博士学位論文

A novel therapeutic approach for pancreatic ductal adenocarcinoma treatment: Study on the role of an ERK-MAPK signaling modulator ACAGT-007a in apoptosis induction in KRAS mutant pancreatic cancer

近畿大学大学院
薬学研究科 薬科学専攻

Golam Iftakhar Khandakar

博士学位論文

A novel therapeutic approach for pancreatic ductal adenocarcinoma treatment: Study on the role of an ERK-MAPK signaling modulator ACAGT-007a in apoptosis induction in KRAS mutant pancreatic cancer

2023 年 1 月 12 日

近畿大学大学院
薬学研究科 薬科学専攻

Golam Iftakhar Khandakar

Table of Contents

Summary

Cell proliferation, cell death, and normal cell turnover are all regulated by signaling pathways that involve many different proteins, including multiple kinases and phosphatases. When a gene is mutated, cell death is not properly induced, causing the unregulated proliferation of cancer cells. Especially, KRAS gene mutations result in the abnormal activity of ERK and AKT, two enzymes that support cell proliferation, in more than 90% of pancreatic cancers. It is thought that this mutation contributes to the uncontrollable growth of cancer cells. By blocking the ERK signaling pathway, many of the conventional anti-cancer medications have reduced the growth of cancer cells, but the therapeutic effect of ERK signaling inhibitors on pancreatic cancer is insufficient. It is essential to develop a new therapeutic strategy that employs different mechanisms. Prof. Sugiura's laboratory has identified the ERK MAPK modulator ACA-28 and its derivative ACAGT-007a (GT-7) both of which induce apoptosis against melanoma cells with high ERK activity. Interestingly, GT-7 suppresses cancer cell growth by further activating the already-activated ERK in melanoma cells. Based on the findings of a prior study in melanoma cells in my lab, I focused on the effects of GT-7 on three pancreatic ductal adenocarcinomas (PDAC) cell lines with different KRAS mutations. My findings revealed that GT-7 increased phosphorylation of both ERK and AKT, and ERK was required for the induction of apoptosis in some types of PDAC. Many of the conventional therapeutic regimens inhibit ERK activation whereas GT-7 induces apoptosis in pancreatic cancer cells by a completely different mechanism. It has been assumed that similar to the compound ACA-28, its potent derivative GT-7 can increase pERK via ROS signaling stimulation. This elevated phosphorylated ERK may translocate to the nucleus by interacting with ERK substrates thereby triggering apoptosis Additionally, it was discovered that

using GT-7 in combination with a medication that prevents AKT activation in pancreatic cancer cells dramatically increases the effect of GT-7's ability to induce apoptosis. Considering the result of this study it is anticipated that the combination of ACAGT-007a (ERK stimulation) and Wortmannin (PI3K/AKT inhibitors) will contribute to the development of a novel therapeutic strategy for the treatment of pancreatic cancer. Moreover, this research is also expected to develop a promising new anticancer drug for the health benefit of pancreatic cancer patients.

Introduction

1.1 Pancreatic Cancer:

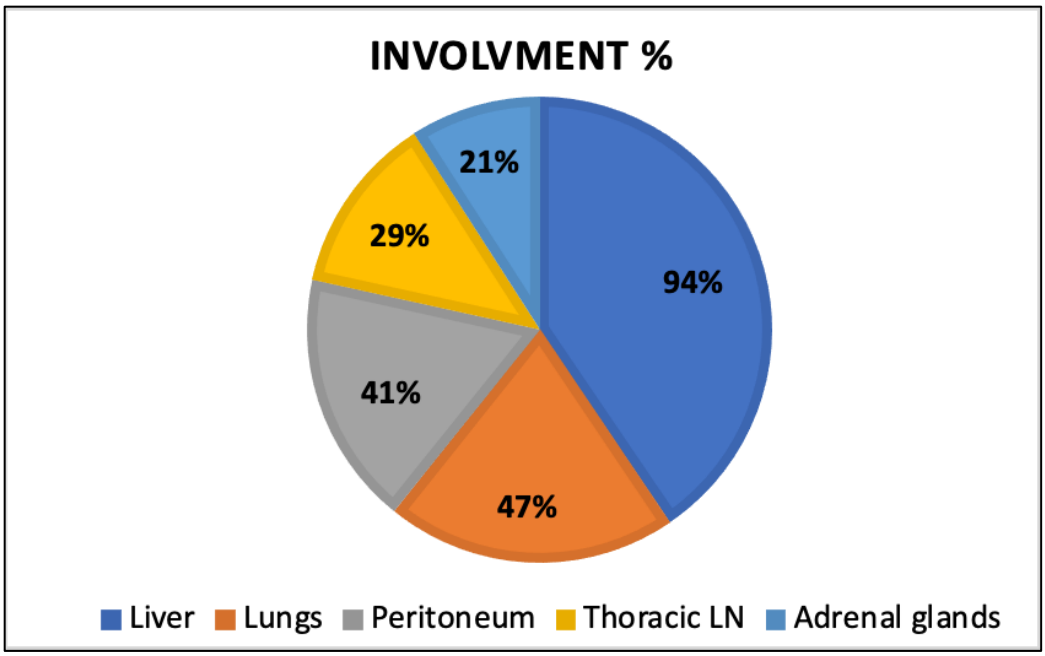
Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies and the fourth leading cause of cancer-related deaths in the world **[1]**. Moreover, it is projected to be the second leading cause of cancer-related death within the next decades **[2]**. Worldwide pancreatic cancers Burden, risk factors, and Trends **[3]**. According to the World Cancer Research Fund International, Hungary had the highest overall rate of pancreatic cancer in 2020, followed by Uruguay and Japan (Figure 1). The majority of pancreatic cancers are discovered to be metastatic at the time of initial diagnosis, making it challenging to make an early diagnosis. Due to the lack of early-stage detection of this cancer, only 10–15% of them can be surgically removed. The majority of individuals with pancreatic cancer will die from metastasis, which mainly occurs in the liver, lungs, and peritoneum (Figure 2) **[4]**. KRAS mutation is the most frequent mutation which initiates the genetic event of PDAC. Almost all PDAC cases have activating mutations of the KRAS protein, an isoform of the RAS protein, which occurs during the early phases of malignant transformation **[5]**. Oncogenic KRAS signaling is the main driving force for the downstream activation of RAF/MEK/ERK and PI3K/AKT signaling, which promotes a large array of cellular activities including proliferation, apoptosis, differentiation, and transformation **[6]**. These results provide further evidence that mutations in the KRAS proto-oncogene contribute to the pathogenesis of pancreatic cancer and identify downstream ERK and AKT signaling pathways as promising drug targets for the treatment of PDAC **[7]**. Currently, gemcitabine is the only standard treatment for patients with unresectable locally advanced or metastatic pancreatic cancer for over a decade. Gemcitabine and nab-paclitaxel, along with the FOLFIRINOX treatment, have recently increased survival rates compared to gemcitabine alone.

Unfortunately, PDAC responds poorly to all current treatments because of its notorious characteristic resistance to all conventional chemotherapeutic regimens **[8]**. Moreover, therapies targeting PDAC-associated molecular pathways, such as RAF/MEK/ERK signaling inhibitors, have not provided satisfactory results **[9]** partly due to the rapid up-regulation of compensatory alternative pathways and feedback loops within tumor cells. As a result, there is an urgent requirement for the discovery of novel therapeutic strategies to combat this lethal cancer.

Rank	Country	Number	ASR/100,000
	World	*495,773*	*4.9*
1	Hungary	2,499	11.2
2	Uruguay	732	10.7
3	**Japan**	**44,307**	**9.9**
4	Slovakia	1,078	9.6
5	Czechia	2,466	9.5
6	Austria	2,011	9
7	Armenia	437	8.9
8	Estonia	295	8.9
9	Malta	98	8.9
10	Germany	21,541	8.8

Ref: World Cancer Research Fund

Figure 1: Total global pancreatic cancer incidence and rates in 2020 according to World Cancer Research Fund



Ref: Embuscado, Erlinda E et al.

Figure 2: Frequency of metastatic involvement by pancreatic cancer to various organ sites. The liver, then the lungs, and finally the adrenal glands, are the most common places where cancer spreads.

1.2 The molecular biology of pancreatic cancer:

The molecular mechanisms of solid cancer are extremely complicated, with different mechanisms taking place and influencing the tissue at various phases of the disease. Since the fundamental causes of pancreatic cancer were discovered in the 1970s and 1980s **[10]**, to understand pancreatic cancer in evolutionary terms, Makohon-Moore and Iacobuzio-Donahue *et al*. characterize pancreatic cancer in three broad stages **[11]**.

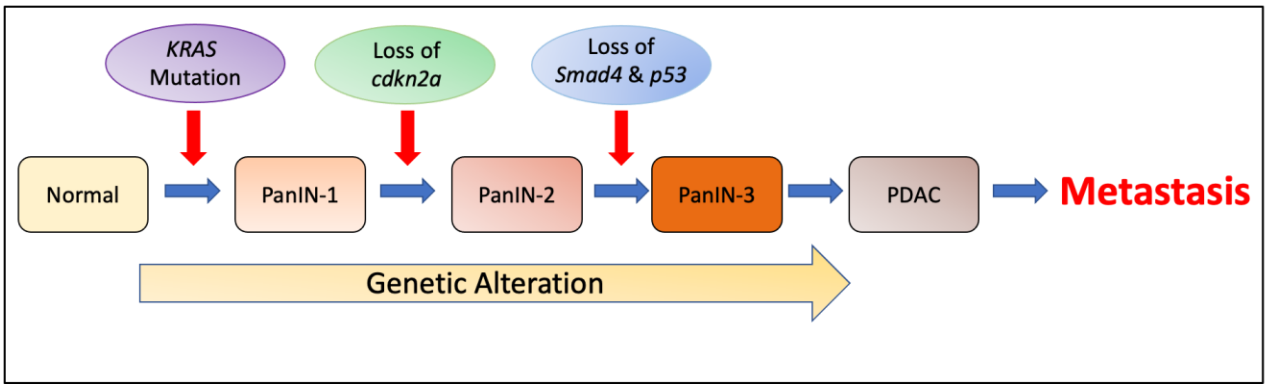
- **Initiation stage:** Environmental exposure or DNA repair failure causes a normal pancreatic cell's initial driver gene mutation. In most cases, the mutation causes apoptosis, immune surveillance, or tissue turnover. When

this mechanism fails, cells with the initiating mutation escape senescence and immunosuppression, maintaining fixation for survival or growth.

- **Clonal expansion:** In this stage, the cell carrying the driver gene mutation continues to divide, and its descendants eventually pick up more driver gene alterations and passenger mutations.
- **Introduction to foreign microenvironments:** At this stage, continuous clonal proliferation might result in a population of cells that penetrate the basement membrane and enter the stroma. These disseminated cells have the best chance of colonizing other microenvironments, such as the liver, lung, or peritoneum.

1.3 Progression of pancreatic cancer:

Pancreatic intraepithelial neoplasia, or PanINs, are precursor lesions that lead to pancreatic cancer. These lesions affect the exocrine pancreas tiny ducts and can be categorized as PanIN-1 (low-grade dysplasia), PanIN-2 (moderate dysplasia), or PanIN-3 (high-grade dysplasia) lesions depending on the degree of cytologic atypia. Genetic alterations due to microdissection of these lesions indicate that activation of KRAS mutation is a relatively early event and found in >99% of PanIN-1 lesions followed by inactivation mutation in CDKN2A in PanIN-2 lesions and PanIN-3 lesions show inactivating mutations in TP53 and SMAD4. Taken as a whole, these findings are in favor of a genetic progression model of pancreatic carcinogenesis that results in the development of an invading tumor (Figure 3) **[12]**.



Ref: Yachida, S. *et al.*

Figure 3: Genetic progression model of pancreatic carcinogenesis. Early events include telomere shortening and activating mutations in KRAS (PanIN-1), intermediate events involve inactivating mutations or epigenetic silencing of CDKN2A (PanIN-2), and late events involve inactivating mutations of TP53 and SMAD4 (PanIN-3).

