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α, γ-Mangostins Induce Autophagy and Show Synergistic Effect with Gemcitabine in Pancreatic Cancer Cell Lines

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Abstract

Pancreatic cancer is one of the most lethal and aggressive cancers in the world. However, no effective treatment is currently available for pancreatic cancer. The objective of this study was to determine the anti-pancreatic cancer effect of α-mangostin (αM) and γ-mangostin (γM) extracted from the pericarp of *Garcinia mangostana L.*. Both αM and γM reduced the viability of pancreatic cancer cells MIA PaCa-2 and PANC-1 in a dose-dependent manner. These compounds induced apoptosis by increasing c-PARP and c-Caspase 3 levels. They also induced autophagy by increasing levels of microtubule-associated protein 1A/1B light chain 3B (LC3II) in both cell lines while decreasing sequestosome 1 (p62) in MIA PaCa-2. Both αM and γM induced autophagy through increasing phosphorylation levels of AMP-activated protein kinase (p-AMPK) and p38-mitogen activated protein kinase (p-p38) while decreasing phosphorylation level of mammalian target of rapamycin complex 1 (p-mTOR). Of various microRNAs (miRNA), miR-18a was found to be a putative regulatory miRNA for autophagy induced by αM or γM. In combination with gemcitabine, a compound frequently used in pancreatic cancer treatment, αM and γM showed synergistic anti-cancer effects in MIA PaCa-2. Collectively, these results suggest that αM and γM can induce apoptosis and autophagy in pancreatic cancer cells and that their anti-cancer effect is likely to be associated with miR-18a. In conclusion, αM and γM might be used as a potential new therapy for pancreatic cancer.

Key Words: α, γ-Mangostins, Pancreatic cancer, Apoptosis, Autophagy, Gemcitabine, microRNA

INTRODUCTION

Pancreatic cancer is the most fatal form of cancer, ranking the fourth among all cancers related to death (Siegel *et al*., 2017). Of 53,670 newly recorded pancreatic cancer cases in the United States, 43,090 cases resulted in death by 2014 (Siegel *et al*., 2017). Although 5-year relative survival rate of pancreatic cancer has been increasing steadily, it accounts for only 8% of 5-year survival rate for all kinds of cancers worldwide (Siegel *et al*., 2017). Although resection is a unique method for complete cure of pancreatic cancer, it is unusable in most patients due to poor prognosis, late diagnosis, and early metastasis. Therefore, the best treatment option available for pancreatic cancer is chemotherapy. Although gemcitabine is the most effective chemotherapeutic treatment against pancreatic cancer, it shows a tumor response rate of only 12% (Storniolo *et al*., 1999). Combination of gemcitabine with other drugs such as capecitabine (Herrmann *et al*., 2007), cisplatin (Heinemann *et al*., 2006), fluorouracil (Berlin *et al*., 2002), and erlotinib (Moore *et al*., 2007), and others on phase III pancreatic cancer patients has revealed little synergistic effect in enhancing the efficacy of gemcitabine. Thus, development of new first-line chemotherapy drugs or combination drugs against pancreatic cancer is urgently needed.

In Southeast Asia, the pericarp of *Garcinia mangostana L*. or mangosteen, also known as the queen of tropical fruits, has been used as a traditional medicine to treat a number of diseases, including dysentery, diarrhea, wound, skin infection, and others (Pedraza-Chaverri *et al*., 2008; Obolskiy *et al*., 2009). Over the past few decades, it has been demonstrated that mangosteen contains high amounts of xanthones showing significant biological activities (Obolskiy *et al*., 2009). Of these xanthones, α-mangostin (αM) and γ-mangostin (γM) have been found to exhibit many activities, including anti-fun-

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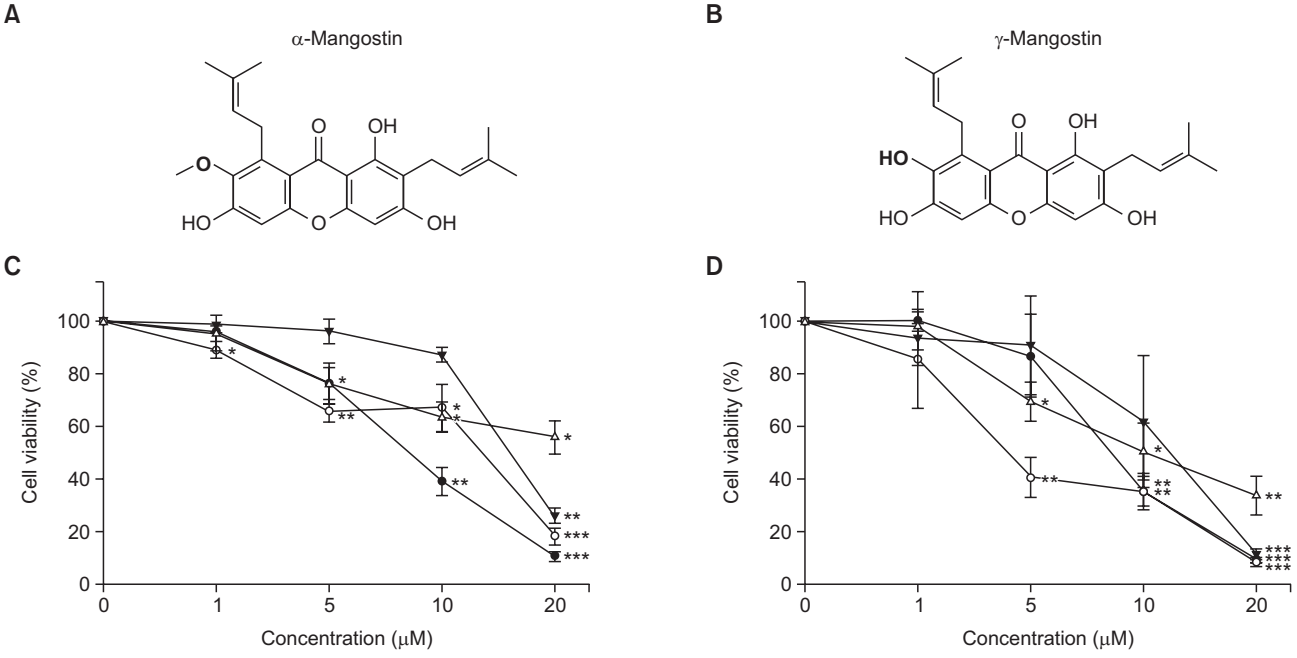

