. Bond-Smith G, Banga N, Hammond TM, Imber CJ. Pancreatic adenocarcinoma. BMJ 2012;344:e2476. doi: 10.1136/bmj.e2476.
6. Siegel RL, Miller KD, Goding Sauer A, Fedewa SA, Butterly LF, Anderson JC, *et al.* Colorectal cancer statistics, 2020. CA Cancer J Clin 2020;70:145-64.
7. Cheng CY, Chiang CJ, Hsieh CH, Chang YK, Lai MS. Is quality of registry treatment data related to registrar experience and workload? A study of Taiwan cancer registry data. J Formos Med Assoc 2018;117:1093-100.
8. Ahmed AA, Marchetti C, Ohnmacht SA, Neidle S. A G-quadruplex-binding compound shows potent activity in human gemcitabine-resistant pancreatic cancer cells. Sci Rep 2020;10:12192.
9. Bhutia YD, Hung SW, Patel B, Lovin D, Govindarajan R. CNT1 expression influences proliferation and chemosensitivity in drug-resistant pancreatic cancer cells. Cancer Res 2011;71:1825-35.
10. Lin JH, Chen SY, Lu CC, Lin JA, Yen GC. Ursolic acid promotes apoptosis, autophagy, and chemosensitivity in gemcitabine-resistant human pancreatic cancer cells. Phytother Res 2020;34:2053-66.
11. Zhu W, Li J, Wu S, Li S, Le L, Su X, *et al.* Triptolide cooperates with cisplatin to induce apoptosis in gemcitabine-resistant pancreatic cancer. Pancreas 2012;41:1029-38.
12. Wu X, Zhou QH, Xu K. Are isothiocyanates potential anti-cancer drugs? Acta Pharmacol Sin 2009;30:501-12.
13. Lam TK, Gallicchio L, Lindsley K, Shiels M, Hammond E, Tao XG, *et al.* Cruciferous vegetable consumption and lung cancer risk: A systematic review. Cancer Epidemiol Biomarkers Prev 2009;18:184-95.
14. Lai IL, Chou CC, Lai PT, Fang CS, Shirley LA, Yan R, *et al.* Targeting the Warburg effect with a novel glucose transporter inhibitor to overcome gemcitabine resistance in pancreatic cancer cells. Carcinogenesis 2014;35:2203-13.
15. Chen C, Yang JS, Lu CC, Wu YT, Chen FA. Effect of quercetin on injury to indomethacin-treated human embryonic kidney 293 cells. Life (Basel) 2021;11:1134.
16. Kuo CN, Liao YM, Kuo LN, Tsai HJ, Chang WC, Yen Y. Cancers in Taiwan: Practical insight from epidemiology, treatments, biomarkers, and cost. J Formos Med Assoc 2020;119:1731-41.
17. Rawla P, Sunkara T, Gaduputi V. Epidemiology of pancreatic cancer: Global trends, etiology and risk factors. World J Oncol 2019;10:10-27.
18. Amrutkar M, Gladhaug IP. Pancreatic cancer chemoresistance to gemcitabine. Cancers (Basel) 2017;9:157.
19. Heinemann V. Gemcitabine: Progress in the treatment of pancreatic cancer. Oncology 2001;60:8-18.
20. Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, *et al.* Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med 2013;369:1691-703.
21. Abbruzzese JL. New applications of gemcitabine and future directions in the management of pancreatic cancer. Cancer 2002;95 (4 Suppl):941-5.
22. Di Marco M, Di Cicilia R, Macchini M, Nobili E, Vecchiarelli S, Brandi G, *et al.* Metastatic pancreatic cancer: Is gemcitabine still the best standard treatment? Oncol Rep 2010;23:1183-92.
23. Shukla SK, Purohit V, Mehla K, Gunda V, Chaika NV, Vernucci E, *et al.* MUC1 and HIF-1alpha signaling crosstalk induces anabolic glucose metabolism to impart gemcitabine resistance to pancreatic cancer. Cancer Cell 2017;32:71-87.e7.
24. Fryer RA, Barlett B, Galustian C, Dalgleish AG. Mechanisms underlying gemcitabine resistance in pancreatic cancer and sensitisation by the iMiD™ lenalidomide. Anticancer Res 2011;31:3747-56.
25. Wang Z, Li Y, Ahmad A, Banerjee S, Azmi AS, Kong D, *et al.* Pancreatic cancer: Understanding and overcoming chemoresistance. Nat Rev Gastroenterol Hepatol 2011;8:27-33.
26. Martinvalet D. ROS signaling during granzyme B-mediated apoptosis. Mol Cell Oncol 2015;2:e992639. doi: 10.4161/23723556.2014.992639.
27. Tian B, Yang Q, Mao Z. Phosphorylation of ATM by Cdk5 mediates DNA damage signalling and regulates neuronal death. Nat Cell Biol 2009;11:211-8.
28. Kozlov SV, Graham ME, Jakob B, Tobias F, Kijas AW, Tanuji M, *et al.* Autophosphorylation and ATM activation: Additional sites add to the complexity. J Biol Chem 2011;286:9107-19.