KRAS: Pancreatic ductal adenocarcinoma solely harbors the KRAS mutation, which is the most common isoform mutated in PDAC malignancy. Oncogenic KRAS mutations are found in approximately 90% of PDAC and may contribute to the poor prognosis of these tumors **[13]**. Activated KRAS initiates many different pro-survivals signaling mechanisms including ERK mitogen-activated protein kinase (MAPK) and PI3K pathways **[14]**. KRAS mutations have been thoroughly described as drivers of pancreatic cancer pathogenesis and are among the initial genetic anomalies in the PDAC development model **[15]**. The most common activating point mutations (most often residing in codons 12 and 13) produce a constitutively active protein resulting in unrestricted growth signaling **[16]**. KRAS alterations are assumed to be an early event in IPMN and PanIN biogenesis because they are present in all types without significant differences **[17]**. KRAS mutations predominate pancreatic cancer, and lung and colon cancer. on the other hand, NRAS mutations predominate in cutaneous melanomas and acute myelogenous leukemia, and HRAS mutations are found in bladder and head and neck squamous cell carcinomas **[7]**.

TP53: The TP53 gene encodes a tumor suppressor protein. By affecting multiple genes expression, TP53 can also have an impact on the microenvironment of PDAC. The PDAC microenvironment is altered by the loss of TP53's normal functions, which encourages inflammation that is tumorigenic. Tumor growth and metastasis are accelerated by mutated TP53, which can change the PDAC microenvironment's immunosuppressive characteristics **[18]**. By increasing the number of T cells, TP53 boosts the immune response in the PDAC microenvironment and makes dendritic cells (DC) more effective **[19]**. The inhibitor of mouse double minute 2 (MDM2), nutlin-3a, was used to determine this. WT TP53 inhibits IL-6 expression, while mut-TP53 cells exhibit greater levels of IL-6 in the PDAC microenvironment. Metastasis is associated with elevated IL-6 expression **[20]**. Mut-TP53 has been shown to promote metastasis by inducing NF-B activity, which in turn promotes the expression of inflammatory cytokines like IL-6 and tumor necrosis factor- (TNF-) **[21].**

CDKN2A*:* The expression of CDKN2A is another gene that is frequently changed in PDAC. CDKN2A is a gene on chromosome 9q21 that encodes the highly complex tumor suppressor proteins p16/INK4A and p14ARF. p16/INK4A and p14ARF, which are typically tumor suppressor proteins, inhibit the progression of the cell cycle. By reducing the activity of CDK4 and CDK6, p16/INK4a normally prevents the progression of the cell cycle. By preventing MDM2 from being degraded and thereby disrupting the TP53 protein, the p14ARF protein causes cell cycle arrest. Reduced T and B cell infiltration in the PDAC microenvironment has been linked to CDKN2A mutation **[22]**. Loss of CDKN2A causes more genomic instability, thereby allowing for the accumulation of additional mutations **[23]**.

SMAD4: SMAD4 is one of the tumor suppressor proteins. In 60% of PDAC cases, the SMAD4 gene is inactivated due to homozygous deletion or mutation. When SMAD4 is inactive, TGF responses are altered, favoring non-canonical TGF

signaling, which is associated with pro-tumorigenic responses **[24]**. Loss of SMAD4 occurs mainly in the later stages, PanINs 3-4. A significant remodeling of the tumor microenvironment occurs concurrently with the genetic alterations that support oncogenic transformation and ultimately give rise to PDAC.

1.4 MAPK signaling and tumorigenesis

Receptor tyrosine kinases and G protein-coupled receptors both induce a RAS conformational change, which in turn activates the MAPK/ERK pathway **[22]**. The Ras family of small GTP-binding proteins includes the well-known proteins HRAS, KRAS, and NRAS **[25,26]**. Receptor activation via ligand interaction transforms GDP-bound Ras (inactive) into GTP-bound Ras (active). Activated Ras recruits and activates Raf, triggering the MAPK/ERK pathway. MAPK cascades are important signaling pathways that control a variety of cellular processes, such as proliferation, differentiation, apoptosis, and stress responses **[27]**. The Ras/Ras/MEK/ERK MAPK pathway is frequently deregulated in numerous human cancers as a result of genetic alterations in their components **[28]**. Approximately 30% of human cancers are caused by activating mutations in Ras codons 12, 13, 59, or 61, which results in Ras's constitutive activation and carcinogenesis **[29]**. With this knowledge, the Ras/Raf/MEK/ERK signaling pathway has been considered a promising therapeutic target for cancer therapy.

1.5 The phosphoinositide 3-kinase (PI3K) pathway & tumorigenesis

The PI3K/AKT pathway is also activated by receptor tyrosine kinases (RTKs) or G-proteins coupled receptors (GPCRs). After being activated, PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce phosphatidylinositol 3,4,5-trisphosphate (PIP_3) **[30]**. PIP_3 acts as a scaffold to recruit PDK1 and AKT through the pleckstrin homology (PH) domain to the plasma membrane, where PDK1 phosphorylates AKT at Thr308 in the kinase domain **[31]**. Activated AKT phosphorylates downstream effectors to drive carcinogenesis. In the pancreas,

AKT contributes as a regulator of cell plasticity, and research has shown that pancreatic cancer frequently exhibits its overexpression **[32]**. PDAC is frequently associated with increased AKT activity, which has been detected in 60% of PDAC samples, with AKT2 oncogene amplification occurring in 10%-20% of PDAC cases. Furthermore, PDAC tumors have been shown to have an activating mutation in PIK3CA and/or loss of the tumor suppressor phosphatase and tensin homolog (PTEN) in 4% and 25%-70% of cases, respectively. Interestingly, patients with low PTEN expression have a much higher incidence of recurrence or metastasis than those with high PTEN **[33]**. Moreover, it has been reported that PDAC patients with high PI3K pathway activity have a significantly lower survival rate than those with low PI3K pathway activation **[33]**. Therefore, PI3K/AKT hyperactivation is a negative prognostic marker of cancer, as it is linked to poor differentiation and a poorer prognosis.

1.6 Regulation of Ras/Raf/MEK/ERK & PI3K/AKT pathway:

Ras is a small GTP-binding protein that functions as the upstream molecule in several signaling pathways, including Raf/MEK/ERK, PI3K/AKT, and RalEGF/Ral **[34]**. The Ras/Raf/MEK/ERK signaling pathway contains four core protein kinases: Ras, Raf, MEK, and ERK. Ras, Raf, and MEK are members of multi-gene families; Ras has three members (KRAS, NRAS, and HRAS), Raf has three members (A-Raf, B-Raf, and Raf-1) and MEK has five gene family members (MEK1, MEK2, MEK3, MEK4, and MEK5) **[35]**. The Ras/Raf/MEK/ERK signaling pathway is activated at the cell surface by ligand binding to receptor tyrosine kinases (RTK), followed by phosphorylation of four core protein kinases, Ras, Raf, MEK, and ERK, which regulates gene transcription **[36]**. Ras is a membrane-bound small GTPase that is essential for transducing responses to various extracellular signals that influence various cellular processes, most notably cell proliferation, differentiation, and apoptosis. Activation of Ras proteins by numerous cell surface molecules in turn activates MAPK cascades such as RAF/MEK/ERK and PI3K/AKT, a major effector

pathway of Ras that has a well-defined role in cancer (Figure 4). The key negative feedback regulator of the MAPK/ERK and PI3K/AKT signaling pathway members associated with cellular proliferation and differentiation is dual-specificity phosphatase (DUSPs) and PTEN. The PI3K/AKT/mTOR pathway is activated by Ras-dependent and Ras-independent mechanisms and shares a convergence point with MAPK pathways downstream of growth factors and G protein-coupled receptors **[6]**. The AKT gene and its downstream regulatory effector genes, including receptor tyrosine kinases (RTKs), PI3Ks, PTEN, and Ras, are frequently amplified or mutated in human tumors **[37]**. Signaling events are initiated by Ras introduced through Raf, MEK, and ERK1/2 (also called MAPK1/3) and transduced to the nucleus. During tumorigenesis, the cell loses control over its growth and develops the ability to invade. Uncontrolled cell growth and proliferation are encouraged by aberrant activation of the PI3K/AKT and/or MAPK/ERK pathways, which drive the development of cancer **[37]**.

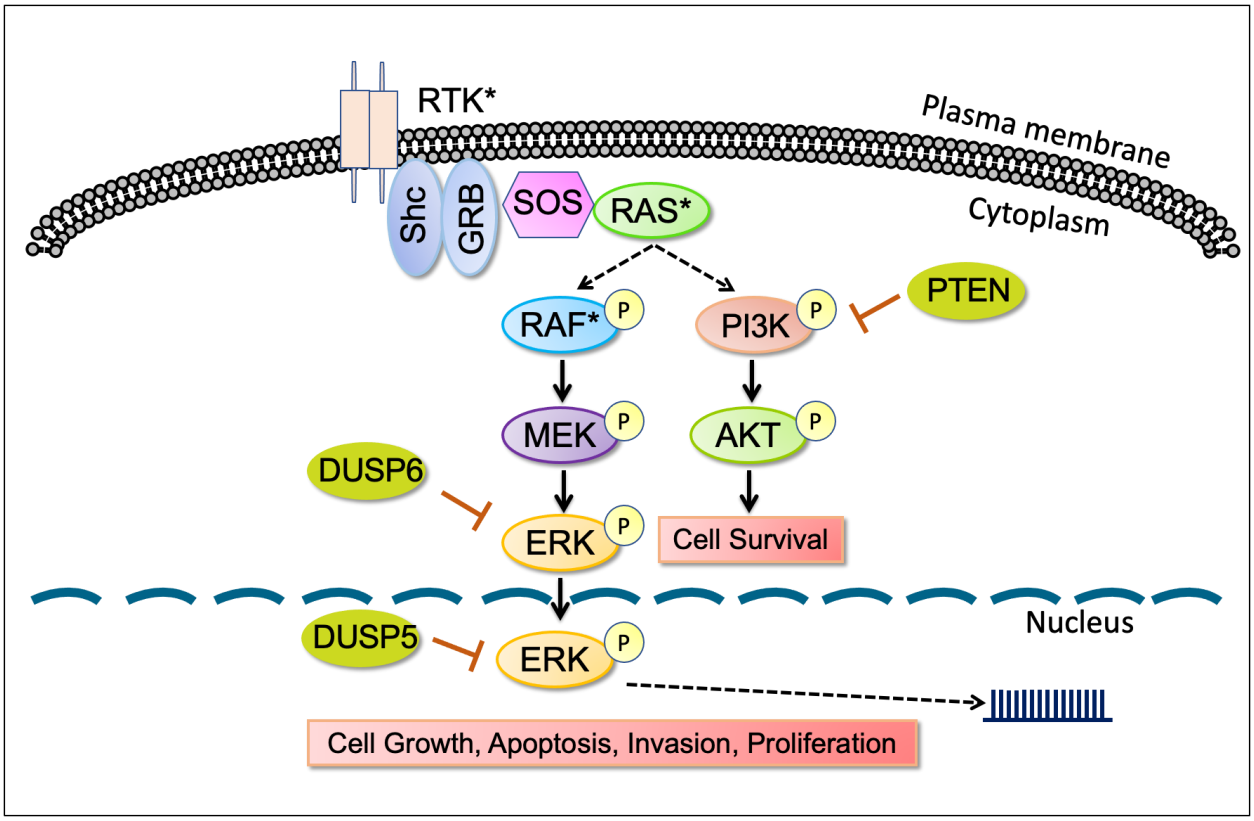


Figure 4: Signal transduction of MEK/ERK and PI3K AKT pathway. The PI3K/AKT and MAPK/ERK pathways are both important cellular signaling cascades with large functional overlaps in cell growth, proliferation, and survival/apoptosis, and are both activated by RTKs. Ras act as a node of AKT and ERK signaling functions as a binary switch. Activated RAS recruits and activates Raf, triggering the MAPK/ERK and PI3K/AKT pathways.