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	% Viability (48 h)				IC_{50}	% Viability (72 h)				IC_{50}
	1 μM	5 μM	10 μM	20 μM		1 μM	5 μM	10 μM	20 μM	
MIA PaCa-2 αM	96.1	76.2	39.0	10.7	8.4	100.2	87.0	35.2	9.1	8.5
MIA PaCa-2 γM	89.0	65.9	66.8	18.1	11.7	85.3	40.6	35.4	8.0	4.2
PANC-1 αM	98.7	96.1	87.3	26.2	15	93.9	91.0	62.0	11.6	11.7
PANC-1 γM	95.3	76.2	63.5	55.8	25	97.8	69.4	50.4	33.7	10.2

Fig. 1. Structures of αM and γM and anti-cancer effect of αM and γM. Structures of αM (A) and γM (B). Cell viability of pancreatic cancer cells was determined using WST-1 assay at 48 h (C) and 72 h (D) after treatment with αM or γM. It was tabulated with additional IC_{50} value (E). All experiments were repeated three times. Circle : MIA PaCa-2; Triangle : PANC-1; Filled : αM; Unfilled : γM. **p*<0.05, ***p*<0.01, and ****p*<0.001.

gal (Gopalakrishnan *et al*., 1997), anti-bacterial (Suksamrarn *et al*., 2003), neuroprotective (Chairungsrilerd *et al*., 1998; Pedraza-Chaverri *et al*., 2009), and cardioprotective activities (Devi Sampath and Vijayaraghavan, 2007). In addition, it has been reported that αM and γM have potential anti-cancer effects by inducing apoptosis (Matsumoto *et al*., 2004; Moongkarndi *et al*., 2004; Sato *et al*., 2004; Matsumoto *et al*., 2005), cell cycle arrest (Matsumoto *et al*., 2005; Johnson *et al*., 2012; Xu *et al*., 2014), and autophagic cell death (Chao *et al*., 2011). In addition, they can reduce oxidation (Moongkarndi *et al*., 2004; Jung *et al*., 2006), inflammation (Nakatani *et al*., 2002; Gutierrez-Orozco *et al*., 2013), metastasis and invasion (Shibata *et al*., 2011; Wang *et al*., 2012) of several cancer cell lines. However, molecular mechanisms of both compounds in pancreatic cancer cells have been reported yet.

Therefore, the objective of this study was to determine the anti-cancer effect of αM and γM extracted from the pericarp of *Garcinia mangostana L*. on pancreatic cancer cell lines and decipher molecular mechanisms associated with their anti-cancer effect using TUNEL assay, western blotting, and miRNA assay. In addition, anti-cancer effect of a combination of gemcitabine with αM and γM in pancreatic cancer cell lines was determined in this study.

MATERIALS AND METHODS

Cell lines and culture

Human pancreatic cancer cell lines MIA PaCa-2 and PANC-1 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in DMEM (Hyclone, Waltham, MA, USA) supplemented with 10% FBS (Hyclone, Waltham, MA, USA) and 1% penicillin/streptomycin (Gibco, Invitrogen Inc., Carlsbad, CA, USA) and incubated at 37°C in humidified atmosphere with 5% CO_2.

Cell viability

Separation and identification of αM and γM were conducted as described previously (Chae *et al*., 2016). Their structures are shown in Fig. 1A and 1B. αM and γM were dissolved in dimethylsulfoxide (DMSO) at 20 mM and stored at 4°C. Cell viability was measured using WST-1 reagent (Roche, Mannheim, Germany). Cells were seeded into 96-well cell culture plates at density of 4,000 to 5,000 cells per well. After incubation for 24 h, cells were treated with αM or γM at various concentrations (1, 5, 10, and 20 μM). DMSO (0.1% v/v) was used as a negative control. After 48 h or 72 h of treatment, absorbance was measured at wavelength of 450 nm using a microplate reader after 2 h of incubation with 10 μl of WST-1 reagent added to

each well. Cell viability was calculated from mean values of three wells. The experiment was repeated three times.