1.7 Current hurdles of PDAC treatment:

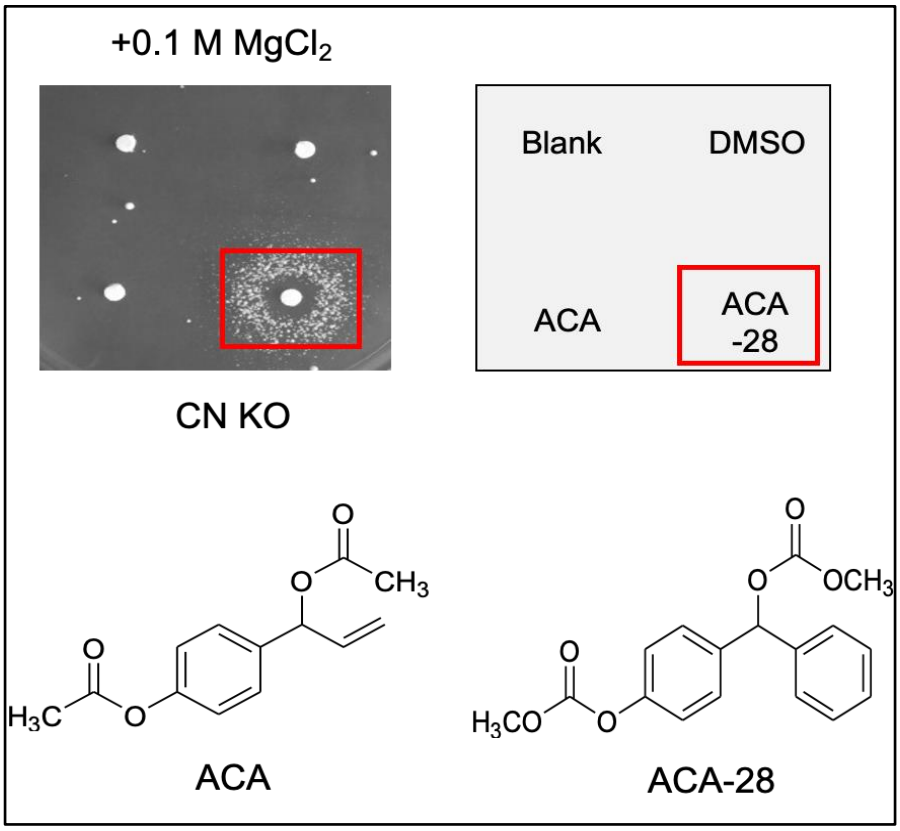
Pancreatic cancer is notoriously a "silent" disease in its early stages, with no symptoms, making diagnosis even more challenging. Physical effects don't show up until cancer has spread throughout the body and reached an advanced stage. Once detected, medicine must be able to offer a treatment that provides a meaningful extension of life to the patients suffering from this dreaded disease. Surgical resection of the primary tumor remains the only curative option for

pancreatic cancer. Over the past 30 years, PDAC patients' life expectancy has only slightly increased by a few months **[38]**. Even with combination regimens, the 5-year survival rate for PDAC patients in the SEER database (2012-2018) is incredibly low (11.5%), despite the fact that the current therapeutic strategies (i.e., surgery, chemotherapy, and radiation therapy) do confer an improvement in outcomes compared to no treatment. Gemcitabine and Nab-Paclitaxel have poor clinical outcomes on overall survival when compared to FOLFIRINOX **[39].** FOLFIRINOX therapy, while more effective, has its uses constrained by its associated toxicities and poor quality of life **[40].** The heterogeneity of pancreatic cancer is greatly influenced by abnormal activation or deregulation of various signaling pathways. The MAPK signaling cascade is one such complex interconnected signaling cascade that is frequently involved in oncogenesis, tumor progression, and drug resistance **[41]**. The MAPK family consists of a large number of kinases that are altered in cancers for which many targeted therapies have been developed. Targeting KRAS, EGFR, PI3K-AKT and other genes have been shown to be an effective strategy for the treatment of PDAC **[42].** Targeted therapy, using different small molecules such as Vemurafenib exert their effects by inhibiting ERK activation **[43]**, can induce resistance, in some cases even from the first doses, and long-term administration of these drugs often results in the appearance of drug tolerance, which greatly hampers the efficacy of the drugs and the survival of patients. In addition, the FDA has approved the PI3K selective inhibitor Idelalisib as a monotherapy for relapsed follicular B cell non-lymphoma Hodgkin's and relapsed small lymphocytic leukemia (SLL), but more than 50% of patients experienced serious side effects **[37]**. Many small molecule inhibitors that target both PI3K/AKT and MAPK/ERK signaling cascades combinedly have been developed and tested in preclinical models due to their potential to combat cancer's oncogenic role and aberrant activation of these pathways. However, this treatment approach has yet to yield promising results in clinical practice due to the presence of unacceptable side effects and the limitation of exposure

doses **[37]**. Resistance to MAPK inhibitors is a current issue, owing to the high number of interactions and potential compensatory responses. Therefore, developing novel strategies for cancer therapy is imperative.

1.8: Compound Introduction:

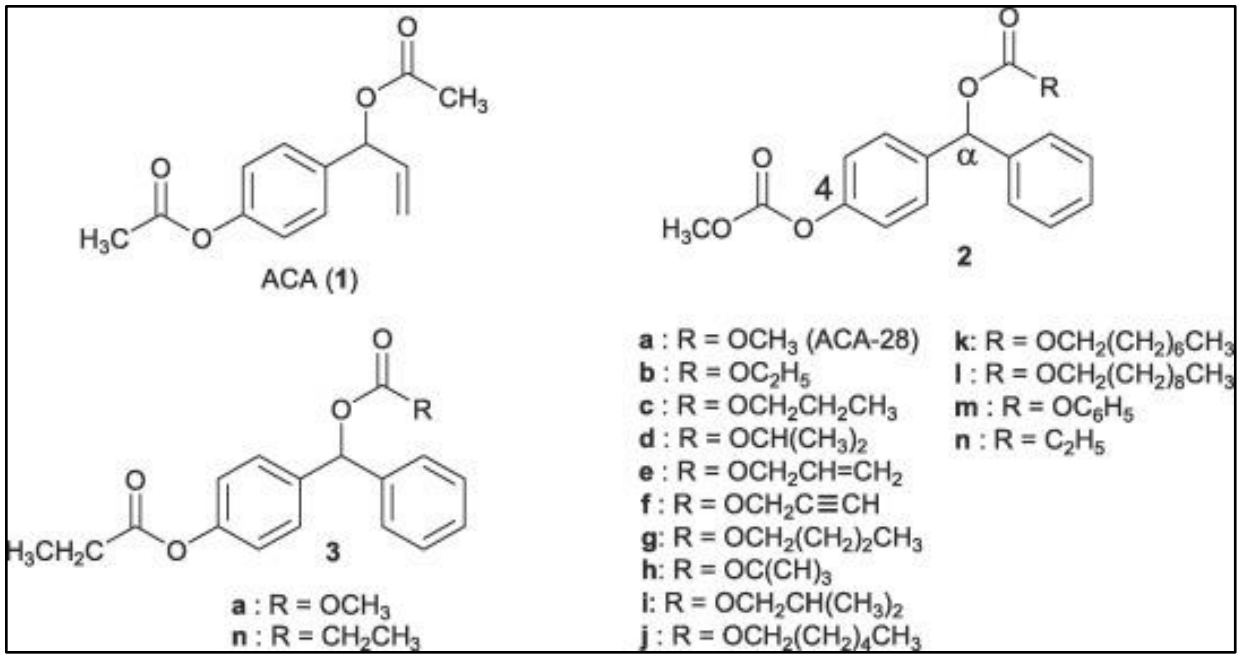
ACAGT-007a (GT-7): GT-7 was discovered through a molecular genetic screen employing a fission yeast phenotypic assay **[44]**. This test involves the calcineurin (CN) knockout phenotype and its reversal through MAPK inhibition **[44]**. In our previous research, Sugiura's lab conducted a chemical genetic screening against a pool of 1'-acetoxychavicol acetate (ACA) derivative compounds. This screening was performed because ACA has been shown to have a variety of important biological activities, such as the ability to combat cancer and inflammation. This screen has identified 4-(methoxycarbonyloxy) benzhydryl methyl carbonate (ACA-28 **(2a)**) as a novel ERK signaling modulator and displayed that ACA-28 kills human melanoma cancer cells more effectively than it kills normal human melanocytes **[44]**.



Ref: Satoh. *et al. 2017*

Figure 5: Effects of ACA and its derivative ACA-28 on the Cl^--sensitive cell growth of CN KO cells. Detection of cell growth recovery of CN KO cells in the presence of 0.1M $MgCl_2$ by an ACA derivative ACA-28, but not by ACA. Chemical structures of parental 1′-acetoxychavicol acetate (ACA) (left) and its derivative ACA-28 (right).

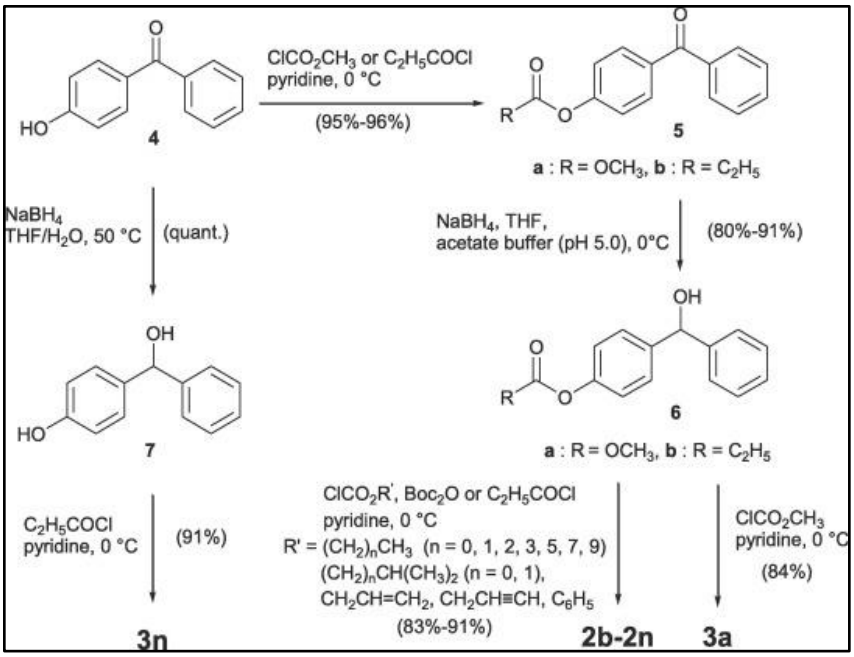
In order to develop new derivatives of ACA-28 (**2a**), the structural basis of ACA-28, which offers ERK-dependent apoptosis in cancer cells in a selective manner, was analyzed. This allowed for the development of new derivatives of ACA-28 (**2a**). By concentrating on two carbonate areas in the structure of ACA-28**(2a)**, specifically at the positions of C4 and Ca, a total of fifteen analogs, including **3a** and **3n**, were able to be synthesized (Fig. 6).

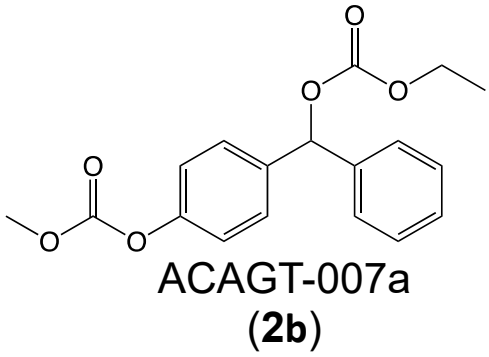


Ref: Satoh. *et al. 2020*

Figure 6: Chemical structures of ACA (**1**), ACA-28 (**2a**), and analogs (**2b**–**2n**, **3a**, and **3n**) of **2a**.

SAR studies were performed where the structure of ACA-28(**2a**) was modified by C2 or C3 alkyl moiety as a component of the carbonate unit at the Ca carbon and synthesized a potent derivative of ACA-28 **(2a)**, ACAGT-007a (**2b**) in terms of growth inhibition as well as induction of apoptosis. (Figure 7) **[45].**





Ref: Satoh. *et al. 2020*

Figure 7: Syntheses of analogs (**2b**–**2n**, **3a**, and **3n**) of **2a**.