Western blotting

MIA PaCa-2 and PANC-1 cells (2×10^5 cells per well in 6-well plate) were treated with 1, 5, 10, and 20 μM of αM or γM. Cells were lysed in 200 μl RIPA Buffer (Thermo Scientific, Waltham, MA, USA) containing phosphatase inhibitor and protease inhibitor (Roche) for 20 min on ice following the manufacturer's protocol. Protein concentrations were measured using BCA Protein Assay Kit (Thermo Scientific). Total protein (30 μg) was separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% of nonfat dry milk in TBST at room temperature for more than 1 h and incubated at 4°C overnight with the following primary antibodies: anti-human-Bax, PAPR, cleaved PARP, caspase-3, cleaved caspase-3, LC3II, SQSTM1/P62, AMPKα, phospho-AMPKα, mTOR, phospho-mTOR, p38 MAPK, and phospho-p38 MAPK from Cell Signaling Technology (Beverly, MA, USA), β-Actin from Bioworld Technology (St. Louis Park, MN, USA). Blots were then probed with HRP-conjugated anti-rabbit antibodies from Thermo Scientific or anti-mouse antibodies from Bethyl Laboratories (Montgomery, TX, USA). Blots were visualized using ECL solution (Thermo Scientific).

TUNEL assay

MIA PaCa-2 and PANC-1 cells (1.5×10^4 cells per chamber) were seeded into Chamber Slide (Lab Tek Chamber Slide) for 24 h. Cells were fixed with 4% paraformaldehyde after incubation with αM or γM at IC_{50} for 48h. They were then subjected to TUNEL assay using In Situ Cell Death Detection Kit, AP (Roche). NBT/BCIP (Roche) was used for staining. DMSO (0.1% v/v) was used as a negative control. IC_{50} of HS-345 (Seo *et al*., 2013) was used as a positive control for apoptosis.

RNA extraction

MIA PaCa-2 cells were grown in DMEM media as described above and seeded into 6-well cell culture plates at density of 2×10^5 cells per well. On the following day, cells were treated with αM or γM at 20 μM for 48 h. Total RNA was extracted from cells using Trizol reagent (Invitrogen Inc.) following the manufacturer's instructions. DNA was subsequently removed using DNA-free™ Kit (Invitrogen Inc.) following the manufacturer's protocol.

miRNA PCR array

miScript® miRNA PCR Array Human miFinder Kit (QIAGEN Inc., Valencia, CA, USA) was used for profiling microRNAs in MIA PaCa-2. Total RNA (250 ng) was used for this experiment. cDNA synthesis and miRNA PCR Array were conducted using Pathway-Focused miScript® miRNA PCR Arrays (QIAGEN Inc.) following the manufacturer's protocols. SNORD95 was used as an internal control gene. This experiment was repeated twice.

TaqMan miRNA assay

To confirm miRNA PCR Array data, TaqMan miRNA assay was performed. Briefly, 50 ng of total RNA was converted into cDNA using TaqMan® microRNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA, USA) following the manufacturer's protocol. Real-time PCR was performed in triplicates and $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used for relative quantitation of genes. Data were normalized with RNU6B.

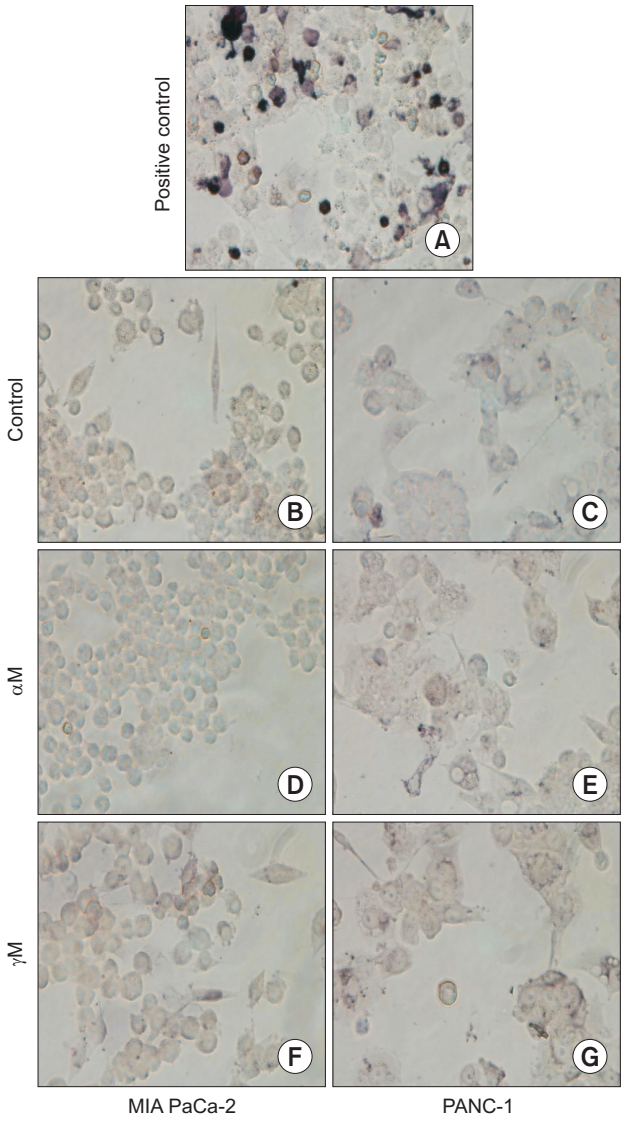


Fig. 2. Effect of αM or γM on apoptosis of pancreatic cancer cells assessed by TUNEL Assay. MIA PaCa-2 and PANC-1 cells were treated with αM or γM at a concentration equal to their IC_{50} values for 48 h. HS-345 was used as a positive control. Cells were photographed at 200X magnification.

Combination study with gemcitabine

Gemcitabine was purchased from Sigma-Aldrich (St. Louis, Mo, USA) and dissolved in DMSO at 10 mM. MIA PaCa-2 and PANC-1 cells were seeded into 96-well cell culture plates at density of 4,000 to 5,000 cells per well. Cells were incubated with gemcitabine only for 48 or 72 h to obtain IC_{50} value of gemcitabine. Cells were then incubated with a combination of gemcitabine and αM or γM based on the derived IC_{50} value. Measurement of cell viability was done using WST-1 Assay. Their combinatorial effect was evaluated using combination index (CI) calculated with the following equation:

$$CI = \frac{D_A}{(D_{50})_A} + \frac{D_B}{(D_{50})_B} + \alpha\frac{D_A}{(D_{50})_A}\frac{D_B}{(D_{50})_B}$$

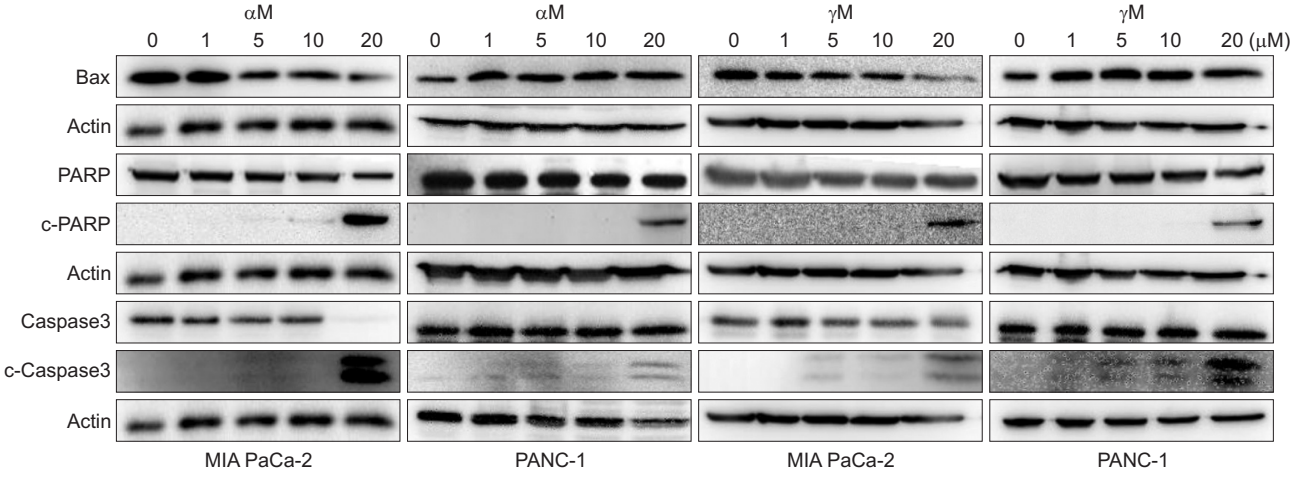


Fig. 3. Effect of αM or γM on apoptosis of pancreatic cancer cells assessed by western blotting. Cells were treated with αM or γM at the concentrations indicated above for 48 h. Actin was used as a loading control.

Where $(D_{50})_A$ and $(D_{50})_B$ were concentrations of drug A and drug B alone that reduced cell viability by 50% compared to the control. (D_A) and (D_B) were concentrations of drug A and drug B in combination producing a 50% reduction in cell viability compared to the control. α was 0 when drug A and B were mutually exclusive. It was 1 when they were mutually non-exclusive. CI<0.8 was considered as having a synergistic interaction. Additive interaction was considered at 0.8<CI<1.2 while antagonistic interaction was considered at CI>1.2 (Chou and Talalay, 1984).

Statistical analysis

miRNA PCR array was repeated twice. Other experiments were repeated three times. All data are represented as mean ± SD. For comparison between two groups, student *t*-test was used. Statistical analysis was performed using Microsoft Excel (Microsoft Corporation, Seattle, WA, USA). *p*-value of less than 0.05, 0.01, and 0.001 were considered statistically significant (**p*<0.05; ***p*<0.01, and ****p*<0.001).

RESULTS

αM and γM reduced viability of pancreatic cancer cells

Effect of αM and γM on viability of pancreatic cancer cells was investigated using WST-1 Assay. αM and γM reduced cell viabilities (Fig. 1C, 1D). Cell viability and IC_{50} values are shown in Fig. 1E. Their cytotoxicity effects were more efficient for MIA PaCa-2 than for PANC-1. Since their cytotoxicity effects appeared sufficiently after 48 h of incubation, further experiments were performed with 48 h of incubation.