In this thesis, I showed that GT-7 induced apoptosis in T3M4, a PDAC cell line containing the KRAS Q61H mutant allele, by stimulating ERK activation. In pancreatic ductal adenocarcinoma (PDAC), the presence of multiple KRAS mutations has been linked to a wide range of clinically significant phenotypes and drug resistance **[46]**. I examined three PDAC cell lines with different KRAS mutations MIA-Pa-Ca-2 (KRAS G12C), T3M4 (KRAS Q61H), and PANC-1 (KRAS G12D)). T3M4 cells, but not the other two PDAC cell lines, were successfully subjected to GT-7-induced ERK-dependent apoptosis. In order to determine whether the cells are resistant to apoptosis, I also demonstrated the variations in AKT phosphorylation levels following treatment with GT-7. Consistently, apoptosis was induced more strongly by co-treatment with GT-7 and the PI3K/AKT signaling inhibitor Wortmannin than by GT-7 alone. These results showed the therapeutic potential of the GT-7 combination therapy for PDAC.

In this thesis, I will discuss the possible mechanisms by which GT-7 induces apoptosis in PDAC cell lines in relation to the relevant KRAS mutations and the downstream ERK and AKT signaling.

2. Materials and Methods

2.1. Cell Culture

The human pancreatic ductal adenocarcinoma (PDAC) cell lines MIA-Pa-Ca-2, T3M4, and PANC-1 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque, Kyoto, Japan), supplemented with 10% fetal bovine serum (BioWest, Nuaillé, Pays De La Loire, France), sodium pyruvate, phenol red, and L-glutamine. The cells were maintained in a humidified incubator containing 10% CO_2 at 37°C. Every second-third day, the medium was changed, and cells were subcultured with 0.25% Trypsin-EDTA (Gibco, Invitrogen, Carlsbad, CA, USA) at 37°C when con-fluency reached 70-80%. Their genetic profiles are shown in table. **[46,47,48]**

Feature	MIA-Pa-Ca-2	PANC-1	T3M4
Mutation Status	p.G12C	p.G12D	p.Q61H
Source of Tumor	Primary	Primary	Lymphatic Metastasis
Differentiation stage	Poorly	Poorly	Moderately
Grade	G3	G3	G2
Migration	-	Yes	-
ERK Activation	Able to activate	Low	High
AKT Activation	Low	Strong	Low

Figure 8: The genetic profile of Pancreatic cancer cells

2.2. Chemicals and Reagents

A) ACAGT-007a (GT-7) was synthesized as described above (described as **2b**) and dissolved in DMSO.

B) Honokiol (H669560) was purchased from Toronto Research Chemicals (Toronto, ON, Canada) and dissolved in DMSO.

C) U0126 (U-6770) was purchased from LC Laboratories (Woburn, MA, USA) and dissolved in DMSO.

D) Wortmannin (HY-10197) was purchased from MedChemExpress (Monmouth Junction, NJ, USA) and dissolved in DMSO.

E) Perifosine (P-6522) was purchased from LC Laboratories and dissolved in water.

2.3. WST-8 Assay

The assay was carried out using the Cell Count Reagent SF (Nacalai tesque, Kyoto, Japan) according to the manufacturer's instructions with small modifications. Briefly, 100 µL of mammalian cell suspension at a cell density of 5.0×10^4 cells/mL was seeded in a 96-well plate (IWAKI, Shizuoka, Japan) and incubated for 24 h. In each medium, the compounds in solution were diluted 1:1000, and 100 µL of the diluted compounds were added to the cell culture. Cells treated with solvent (DMSO) were used as controls. After incubation for 48 h, 6 µL of the Cell Count Reagent SF and 40 µL of the medium were mixed and added to the plate, followed by incubation for an additional 3 h. Then, a Sunrise microplate reader measured the absorbance at 450 nm (Tecan, Männedorf, Switzerland). Absorbance at 600 nm was also measured as the reference.

2.4. Protein Extraction and Western Blot Analysis

One milliliter of the PDAC cell suspension at a cell density of 2.0×10^5 cells/mL was seeded in a 6-well plate (IWAKI, Shizuoka, Japan) and incubated for 24 h. After 24 h incubation, the medium was aspirated. Then, the cells were treated with chemicals required for the respective experiments as described in each figure

legend. Harvested cells were lysed as previously described **[44]**. The following primary antibodies were used: anti-GAPDH (14C10) Rabbit mAb (Cell Signaling Technology, Danvers, MA, USA, #2118), anti-Caspase-3 Antibody (Cell Signaling Technology, Danvers, MA, USA, #9662), anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody (Cell Signaling Technology, Danvers, MA, USA, #9101), anti-p44/42 MAPK (Erk1/2) Antibody (Cell Signaling Technology, Danvers, MA, USA, #9102), anti-Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb (Cell Signaling Technology, Danvers, MA, USA, #4060), anti-Akt (pan) (C67E7) Rabbit mAb (Cell Signaling Technology, Danvers, MA, USA #4691), anti-MKP-3 Antibody (G-4) (Santa Cruz Biotechnology, Dallas, TX, USA, sc-137246), and anti-PTEN (138G6) Rabbit mAb (Cell Signaling Technology, Danvers, MA, USA, #9559). As secondary antibodies, anti-rabbit (#7074) or anti-mouse (#7076) IgG HRP-linked antibodies (Cell signaling Technology, Danvers, MA, USA) were used. The proteins were detected by Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan) or ECL Select (Cytiva, Marlborough MA, USA). Relative intensities of all bands were quantified using MULTI GAUGE Ver. 3.2 software (Fujifilm, Tokyo, Japan).

2.5. Small Interfering RNA Transfection

For small interfering RNA (siRNA) experiments, a human PTEN siRNA (5′-CCACACGACGGGAAGACAAGUUCAU-3′, 5′-AUGAACUUGUCUUCCCGUCGUGUGG-3′) and a control siRNA (Stealth RNAi™ Negative Control Med GC Duplex) were purchased from Invitrogen USA (PTEN siRNA; VHS41286 and control siRNA; 12935300). One milliliter of 1.25×10^5 cells/mL was seeded in a 6-well plate (IWAKI, Shizuoka, Japan). A total of 7.5 µL of Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA; 13778150) was diluted in 125 µL of Opti-MEM® Reduced Serum Media (Gibco, Invitrogen, Carlsbad, CA, USA; 31985062). Simultaneously, 2.5 µL of 20 µM siRNA was also diluted in 125 µL of Opti-MEM® Reduced Serum Media. Then, diluted Lipofectamine™ and diluted siRNA were mixed (1:1 ratio) and incubated for 10

min at room temperature. Finally, the siRNA–lipid complex was added to the seeded cells, and the transfected cells were incubated for 48 h.

2.6. Microscopy

One milliliter of the PDAC cells suspension at a cell density of 2.0 × 10^5 cells/mL was seeded in a 35 mm glass-bottom dish (Figure 11C; D11130H, MATSUNAMI, Osaka, Japan) or a 6-well plate (Figure 12F; IWAKI, Shizuoka, Japan), and incubated for 24 h. After the incubation, the compounds in solution were diluted at 1:2,000 by medium, and 1 mL of the diluted compounds were added to the cell culture. After an additional 24 h incubation, cells were observed by phase-contrast microscopy (BZ-X700/710, KEYENCE, Osaka, Japan) with a 10× (Figure 11C) or 4× (Figure 12F) magnification.

2.6. Flow Cytometry (FCM) Analysis of Apoptotic Cell

One milliliter of the PDAC cells suspension at a cell density of 2.0 × 10^5 cells/mL was seeded in a 6-well plate (IWAKI, Shizuoka, Japan) and incubated for 24 h. After 24 h incubation, the cells were treated with chemicals required for the respective experiments as described in each figure legend. Apoptotic cells were stained by an eBioscience™ Annexin V-FITC Apoptosis Detection Kit (eBioscience, San Diego, CA, USA). Chemi-cal-treated cells were washed by 1 mL of PBS(–), then resuspended by 100 µL of Binding Buffer (1×). In total, 2.5 µL of Annexin V-FITC and 5 µL of Propidium Iodide (PI) (20 µg/mL) were added. After 10 min incubation at room temperature, cells were diluted by 500 µL of PBS(–) and analyzed using FACS Calibur and LSR-Fortessa flow cytometers (BD Bio-sciences, Franklin Lakes, NJ, USA) and FlowJo (BD Biosciences, Franklin Lakes, NJ, USA).

2.8. RNA Isolation and qRT-PCR

Total RNA was isolated from PDAC cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In total, 0.1 μg of total RNA was reverse transcribed using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka Japan) according to the manufacturer's instructions. Finally, 500-fold diluted cDNA was amplified using LightCycler® 480 SYBR Green I Master (Roche, Basel, Switzerland) and primer sets, and the amplicon was detected and analyzed by LightCycler480 System II (Roche, Basel, Switzerland).

2.9. Statistical Analysis

Each experiment was performed 3 times. Representative data of at least three individual experiments were shown in Figures 9B, 10A, C, 11A, C and 12A, C, D, F. A Student's *t*-test was used to examine the differences between the two conditions in Figures 9A, 10B, and 12E. Repeated ANOVA was used for multiple comparisons in Figures 11B and 12B. *p* values of less than 0.05 are judged statistically significant. Values are shown as means ± standard error of the mean (SEM).

2.10. Data Access

Gene expression and mutation data were downloaded from the Cancer Cell Line Encyclopedia database (https://sites.broadinstitute.org/ccle/) (accessed on 29 August 2021).

3. Results

3.1 ACAGT-007a (GT-7) Reduces Cell Viability and Induces Apoptosis in PDAC Cells

Using an in vitro cell viability assay, the sensitivity to GT-7 of three PDAC cell lines with various genetic backgrounds for KRAS mutations (MIA-Pa-Ca-2, T3M4, and PANC-1) was evaluated (Figure 9A). T3M4 cells carry the KRAS Q61H mutation, which is found in approximately 5% of PDAC patients and is the most prevalent mutation occurring at codon 61 of KRAS **[47]**. The KRAS G12D mutation is found in PANC-1 cells, which is the most common form of the disease in PDAC. The KRAS G12C mutation is present in MIA-Pa-Ca-2 cells, which accounts for nearly 4% of all PDAC cases **[49]**.

In total, 30 μM of GT-7 treatment for 48 h suppressed the viability of MIA-Pa-Ca-2 cells to 18%, and that of T3M4 cells to 28%. In contrast, the viability of PANC-1 cells remained above 50% when treated with 30 μM of GT-7 as opposed to the vehicle, which indicates a relative resistance to GT-7(Figure 9A). The IC_{50} values of GT-7 for these cell lines were determined 48 h after treatment. (Figure 9A). To summarize, the MIA-Pa-Ca-2 cell line showed the most sensitivity to the GT-7 treatment (IC_{50}: 7.3 μM), whereas the PANC-1 cell line showed the least sensitivity (IC_{50}: 31.0 μM). T3M4 cells, with an IC_{50} value of 21.4 μM, displayed a sensitivity that was somewhere in the middle. I compared the IC_{50} values of GT-7 in these cell lines to those of Honokiol, a promising candidate for cancer prevention and/or treatment of pancreatic cancer **[50]**. GT-7 was shown to exhibit superiority in killing PDAC cell lines in comparison to Honokiol, as shown in Figure 9A. This is because Honokiol requires significantly higher concentrations to kill the three PDAC cell lines, whereas GT-7 only needs a single concentration of the compound to achieve the same result (Figure 9A). In addition, 15 μM of Honokiol did not have an effect on the viability of the three PDAC cell

lines. On the other hand, the same concentrations of GT-7 significantly inhibited the viability of the cells, with the exception of PANC-1. It is important to note that GT-7 effectively reduced the cell viability of MIA-Pa-Ca-2 cells, which have been reported to display malignant phenotypes among PDAC, including the EMT phenotype as well as chemoresistance **[51]**. To investigate whether the effects of GT-7 on the PDAC cell viability was because of the induction of cell apoptosis, the three PDAC cells were treated with 0, 10, and 30 μM GT-7 for 24 h and analyzed by flow cytometry (FCM) using Annexin V and propidium iodide (PI). The result showed that GT-7 caused a dose-dependent increase in apoptotic PDAC cells when stained with annexin V and PI (Figure 9B). At a lower concentration of 10 μM GT-7, the three PDAC cells are more apoptotic. T3M4 was most susceptible (77.1%), followed by MIA-Pa-Ca-2 (47.4%) (Figure 9B). Again, PANC-1 cells are relatively most resistant (32.2%). Higher concentrations of GT-7 (30 μM) caused more severe cell death in almost all cells (except PANC-1, which was 60.2%).