αM and γM induced apoptosis of pancreatic cancer cells

It has been reported that αM and γM can induce apoptosis of several human cancer cell lines (Matsumoto *et al*., 2003; Moongkarndi *et al*., 2004; Sato *et al*., 2004). Furthermore, αM has cytotoxic effect on pancreatic cancer cells BxPC3 and PANC-1 through inducing apoptosis and cell cycle arrest (Xu *et al*., 2014). To confirm the ability of αM and γM to induce apoptosis of MIA PaCa-2 and PANC-1, TUNEL assay and western blotting were performed. Results of TUNEL assay revealed that treatment with αM or γM resulted in little transition to apoptotic cell death compared to the control (Fig. 2). Based on western blotting, levels of cleaved Caspase-3 and cleaved PARP (markers of apoptosis) were increased in groups treated with αM or γM. Bax, another marker of apoptosis, was increased in PANC-1 after treatment with αM or γM (Fig. 3). These results suggest that αM and γM can induce apoptosis of MIA PaCa-2 and PANC-1.

αM and γM induced autophagy through AMPK/mTOR and p38 in pancreatic cancer cells

It has been reported that αM has anti-cancer effect through autophagy in glioblastoma *in vivo* and colon cancer *in vitro* (Chao *et al*., 2011; Kim *et al*., 2012). Therefore, whether αM and γM could induce autophagy in pancreatic cancer was assessed by western blotting. Treatment with αM or γM at 20 μM significantly increased levels of LC3II, a marker of autophagosome formation during autophagy, in both cell lines (Fig. 4A). Levels of p62 (SQSTM1), another marker of autophagy (degraded during autophagy), were decreased in MIA PaCa-2, but not in PANC-1 after treatment with αM or γM at 20 μM (Fig. 4A). These results indicated that autophagy induced by αM or γM might have proceeded to completion only in MIA PaCa-2.

To identify the mechanism of autophagy induced by αM and γM, several proteins were examined. p-AMPK and p-p38 were increased while p-mTOR was decreased at earlier time points (6 h, 12 h, and/or 24 h) after treatment with αM or γM (Fig. 4B). However, these proteins did not show any changes at 48 h after treatment with αM or γM in most cases. These results indicate that αM and γM can induce autophagy through AMPK/mTOR and p38 pathways.

αM and γM changed expression levels of miRNAs and miR-18a might be related to cell death caused by αM and γM

To profile changes in expression levels of miRNAs after αM and γM treatment, miRNA PCR array was conducted. A total of 84 miRNAs were profiled in MIA PaCa-2. Most of these miRNAs were found to be downregulated in response to αM and γM treatment. A total of 14 miRNAs were downregulated while two miRNAs (miR-146a, 302c) were upregulated at