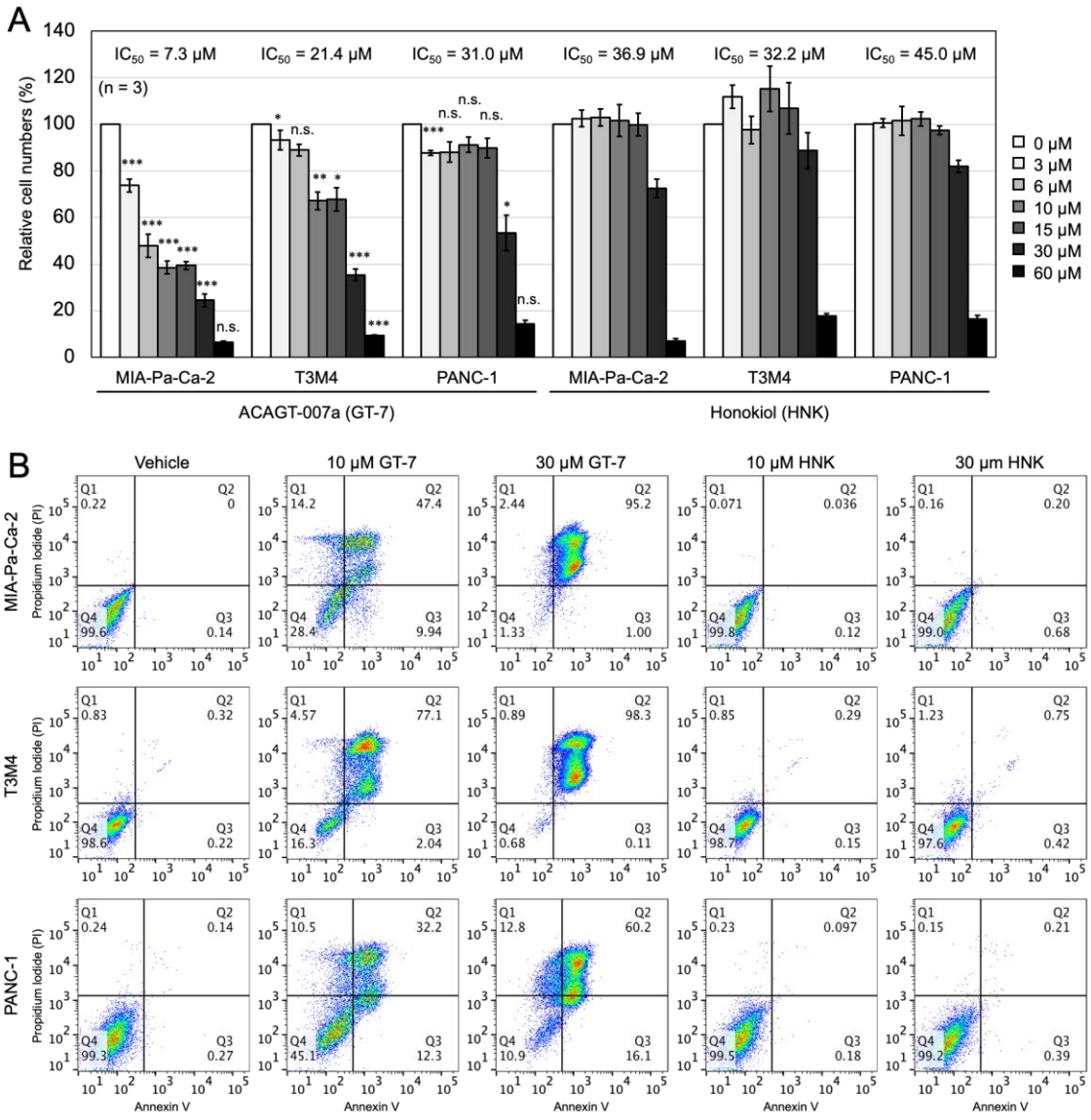


Figure 9: Effects of ACAGT-007a (GT-7) on cell viability and apoptosis of the PDAC cells. (A) Comparison of the cell viability upon GT-7 or Honokiol (HNK) in the PDAC cells. The PDAC cells were treated by GT-7 or Honokiol at serial concentrations (0–60 μM) for 48 h, then the relative living cell numbers (%) were measured by WST-8 assay. The data were averaged from three independent experiments (n = 3). Columns, means; bars, SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, n.s., not significant; significantly different between GT-7 and Honokiol in each concentration using a student's *t*-test. (B) Effect

of GT-7 and Honokiol (HNK) on apoptosis of PDAC cells. The PDAC cells were treated with GT-7 or Honokiol at the concentration indicated for 24 h, and then apoptotic cells were analyzed by FCM.

3.2. ACAGT-007 Induced ERK-Dependent Apoptosis in T3M4 but not in MIA-Pa-Ca-2 and PANC-1 Cells

To better understand the mechanism underlying GT-7-mediated apoptosis in the three distinct PDAC cell lines, I investigated its effect on ERK signaling and its relevance to apoptosis. Immunoblot analysis was performed utilizing anti-ERK and anti-phospho-ERK antibodies. All three PDAC cell lines showed dose-dependent stimulation of ERK phosphorylation in response to GT-7 treatment (Figure 10A, B). PDAC cells were exposed to GT-7 for a period of two hours, at which point the GT-7-induced activation of ERK could be observed. I also performed immunoblotting with anti-Caspase-3 antibodies in order to investigate whether GT-7 induced apoptosis in the three PDAC cells. The relative quantification of caspase-3 cleavage demonstrated that significant apoptosis induction was observed in T3M4 and MIA-Pa-Ca-2 cells in a dose-dependent manner mediated by GT-7 (Figure 10B). In contrast, cleaved Caspase-3 was barely detectable in PANC-1 cells, which once again indicated that PANC-1 cells are the most resistant to apoptosis induced by GT-7.

In order to determine whether the GT-7-induced apoptosis in PDAC cells was mediated by the stimulation of ERK phosphorylation, the effect of the MEK inhibitor U0126 was examined. U0126 was able to significantly inhibit the levels of ERK phosphorylation in three different PDAC cell lines both before and after the addition of GT-7 (Figure 10A, B). Notably, relative quantification of caspase-3 cleavage showed that the GT-7-mediated

induction of caspase-3 cleavage was blocked by the MEK inhibitor U0126 only in T3M4, but not in any of the other cell lines (Figure 10A, B).

FCM analysis was also carried out in order to determine the significance of ERK activation in the GT-7-mediated cell death mechanism (Figure 10C). For the purpose of determining whether three PDAC cell lines were susceptible to GT-7-mediated cell death, 10 μM GT-7 was used. T3M4 cells were the most susceptible, followed by MIA-Pa-Ca-2 cells, and PANC-1 cells were the most resistant, which was consistent with the data that was obtained in Figure 9B. The inhibition of ERK phosphorylation by U0126 resulted in a substantial decrease in the rate of cell death in T3M4 (from 79.5% to 38.6%), whereas in MIA-Pa-Ca-2, the rate of cell death decreased by only a marginal amount (from 47.4% to 44.1%), (Figure 10C). PANC-1 cells were resistant to U0126 (the percentage reduced from 32.0 to 31.0), (Figure 10C). These findings indicate that GT-7-induced ERK stimulation is necessary for the induction of apoptosis in T3M4 cells that harbor the KRAS Q61H mutation, which reproduced the findings in ERK-active melanoma cell lines with a variety of oncogenic mutations, including BRAF **[52]**.

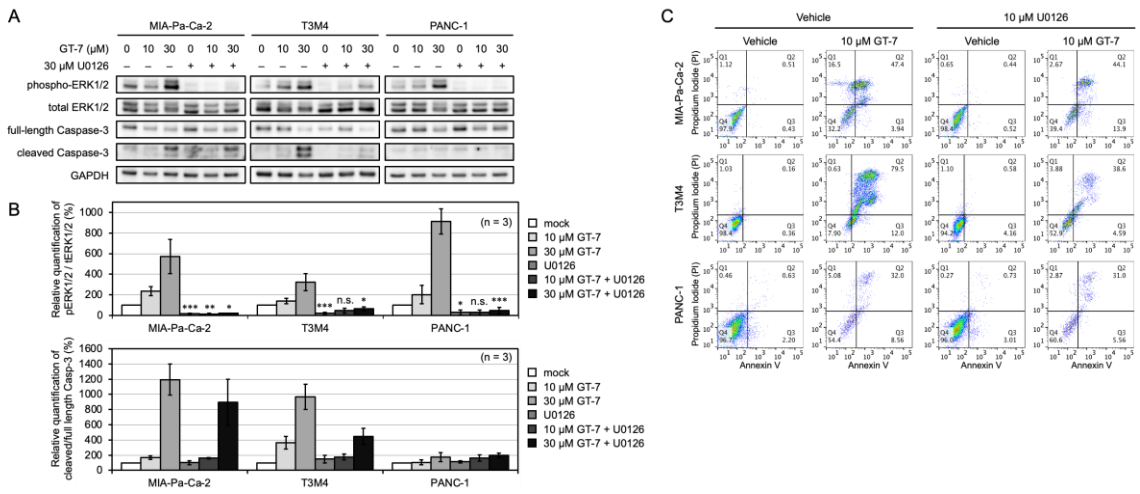


Figure 10: GT-7-induced apoptosis is dependent on ERK activation in T3M4 but not in MIA-Pa-Ca-2 and PANC-1 cells. (A) MEK inhibitor U0126 attenuates GT-7-induced apoptosis in T3M4 cells but not MIA-Pa-Ca-2 and PANC-1 cells. The PDAC cells were treated by GT-7 and/or U0126 for 2 h. The indicated proteins were detected by Western blot analysis. (B) Relative quantification of ERK phosphorylation levels and cleaved/full-length Caspase-3 in PDAC cells upon GT-7 and/or U0126 treatment as shown in (A). Phosphorylation levels (phosphorylated protein intensity/total protein intensity) after the treatment with DMSO (0 μM GT-7 without 30 μM U0126) in each PDAC cell were set as 100%. The data were averaged from three independent experiments (n = 3). Columns, means; bars, SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, n.s., not significant. Comparisons between absence and presence of 30 μM U0126 were made by a student's *t*-test. (C) Apoptosis induced by GT-7 was suppressed by the MEK inhibitor U0126 in T3M4 cells but not in MIA-Pa-Ca-2 and PANC-1 cells. MIA-Pa-Ca-2, T3M4, and PANC-1 cells were treated with DMSO (vehicle), 10 μM GT-7, 10 μM U0126, or the combination of 10 μM GT-7 and 10 μM U0126 for 24 h. Chemical-treated cells were stained by Annexin V-FITC and PI and analyzed by FCM.