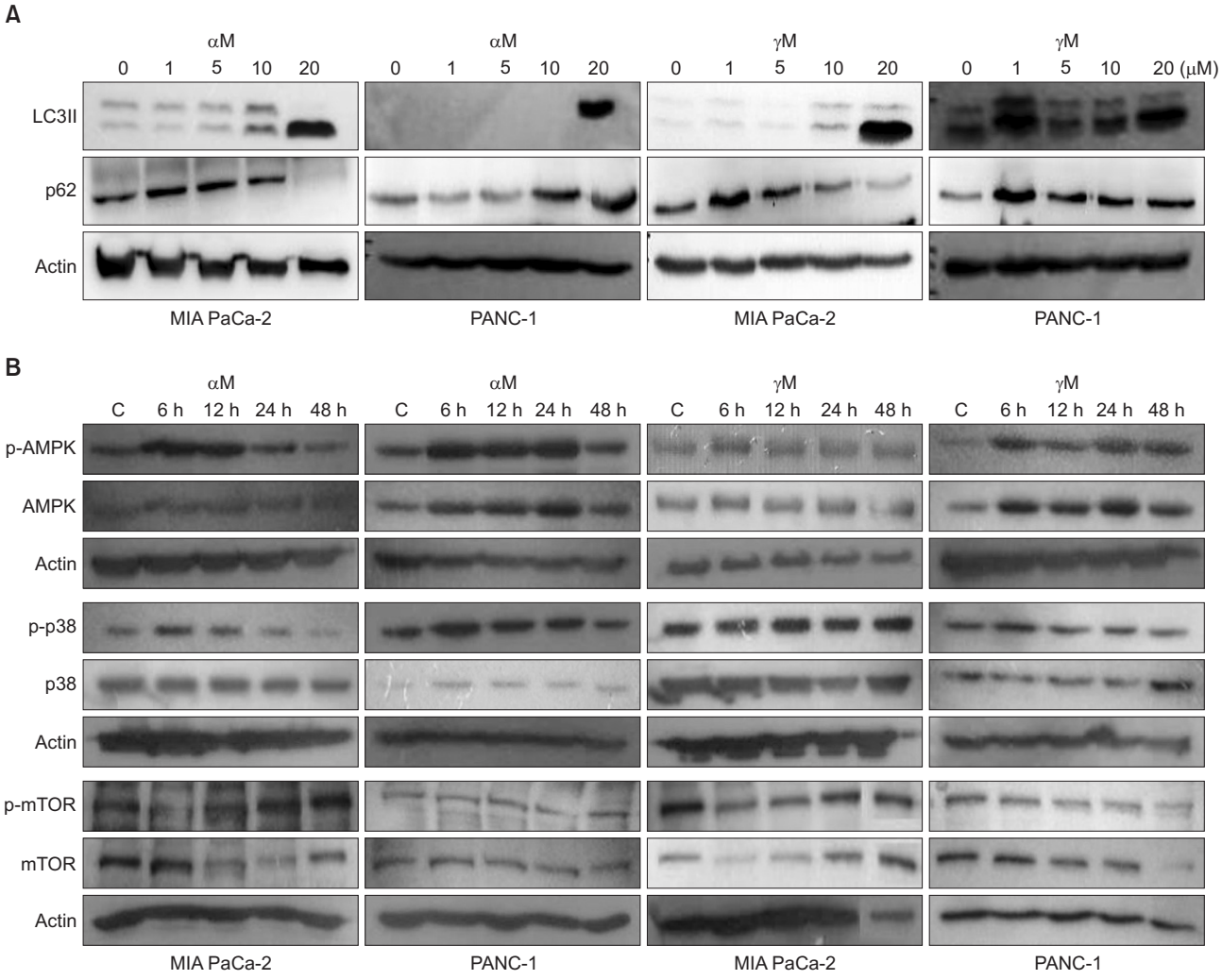


Fig. 4. Effect of αM or γM on autophagy of pancreatic cancer cells assessed by western blotting. LC3II and p62, markers of autophagy, were observed after treatment with αM or γM for 48h at several concentrations indicated above (A). Several proteins involved in autophagy pathway were also observed after treatment with αM or γM at IC_{50} value for certain incubation time indicated above (B). Actin was used as a loading control.

more than 2-fold after treatment with αM or γM (Table 1). TaqMan miRNA assay was carried out to confirm microRNA PCR array results. TaqMan miRNA assay revealed that expression level of miR-18a was decreased after αM and γM treatment (Fig. 5), consistent with miRNA PCR array results.

Anti-cancer effect of a combination of gemcitabine with αM or γM

Gemcitabine is frequently used in pancreatic cancer treatment. However, it is only marginally effective (Storniolo *et al*., 1999). To determine whether αM or γM could sensitize cancer cells to gemcitabine, MIA PaCa-2 or PANC-1 were treated with gemcitabine alone or in combination with αM or γM at IC_{50} for 72 h. Cell viability was measured (Fig. 6A-6F) and tabulated with CI value (Fig. 6G). αM and γM showed synergistic effects for gemcitabine on cell viability of MIA PaCa-2 compared to treatment with gemcitabine alone.

DISCUSSION

Pancreatic cancer is ranked as the fourth leading cause of death (Siegel *et al*., 2017). Characteristics of pancreatic cancer such as poor prognosis, late diagnosis, and early metastasis make pancreatectomy impossible in most cases. Pancreatic cancer also shows chemoresistance. Conventional medication gemcitabine exhibits only 12% tumor responsive rate (Storniolo *et al*., 1999).

It has been reported that αM and γM from *G. mangostana* have anti-cancer effects for various cancers, including prostate cancer, glioblastoma, and colon cancer (Chao *et al*., 2011; Chang and Yang 2012; Johnson *et al*., 2012). Although αM has been assessed in pancreatic cancer cells (Xu *et al*., 2014), mechanisms involved in the anti-cancer effect of αM and γM on pancreatic cancer cells are not fully understood yet. Furthermore, altered miRNA expression after treatment with αM has not yet been studied in pancreatic cancer, although

Table 1. microRNA expression by miScript® miRNA PCR Array (QIAGEN Inc.) in MIA PaCa-2 after treatment with αM or γM

microRNA	Fold change			microRNA	Fold change		
	αM	γM			αM	γM	
let-7a-5p	0.30	0.80		miR-19a-3p	0.21	0.53	
let-7b-5p	0.45	0.73		miR-19b-3p	0.21	0.52	
let-7c-5p	0.39	0.87		miR-200c-3p	0.35	0.73	
let-7d-5p	0.37	0.65		miR-20a-5p	0.20	0.53	
let-7e-5p	0.34	0.82		miR-210-3p	0.16	6.48	
let-7f-5p	0.31	0.93		miR-21-5p	0.43	1.01	
let-7g-5p	0.36	0.74		miR-222-3p	0.58	0.78	
let-7i-5p	0.43	0.82		miR-223-3p	2.65	0.98	
miR-100-5p	0.61	0.71		miR-22-3p	0.52	0.00	
miR-101-3p	0.08	0.56		miR-23a-3p	0.60	0.67	
miR-103a-3p	0.18	0.46	↓	miR-23b-3p	0.51	0.75	
miR-106b-5p	0.19	0.51		miR-24-3p	0.51	0.59	
miR-122-5p	7.62	1.22		miR-25-3p	0.76	0.79	
miR-124-3p	0.56	1.28		miR-26a-5p	0.48	0.83	
miR-125a-5p	0.65	0.69		miR-26b-5p	0.40	0.78	
miR-125b-5p	1.11	0.96		miR-27-3p	0.37	0.63	
miR-126-3p	0.28	0.58		miR-27a-3p	0.28	0.62	
miR-128-3p	0.42	0.53		miR-28-5p	0.24	0.55	
miR-130a-3p	0.19	0.40	↓	miR-29a-3p	0.38	0.80	
miR-140-3p	0.47	0.61		miR-29b-3p	0.06	0.47	↓
miR-141-3p	0.03	0.44	↓	miR-29c-3p	0.36	0.73	
miR-142-3p	0.16	0.59		miR-302a-3p	0.67	0.59	
miR-142-5p	0.57	0.12		miR-302c-3p	2.88	7.11	↑
miR-143-3p	1.05	0.45		miR-30a-5p	0.33	0.74	
miR-144-3p	1.28	0.31		miR-30b-5p	0.27	0.72	
miR-146a-5p	3.29	2.24	↑	miR-30c-5p	0.39	0.54	
miR-150-5p	2.68	1.33		miR-30d-5p	0.46	0.99	
miR-151a-5p	0.47	0.78		miR-30e-5p	0.29	0.76	
miR-155-5p	5.01	1.52		miR-320a	0.65	0.98	
miR-15a-5p	0.07	0.39	↓	miR-32-5p	0.02	0.30	↓
miR-15b-5p	0.38	0.47	↓	miR-374a-5p	0.18	0.65	
miR-16-5p	0.33	0.61		miR-376c-3p	1.58	1.14	
miR-17-5p	0.16	0.43	↓	miR-423-5p	0.72	0.82	
miR-181a-5p	0.17	0.24	↓	miR-424-5p	0.29	0.94	
miR-181b-5p	0.31	0.33	↓	miR-425-5p	0.27	0.42	↓
miR-185-5p	0.35	0.74		miR-7-5p	0.28	0.51	
miR-186-5p	0.27	0.53		miR-92a-3p	0.59	0.65	
miR-18a-5p	0.15	0.46	↓	miR-93-5p	0.31	0.58	
miR-191-5p	0.52	0.68		miR-9-5p	0.29	0.86	
miR-194-5p	0.58	1.09		miR-96-5p	0.08	0.43	↓
miR-195-5p	0.42	0.72		miR-99a-5p	0.84	0.95	
miR-196b-5p	0.78	0.51					