3.3 AKT Signaling Activation Is Associated with PDAC Cells' Resistance to GT-7-Induced Apoptosis

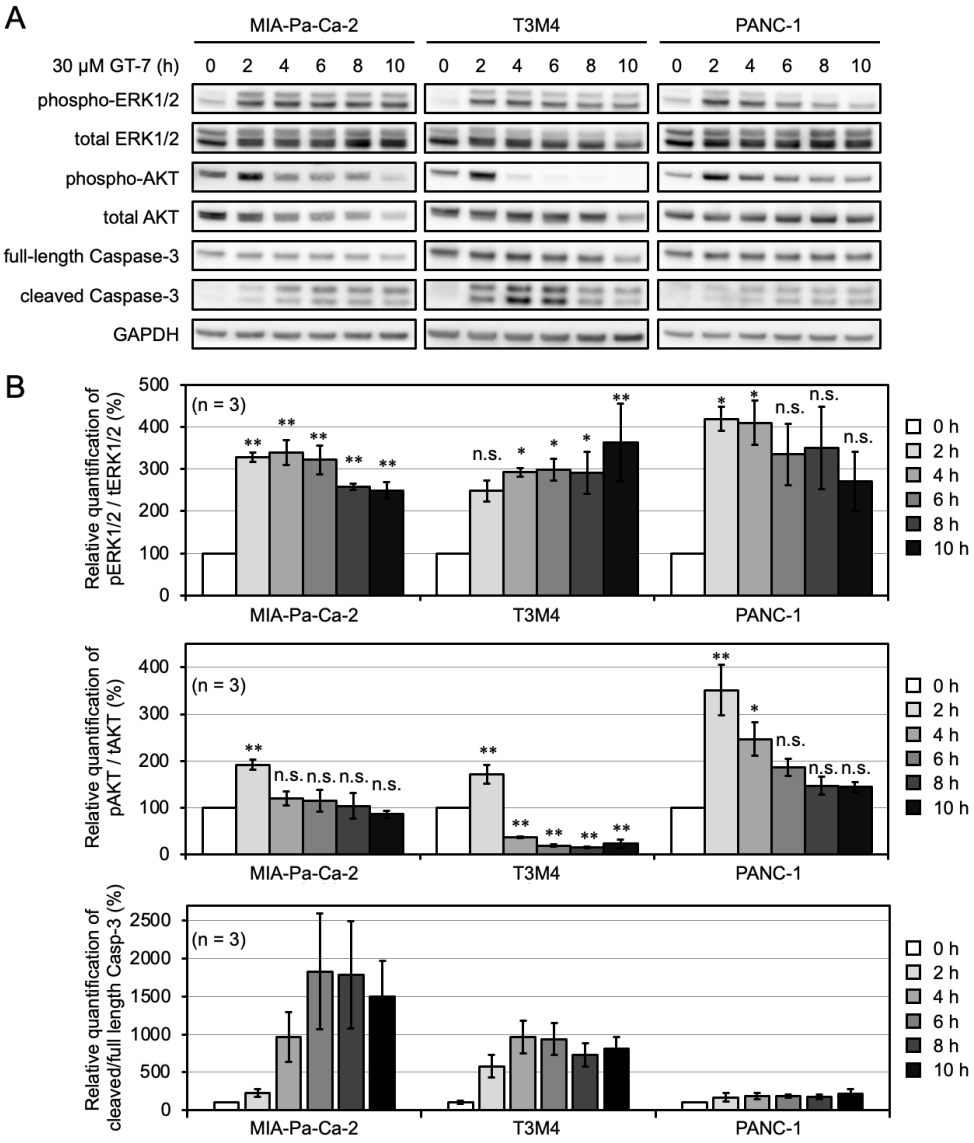
Next, I investigated the effect of GT-7 treatment on both the AKT and ERK signaling, as various KRAS mutations aberrantly activate downstream effector signaling, including the ERK and AKT signaling pathways. I used anti-phospho-ERK and anti-phospho-AKT (Ser473) antibodies, in order to investigate the time course of both ERK and AKT activation following treatment with GT-7. Within two hours of exposing the cells to 30 μM GT-7, ERK activation was observed, and this activation was observed to be sustained for a variable amount of time in three different PDAC cell lines (Figure 11A, B). PANC-1 cells sustained significant ERK activation for 4 hours, whereas MIA-Pa-Ca-2 and T3M4 cells maintained sustained ERK activation even after 10 hours of incubation with GT-7 ($p < 0.01$). Therefore, in terms of sustained ERK activation, which is one of the hallmarks of ERK-dependent apoptosis, PANC-1 showed less favorable ERK signaling dynamics when treated with GT-7 in comparison to MIA-Pa-Ca-2 and T3M4.

After being exposed to GT-7 for two hours, all three PDAC cell lines showed evidence of transient activation of the AKT signaling pathway ($p < 0.01$). (Figure 11A, B). Despite this, each of the three PDAC cells displayed a different magnitude and duration of AKT activation in response to being treated with GT-7. PANC-1 cells exhibited the most robust and long-lasting activation of the AKT pathway. AKT was markedly activated in PANC-1 cells after 2 hours of incubation (Approximately three times the basal level) and sustained and higher levels of AKT signaling activity were seen after extended exposure to GT-7 (Figure 11A, B). In terms of the levels of AKT activation, MIA-Pa-Ca-2 displayed an intermediate phenotype after being exposed to GT-7 for longer than 4 hours (Figure 11A, B). Notably, T3M4

showed a marked down-regulation of AKT phosphorylation levels below the basal levels after a transient increase in the AKT phosphorylation 2 hours after the GT-7 treatment (Figure 11A, B).

Additionally, I performed the time-course analysis of apoptosis induction in these PDAC cells following the addition of GT-7. Quantification of cleaved Caspase-3 demonstrated that significant apoptosis induction was detected in T3M4 and MIA-Pa-Ca-2 four hours after the addition of GT-7 (Figure A, B). Again, PANC-1 cells were not susceptible to the apoptosis that was induced by GT-7 (Figure 11A). Therefore, greater susceptibility to apoptosis induction by GT-7 in T3M4 cells as compared with MIA-Pa-Ca-2 cells is likely to be derived from a significant decrease in AKT phosphorylation in T3M4 cells after a transient peak in AKT phosphorylation. Phase-contrast images of the three PDAC cell lines treated with GT-7 throughout the time course reflect the cell death status determined using immunoblot against cleaved Caspase-3 (Figure 11C).

The time-course analysis of the ERK and AKT activation status in comparison with the cleaved Caspase-3 suggested that ERK activation was commonly observed in all three PDAC cells, which was less sustained in PANC-1 cells. Therefore, the ability of each PDAC cell line to maintain high levels of active AKT signaling correlates with the cells' resistance to the apoptosis-inducing effects of GT-7. PANC-1 showed the most sustained AKT activation, followed by MIA-Pa-Ca-2. In particular, a marked down-regulation of AKT phosphorylation below basal levels was observed in T3M4, coincident with the apoptosis induction that was detected by the Caspase-3 cleavage. Therefore, in order to make cells susceptible to GT-7-mediated apoptosis, activation of ERK may be required, along with inhibition of AKT signaling below a certain level.


A
MIA-Pa-Ca-2
T3M4
PANC-1
30 µM GT-7 (h)
0 2 4 6 8 10 0 2 4 6 8 10 0 2 4 6 8 10
phospho-ERK1/2
total ERK1/2
phospho-AKT
total AKT
full-length Caspase-3
cleaved Caspase-3
GAPDH
B
Relative quantification of pERK1/2 / tERK1/2 (%)
(n = 3)
Relative quantification of pAKT / tAKT (%)
(n = 3)
Relative quantification of cleaved/full length Casp-3 (%)
(n = 3)
MIA-Pa-Ca-2
T3M4
PANC-1
0 h
2 h
4 h
6 h
8 h
10 h
n.s.

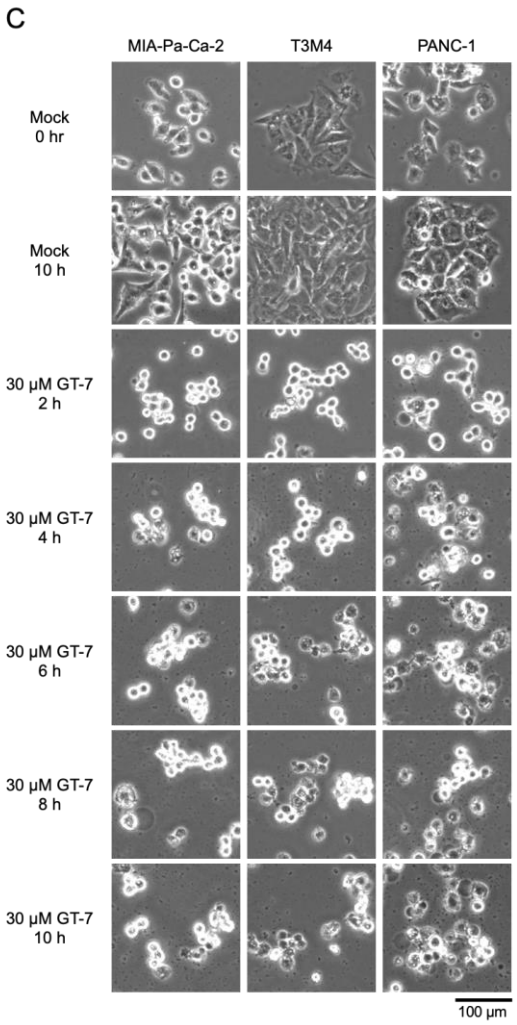


Figure 11: Apoptosis induction and the activation of both ERK and AKT signaling by ACAGT-007a (GT-7) in the PDAC cells. (A) The PDAC cells were treated with 30 μM GT-7 for the time indicated (0–10 h). The indicated proteins were detected by Western blot analysis. (B) Relative quantification of ERK and AKT phosphorylation levels and cleaved/full-length Caspase-3 in the PDAC cells upon GT-7 treatment as shown in (A). Phosphorylation levels (phosphorylated protein intensity/total protein intensity) after the treatment with DMSO (0 h) in each PDAC cell were set as 100%. The data were averaged from three independent experiments (n = 3). Columns, means; bars, SEM. * $p < 0.05$, ** $p < 0.01$, n.s., not significant; significantly

different from DMSO (0 h) in each cell line using one-way ANOVA, followed by a post hoc test using Dunnett's multiple comparisons. (C) Time-dependent (0-10 hours) phase-contrast images of PDAC cell lines as indicated in (A). Cells indicated were cultured as described in (A) in the presence of 30 μM of ACAGT-007a. Bars, 100 μm.

3.4 Inhibition of AKT by Wortmannin Enhanced the ACAGT-007a-Induced Apoptosis in MIA-Pa-Ca-2 and T3M4 Cells but Not in PANC-1 Cells

I investigated the effects of Wortmannin and Perifosine, two well-established inhibitors of PI3K and AKT, respectively, to determine whether the differences in the dynamics of AKT activation had any relationship with the cells' resistance to apoptosis induced by GT-7. Both before and after the addition of GT-7, the AKT activity in MIA-Pa-Ca-2 and T3M4 cells was significantly inhibited by Wortmannin. This was determined by measuring the levels of phospho-AKT at Ser473 in the cells (Figure 12A, B). Notably, in PANC-1 cells, Wortmannin was able to significantly inhibit phospho-AKT at the basal levels but failed to do so when GT-7 was added (Figure 12A, B). In a similar way, the addition of Perifosine to MIA-Pa-Ca-2 and T3M4 cells both before and after the addition of GT-7 resulted in a significant reduction of AKT phosphorylation levels (Figure 12A, B). Perifosine, however, only inhibited AKT phosphorylation at basal levels in PANC-1 cells, and GT-7-induced AKT phosphorylation was resistant to Perifosine (Figure 12A, B). A single treatment with Wortmannin or Perifosine did not induce apoptosis in any of the PDAC cell lines, as shown by the quantification of cleaved Caspase-3. This suggests that inhibition of PI3K/AKT signaling alone is not sufficient to induce apoptosis in PDAC cells (Figure 12A, B).

Importantly, the apoptosis induced by GT-7 was markedly enhanced by Wortmannin treatment in MIA-Pa-Ca-2 and T3M4 cells but not in PANC-1 cells (Figure 12A). The resistance of PANC-1 cells against Wortmannin in terms of enhancement of GT-7-mediated apoptosis may be explained by the residual AKT phosphorylation levels even after the Wortmannin treatment. In three PDAC cells, the addition of perifosine did not significantly increase apoptosis induction by GT-7(Figure 12A). This is probably also because of the residual levels of phosphorylation of AKT caused by the treatment with GT-7. As a result, the inhibitory effects of Wortmannin and Perifosine in

PANC-1 cells appear to be counteracted by the AKT phosphorylation induced by GT-7.