Expression levels of 84 microRNAs were measured by miScript® miRNA PCR array (QIAGEN Inc.) with SNORD95 as internal control gene. This experiment was repeated twice and average values of fold change were compared. Arrow indicates more than 2-fold change caused by treatment with αM or γM.

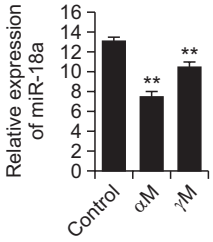
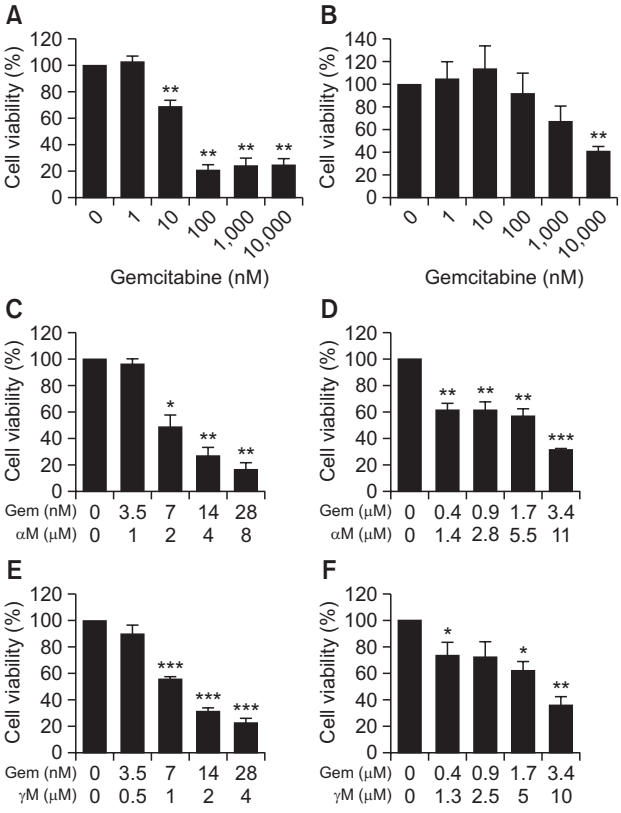


Fig. 5. Expression level of miR-18a assessed by TaqMan miRNA Assay. CT mean was calculated from three wells. RNU6B was used as a control to determine relative miRNA expression. $2^{-\Delta\Delta CT}$ method was used for relative quantitation of miR-18a. Averages were compared to control by Student's *t*-test. A *p*-value of less than 0.01 was considered to be statistically significant (**p<0.01).

there is one such study in human colon cancer (Nakagawa *et al*., 2007). In this study, cytotoxicity effects of αM and γM were determined. Our results confirmed that these compounds had anti-cancer effect on pancreatic cancer cells *in vitro*. Several studies have reported that αM can induce apoptosis of pancreatic cancer cell lines such as BxPC-3, PANC-1, and ASPC-1 (Hafeez *et al*., 2014; Xu *et al*., 2014). The current study also showed that αM and γM could induce apoptosis of MIA PaCa-2 and PANC-1.