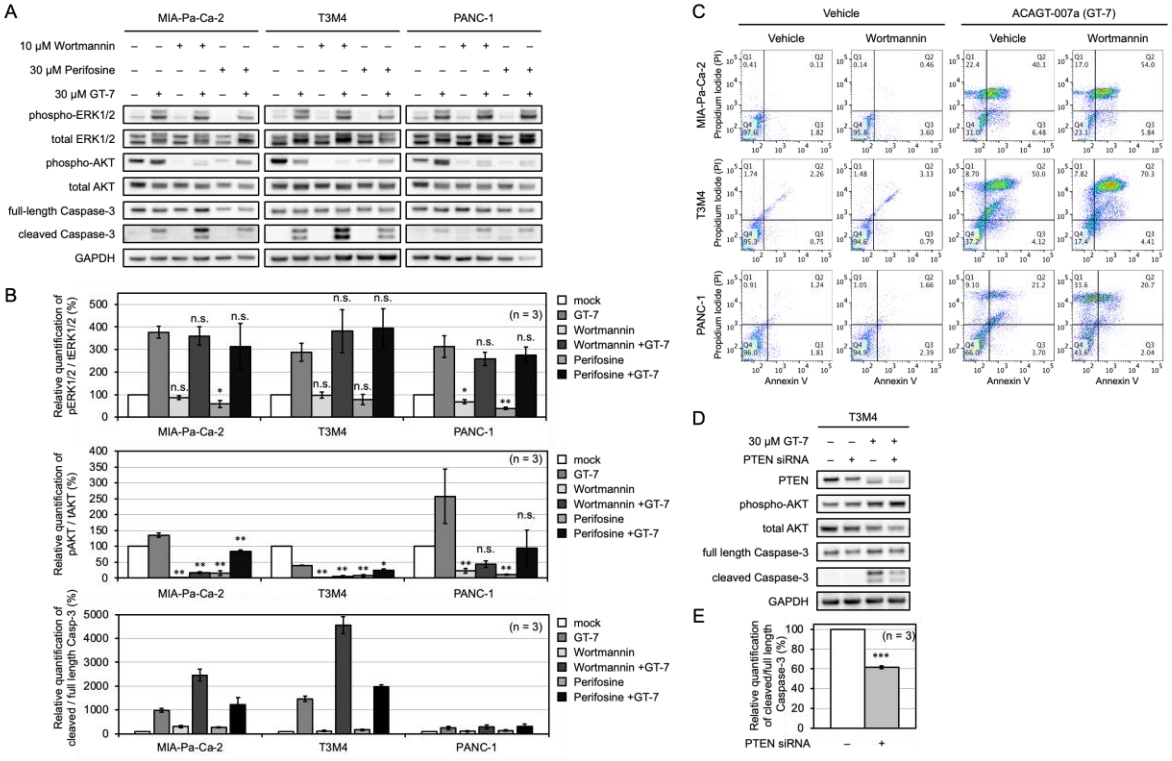
Bondar et al. reported that AsPC-1 cells, which are one of the PDAC cell lines, are resistant to Wortmannin-induced apoptosis and that Wortmannin only marginally down-regulated phospho-AKT levels in AsPC-1. In contrast, a complete down-regulation of phosphorylation was observed in the cells that were caused to undergo apoptosis as a result of Wortmannin treatment **[53]**. When co-treated with GT-7, Wortmannin and Perifosine did not have the expected effect of significantly lowering the levels of AKT phosphorylation (Figure 12B for quantitative analysis of pAKT/tAKT). This residual activity may inhibit apoptosis induction by these PI3K/AKT inhibitors. It is currently unknown why AKT phosphorylation in PANC-1 cells is resistant to the treatment provided by Wortmannin. On the other hand, a recent MS proteomics study that was combined with enrichment for GO categories discovered that the protein phosphatase 2A (PP2A) complex plays an essential part in the viability of PANC-1 cells **[54]**. When compared to MIA-Pa-Ca-2, the expression of the PP2A complex is noticeably lower in PANC-1. Furthermore, activating the PP2A pathway with DT-061 was able to inhibit the viability of PANC-1 cells. PANC-1 cells were able to maintain

higher levels of viability whenever siRNA was used to knock down the PP2A subunit. Therefore, the continued phosphorylation of AKT and viability of PANC-1 cells after treatment with GT-7 may be associated with a lower expression profile of the PP2A complex.

Next, three PDAC cells were treated with 10 μM GT-7 and Wortmannin and then subjected to FCM analysis to determine the effect of Wortmannin on GT-7-mediated cell death. As can be seen in Figure 12C, treatment with Wortmannin significantly increased the cell death population in MIA-Pa-Ca-2 (from 40.1% to 54.0%) and T3M4 (from 50% to 70.3%), but it did not do so in PANC-1 (21.2% to 20.7%), which is consistent with the findings obtained from the quantitative analysis of cleaved Caspase-3. PDAC cells that had been treated with GT-7 produced phase-contrast images that reflected the death status of the cells when immunoblotted against cleaved Caspase-3 (Figure12F).

To further investigate the impact of the AKT signaling activation on GT-7-mediated apoptosis, I examined the effect of PTEN silencing on GT-7-induced apoptosis in T3M4 cells. AKT will become activated if PTEN, a major negative regulator of the PI3K/AKT signaling, is inhibited. PTEN is a major negative regulator of the PI3K/AKT signaling **[55]**. In comparison to the control siRNA, the PTEN siRNAs were able to effectively suppress the targeted gene expression at the protein level in T3M4 cells (Figure 12D). PTEN silencing was shown to consistently result in increased levels of AKT phosphorylation, and the addition of GT-7 to the PTEN-silencing conditions resulted in an even greater increase in AKT phosphorylation levels (Figure 12D, E). Notably, inhibiting PTEN resulted in a significant reduction in the amount of apoptosis induction caused by GT-7. This was determined by comparing the amounts of cleaved Caspase-3 in each sample (Figure 12D, E). Therefore, increasing the levels of AKT phosphorylation by silencing PTEN was able to counteract the effect of GT-7 to induce apoptosis in T3M4 cells.

This finding suggests that the levels of AKT phosphorylation can affect the susceptibility of T3M4 cells to cell death induced by GT-7. Overall, the levels of phosphorylation of AKT found in each PDAC strain are likely to play key roles in dictating the susceptibility to apoptosis induced by GT-7.



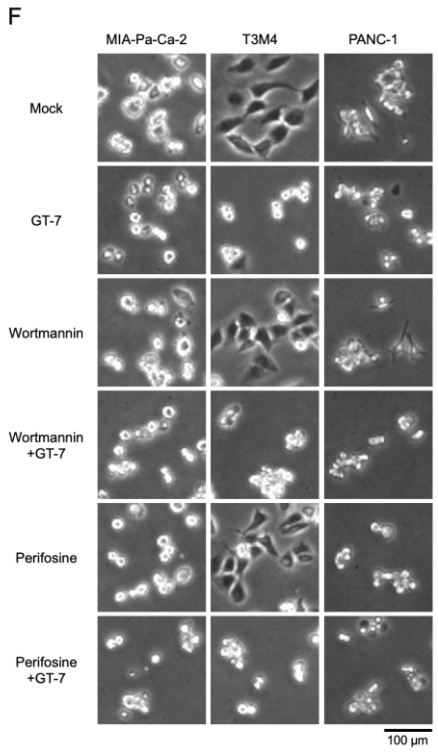


Figure 12. AKT inhibition promotes ACAGT-007a (GT-7)-induced apoptosis in PDAC cells. (A) The effect of PI3K/AKT inhibitors (Wortmannin and Perifosine) on the induction of GT-7-induced apoptosis in the PDAC cells. The PDAC cells were pretreated by 30 μM Wortmannin or Perifosine for 1 h then treated by 30 μM GT-7 for 3 h. The indicated proteins were detected by Western blot analysis. (B) Relative quantification of ERK and AKT phosphorylation levels and cleaved/full-length Caspase-3 in the PDAC cells upon PI3K/AKT inhibitors pretreatment and GT-7 treatment as shown in (A). Phosphorylation levels (phosphorylated protein intensity/total protein intensity) after the treatment with DMSO (without compounds) in each cell were set as 100%. The data were averaged from three independent experiments (n = 3). Columns, means; bars, SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, n.s., not significant. Significant differences from the DMSO treatment (without PI3K/AKT inhibitors) in each absence or presence of GT-7 using one-way ANOVA, followed by a post hoc test using Dunnett's

multiple comparisons (upper and middle panels). Comparisons between absence and presence of GT-7 were made by a student's *t*-test (lower panel). (C) The inhibition of AKT phosphorylation by Wortmannin enhances GT-7 induced apoptosis in MIA-Pa-Ca-2 and T3M4 cells but not in PANC-1 cells. PDAC cells were treated with DMSO (vehicle), 10 μM GT-7, 10 μM Wortmannin, or the combination of 10 μM GT-7 and 10 μM Wortmannin for 6 h. Chemical-treated cells were stained by Annexin V-FITC and PI and analyzed by FCM. (D) The cleaved Caspase-3 was reduced by PTEN knockdown in T3M4 cells. T3M4 cells were pretreated by the negative control siRNA or PTEN siRNA for 48 h and then treated by 30 μM GT-7 for 2 h. The indicated proteins were detected by Western blot analysis. (E) Relative quantification of cleaved Caspase-3 was averaged from three independent experiments (n = 3). Columns, means; bars, SEM. *** $p < 0.005$, significantly different between the treatment with negative control siRNA (PTEN siRNA－) and the treatment with PTEN siRNA (PTEN siRNA +) then GT-7 treatment for 2 h using a student's *t*-test. (F) Effect of AKT inhibitors with or without ACAGT-007a phase-contrast images of pancreatic cancer cells as indicated in (A). Cells indicated were cultured as described in (A) in the single-use of 10 μM of Wortmannin, 30 μM of Perifosine, 30 μM of ACAGT-007a, and in combination with each other.

4. Discussion

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic malignancy, accounting for approximately 90% of all pancreatic cancers. The fact that its early stages are symptomless makes it difficult to diagnose, which in turn leads to delayed diagnosis and a poor prognosis. Strong activation of the ERK, MAPK and AKT signaling pathways is caused by KRAS mutations, which are found in approximately 95% of pancreatic cancers. In patients with pancreatic ductal adenocarcinoma (PDAC), activation of these pathways leads to an increase in cellular proliferation, invasion, and resistance to apoptosis. Thus, targeting MAPK and/or AKT signaling driven by oncogenic KRAS appears to be a valuable approach for PDAC therapy. However, the ERK and AKT signaling inhibitors that are currently available for clinical use are still limited, and there is no evidence that they produce a better outcome for pancreatic cancer patients than conventional cytotoxic chemotherapy. Given the poor clinical outcome of patients with PDAC, together with its resistance to chemoradiotherapy, novel treatment strategies are needed to unlock new treatment possibilities. Inhibiting the MAPK and AKT signaling pathways is one possible strategy that could improve the prognosis of patients who have pancreatic cancer that has spread. Dual pathway targeting has recently emerged as a viable option for overcoming the limitations of currently used clinical drugs. In this study, I demonstrated that GT-7, an anti-cancer compound with ERK-modulating properties, can provide a novel approach to fighting this lethal cancer.

4.1: The Role and Significance of GT-7 in Inducing ERK-Dependent Apoptosis in PDAC

The three PDAC cell lines with different KRAS mutations were treated with GT-7, and the results showed that the drug reduced the viability of these cells and induced apoptosis. The effects of GT-7 on cell viability and the induction of apoptosis were significantly more potent than those of Honokiol, a naturally occurring agent that is known to be effective in the treatment of cancer, including PDAC. It is reported that the growth inhibitory effects of Honokiol on pancreatic cancer cell lines were mediated through G1 phase cell cycle arrest and apoptosis induction **[50]**. The expression of cyclins (D1 and E) and cyclin-dependent kinases (Cdk2 and Cdk4) was significantly downregulated by Honokiol, while the expression of Cdk inhibitors p21 and p27 was significantly upregulated **[50]**. In addition, Honokiol treatment increased the Bax/Bcl-2 and Bax/Bcl-xL ratios in pancreatic cancer cells that are responsible for the induction of apoptosis **[50]**. On the other hand, the uniqueness of GT-7 as an inducer of apoptosis relies on its property to stimulate ERK phosphorylation. Notably, ERK activation stimulated by GT-7 is required for apoptosis induction in T3M4 cells harboring the KRAS Q61H mutation. This is evidenced by the significant cancellation of apoptosis induction by the MEK inhibitor U0126. Numerous studies have shown that over 50 compounds can trigger ERK-dependent cell death in a wide range of tumor cell types **[56,57]**. However, thus far, no reports have been made on an anti-cancer compound capable of inducing ERK-dependent apoptosis. What types of tumors or oncogenic context is more susceptible to ERK-dependent cell death? In melanoma cell lines that carried an oncogenic BRAF or NRAS mutation, GT-7 and its original compound, ACA-28, were shown to induce ERK-dependent apoptosis. However, this effect was not observed in the normal melanocyte NHEM **[44]**. In addition, it was demonstrated that ACA-28 can induce ERK-