Chao *et al*. (2011) have reported that αM can induce cell death through autophagy in glioblastoma cells. However, the effect of αM and γM on autophagy in pancreatic cancer cells has not been reported yet. Autophagy is generally known as a protective and survival pathway against starvation. However, it has been reported that autophagy can induce cell death. This is referred to as type II programmed cell death (Ouyang *et al*., 2012). During autophagy, double-membraned vesicles called autophagosomes are made with long-lived proteins and organelles. These autophagolysosomes are then fused with lysosomes to form autophagosomes. Autophagolysosomes are organelles that can digest their components for recycling (Benbrook and Long, 2012). There are three modes by which autophagy is initiated to induce formation of autophagosomes. One is dependent on Beclin-1, another marker of autophagy. Another is dependent on Ulk1 complex that is regulated through upstream factors such as AKT/mTOR and AMPK/mTOR. The third mode is activated by endoplasmic reticulum stress (ER stress) (Schleicher *et al*., 2010; Macintosh and Ryan, 2013). In this study, autophagy induced by αM and γM occurred through AMPK/mTOR and p-p38 pathways. LC3I is cleaved to form LC3II which is involved in the formation of autophagosomes. Therefore, LC3II has been used as a main indicator of autophagy (Kabeya *et al*., 2000). p62 (SQSTM1) is a ubiquitin binding protein. It is a secondary marker for autophagy. It directly interacts with LC3II in autophagosomes. It is degraded during autophagy processing. A decrease in p62 level means that autophagy is completely done. Incomplete autophagy will lead to accumulation of p62 (Bjorkoy *et al*., 2006; Pankiv *et al*., 2007). To examine whether αM and γM induced autophagy against pancreatic cancer cell lines, expression levels of LC3II and p62 were determined in this study. αM and γM at 20 μM significantly increased LC3II levels in both cell lines. However, p62 level was decreased in MIA PaCa-2, but not in PANC-1. Although αM and γM induced autophagy in both pancreatic cancer cells, autophagy was only completed



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	MIA PaCa-2		PANC-1	
	Alone	Combination	Alone	Combination
Gemcitabine	28.95	7.86	3.41	3.09
αM	8.71	2.24	11.68	9.09
CI value	0.53 Synergy		1.68 Antagonism	
Gemcitabine	28.95	9.04	3.41	2.30
γM	4.27	1.29	10.55	7.45
CI value	0.62 Synergy		1.38 Antagonism	

Fig. 6. Combinatorial effect of αM or γM with gemcitabine against pancreatic cancer cells. To evaluate IC_{50} of gemcitabine, MIA PaCa-2 (A) and PANC-1 (B) were treated with gemcitabine for 72 h. Gemcitabine in combination with αM (C, D) or γM (E, F) were added to MIA PaCa-2 (C, E) or PANC-1 (D, F) at a constant rate based on each IC_{50} value. After incubation for 72 h, cell viability was measured using WST-1 reagent and calculated from mean values of three wells. This experiment was repeated three times. CI value was calculated for combinatorial effect (G). *p<0.05, **p<0.01, and ***p<0.001.

in MIA PaCa-2.

miRNAs are small non-coding RNA composed of 20-22 nucleotides (Lagos-Quintana *et al*., 2001). They can bind to the 3'UTR of protein coding genes to regulate translation (Lai, 2002). Expression levels of miRNAs are altered depending on diseases. Numerous studies have shown an association between miRNAs and apoptosis or miRNAs and autophagy (Frankel and Lund, 2012; Li *et al*., 2012). However, the mechanisms are not well-understood yet. In this study, expression levels of 84 miRNAs were monitored using miRNA PCR Array and six miRNA candidates were confirmed by TaqMan miRNA Assay. Among these, miR-18a-5p showed correlation between miRNA PCR Array and TaqMan miRNA Assay results. Several reports have shown that miR-18a-5p is related to cell death. Suppression of miR-18a can increase apoptosis (Fujiya *et al*., 2014; Zhu *et al*., 2015). In addition, miR-18a-5p may promote carcinogenesis by directly targeting IRF2 in lung cancer (Liang *et al*., 2017) and STK4 in prostate cancer (Hsu *et al*., 2014). However, several studies have shown that miR-18a can promote apoptosis and act as tumor suppressor (Humphreys *et al*., 2014; Wu *et al*., 2015). Furthermore, it has been reported that an increase in miR-18a can cause autophagy (Qased *et al*., 2013; Fujiya *et al*., 2014; Fan *et al*., 2016). Since results of the current study on miR-18a and autophagy were different from those of some reports, more experiments are needed to confirm the relation between miR-18a and autophagy. miR-18a-5p has about 50 putative target genes commonly predicted by TargetScan (http://targetscan.org/), miRANDA (http://microRNA.org/), and miRDB (http://miRDB.org/). Out of these possible target genes, homeobox containing 1 (HMBOX1), SH3 binding protein 4 (SH3BP4), and GRB10-interacting GYF protein 1 (GIGYF1) are thought to be associated with autophagy. HMBOX1 is abundantly expressed in the cytoplasm. When it is overexpressed, apoptosis is decreased while autophagy is increased in HUVECs (Ma *et al*., 2015). SH3BP4 is a negative regulator of RagGTPases. It inhibits mTORC1 (Kim and Kim, 2013). It may have potential to induce autophagy. GIGYF1 is a new autophagy regulator through subcellular localization of Atg1-Atg13 complex (Kim *et al*., 2015).

In summary, our results revealed that αM and γM could induce autophagy and apoptosis in MIA PaCa-2 and PANC-1 cancer cells. In combination with gemcitabine, αM and γM showed synergistic effect in MIA PaCa-2. These results suggest that αM and γM might be promising agents for treating pancreatic cancer. Further studies are needed to confirm the target gene of miR-18a-5p related to autophagy.

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