dependent apoptosis in NIH/3T3 cells that are overexpressing active HER2 but failed to do so in NIH/3T3 cells that do not express active HER2 **[44]**. Consequently, apoptosis was induced by ACA-28 and/or GT-7 in cancer cell lines harboring a wide variety of oncogenic mutations, including HER2, BRAF, NRAS, and KRAS **[44]**. Since "sustained ERK activation" is one of the hallmarks of ERK-induced cell death, it seems plausible that cells harboring high-ERK with oncogenic alterations in upstream activators of ERK signaling are more susceptible to apoptosis induction by GT-7. The hypothesis that there is a cellular threshold for active ERK1/2 levels in determining the cell fate for growth arrest versus death responses mediated by ERK1/2 signaling has been presented in several papers on a consistent basis **[56,57]**. This thesis has provided evidence to support this hypothesis in PDAC. Therefore, sustained activation of ERK1/2 above a particular threshold may activate particular pro-apoptotic targets and induce apoptosis in the cell **[56,57]**. Although the precise molecular mechanisms by which phospho-ERK causes cell death are not yet known, a large number of agents have been reported to induce ERK activation-dependent apoptosis through DNA-damaging stimuli-mediated p53 activation **[56,57]**. Several papers have linked the induction of apoptosis by DNA-damaging agents to an increase in p53 expression, suggesting that ERK activation stimulates p53 transcriptional activity, which in turn increases the expression of pro-apoptotic genes like Bcl-2, Bax, and Noxa **[56,57]**. Again, SMAD4 encodes a tumor suppressor protein that plays a critical role in the development of PDAC **[58]**. Several studies reported that the Inactivation of SMAD4 occurs in approximately 50% of PDACs, and its absence has been linked to poor overall survival and metastasis **[59]**. This lack of SMAD4 expression in PDAC was also found to be highly correlated with tumor size, tumor stage, lymph node metastasis, and tumor differentiation **[60]**. Liu, Xinwei et al. reported that the nuclear translocation of SMAD4 is controlled by the ERK signaling

pathway, and SMAD4 has been shown to be directly phosphorylated by ERK at Thr276. This localization of SMAD4 by ERK critically regulates CD8+ T cell cytotoxic function **[61]**. In addition, ACA has been reported to induce anti-proliferation and apoptosis by enhancing the overexpression of SMAD4 in cervical cancer cells **[62]**.

In contrast, a common feature among compounds that can trigger ERK-mediated apoptosis is the enhancement of reactive oxygen species (ROS) **[56,57]**. According to a study published by **Wang, X et al.** Cisplatin increases ROS activity which in turn activates the depicted cascade of events, including MEK1/2 and ERK activation, and ultimately results in cell death **[63]**.

Additional important findings from my lab include GT-7-mediated downregulation of DUSP6. The original compound of GT-7, ACA-28, was shown to preferentially induce ERK-dependent apoptosis via DUSP6 down-regulation in HER2/ErbB2 overexpressing cells with high ERK and high DUSP6 expression **[64]**. Consistent with DUSP6's role as a negative regulator of ERK1/2, DUSP6 protein levels were dependent on ERK activity, and DUSP6 expression was high in all three PDAC cell lines. DUSP6 protein levels are significantly decreased in T3M4 and MIA-Pa-Ca-2 cells by the addition of GT-7. PANC-1 cells, on the other hand, exhibited resistance to the downregulation of the DUSP6 protein that was mediated by GT-7. Instead, it appears that the levels of DUSP6 protein are modestly increased in PANC-1 cells. After prolonged exposure to GT-7 for 10 hours, there was a gradual decrease in ERK activation, which may have been caused by the steady levels of DUSP6 protein that were maintained in PANC-1 cells. (Figure 13A, B)

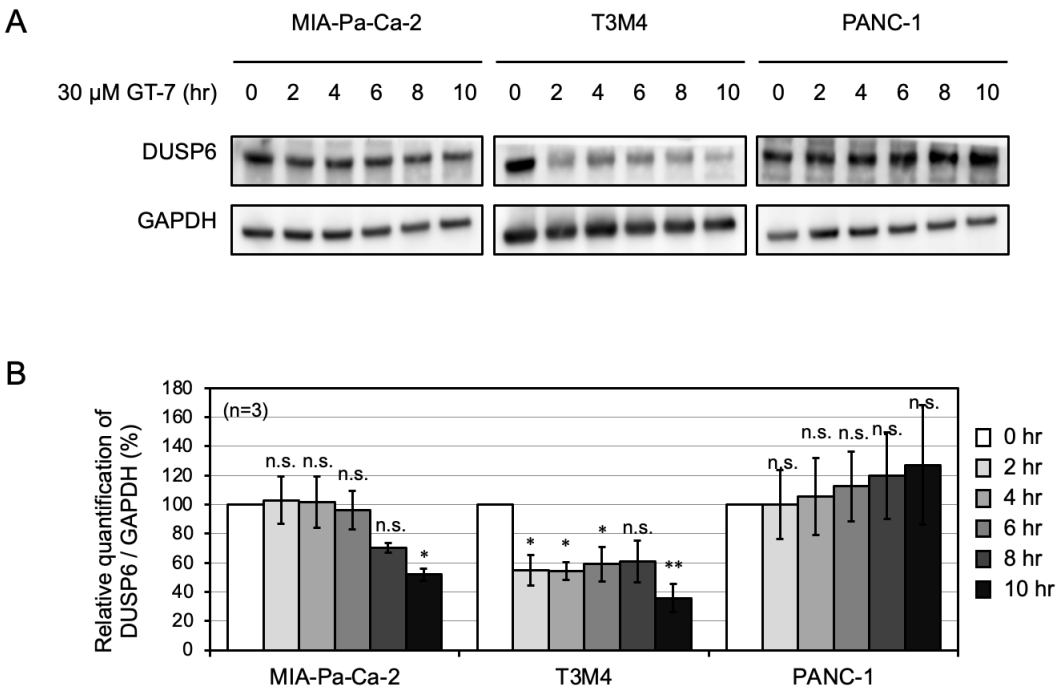


Figure 13: DUSP6 is abundantly expressed and downregulated by GT-7 in pancreatic cancer cells. (A) DUSP6 protein levels are downregulated by GT-7 in a time-dependent manner in MIA-Pa-Ca-2 and T3M4 cells but not in PANC-1 cells. The PDAC cells were treated by 30 μM GT-7 for the time indicated (0-10 h). The indicated proteins were detected by western blot analysis. (B) Relative quantification of DUSP6 expression levels in PDAC cells. Expression levels of DUSP6 (DUSP6 protein intensity/GAPDH protein intensity) after the treatment with DMSO (0 h) in each PDAC cell were set as 100%. The data were averaged from three independent experiments (n=3). Columns, means; bars, SEM. *$p < 0.05$, **$p < 0.01$, n.s., not significant; significantly different from the DMSO treatment (0 h) using one-way ANOVA, followed by a post hoc test using Dunnett's multiple comparisons.

Therefore, ROS can be used to explain the downregulation of DUSP6 that is caused by GT-7, as well as the effect that this has on the activity of ERK. ROS can stimulate ERK activity in one of two ways: either by activating EGFRs or by inhibiting MAPK phosphatases. Firstly, how does ROS activate

EGFRs? EGFRs, which are upstream of ERK, are frequently activated through ligand-induced dimerization or oligomerization and are frequently involved in the control of cell proliferation, survival, migration, and differentiation. It is interesting to note that ROS have been shown to activate EGFRs through a process known as "receptor transactivation" even in the absence of its ligand. There is also evidence that ROS can activate RAS, RAF, or PKC, the upstream activators of ERK MAPK by attacking the cysteine residues of a specific group of target proteins and oxidizing the reactive thiol groups in the process can lead to the formation of a disulfide bond **[56,57]**. Secondly, how does ROS inhibit MAPK phosphatase? The phosphatase activities of the DUSPs, as well as several tyrosine phosphatases that inactivate upstream regulators of the ERK signaling pathway, are abolished when the catalytic cysteine within the catalytic site of the DUSPs is oxidized **[56,57]**. It has been demonstrated that ROS can inhibit the phosphatase activities of ERK-directed phosphatases. This is accomplished by causing oxidation of the catalytic cysteine residues in DUSP5 and DUSP6 **[56,57]**. In addition, the Intracellular accumulation of ROS such as hydrogen peroxide causes DUSP6 phosphorylation on Ser159 and Ser197 residues, which in turn causes the proteasomal degradation of DUSPs that ultimately stimulates the activation of ERK. This chain of events is caused by the accumulation of DUSPs. In a recently released study, it was discovered that blocking the neuroendocrine-like protein Synaptophysin-like 1, also known as SYLP1, caused sustained ERK activation, which in turn caused cell death. This finding was made possible by knocking down Synaptophysin-like 1. It was discovered that the downregulation of SYLP1 led to the production of ROS, which in turn led to the activation of ERK and cell death **[65]**. The action of GT-7 may be associated with a mechanism for cell death that is analogous to the one described above and that is mediated by ROS and dependent on ERK. It is important to note that the GT-7-mediated increase in pERK was

inhibited by the MEK inhibitor U0126 in all three PDAC; however, apoptosis was only inhibited by U0126 in T3M4 cells, and not in MIA-Pa-Ca-2 or PANC-1 cells. (Figure 10A, B).

What is the possible reason for the differential effect of pERK on apoptosis induction in these PDAC cell lines? Several papers have reported that the fate of the cells, either anti- or pro-apoptosis, is determined not only by the kinetics of ERK phosphorylation (transient versus sustained) but also by the spatial distribution of phosphorylated ERK and its substrates. Importantly, it has been found that scaffold proteins, such as DAPK and PEA-15, can regulate the spatial distribution of phosphorylated ERK by anchoring the phosphorylated ERK protein in the cytosol **[66,67]**. For example, activated ERK1/2 is sequestered in the cytoplasm via interaction with PEA-15 and DAPK. Inhibition of ERK1/2 nuclear translocation impairs ERK1/2-mediated proliferation and augments the pro-apoptotic signals of DAPK by phosphorylating the cytoplasmic DAPK. Further, DUSPs are critical in determining where phosphorylated ERK1/2 is dispersed. DUSP6 serves as an anchor for inactive ERK in the cytosol **[68]**. Interestingly, ROS generated by compounds can induce ERK-dependent apoptosis to inactivate the cytosolic ERK phosphatase DUSP6, leading to the sequestration of active ERK in the cytoplasm **[69]**. For this reason, it is intriguing to consider the possibility that these docking phosphatases (DUSPs) and/or scaffold proteins are differentially expressed in these PDACs, and that T3M4 possesses the most favorable conditions for maintaining ERK phosphorylation distribution to induce apoptosis.

How does GT-7 increase pERK? It has been reported that ACA acts as an inhibitor for the nuclear export of Rev by binding to the cysteine-529 residue of CRM1, which is the receptor for NES, and consequently preventing the nuclear export of Rev **[70]**. Tamura et al. reported that the formation of the quinone methide intermediate of ACA is necessary for the ACA to be able

to exert its inhibitory activity on the nuclear export of Rev **[71]**. In addition, it was discovered that the ACA could be treated with N-acetyl-cysteine (NAC), which would result in the production of the two adducts. As ACA-28 and GT-7 bear a similar structure (two carbonate esters) to ACA regarding the binding to NAC, it is highly probable that ACA-28 and GT-7 via the formation of the quinone methide intermediate, thereby serving as oxidants to attack the Cysteine residues of the target proteins. ACA, the original compound, has been reported to induce ROS on a consistent basis **[72]**. When ACA and sodium butyrate were used to treat HepG2 human hepatocellular carcinoma cells, both intracellular ROS levels and NADPH oxidase activities increased. Cells pretreated with catalase, a powerful antioxidant enzyme that breaks down ROS, were less likely to experience a decrease in number in response to a combination of ACA and sodium butyrate. In addition, the authors also showed that ACA, alone or in combination with sodium butyrate together increased pERK activity. In other cancer cell lines, such as NB4 promyelocytic leukemia cells, similar observations of the ACA-mediated induction of ROS and the inhibition of apoptosis induction by a thiol antioxidant NAC have been reported **[73].** As a result, I assume that the compounds GT-7 and ACA-28, which are structurally related to the original compound ACA, are capable of elevating pERK through the stimulation of ROS signaling. As was discussed earlier, ROS can target a variety of phosphatases, including those that inactivate the upstream kinases in the ERK signaling pathway as well as phosphatases against ERK. So, depending on the genomic context or gene expression profiling in each cancer cell line, GT-7's action can either increase MEK activity or decrease phosphatase activity against pERK.

4.2. GT-7-Mediated Apoptosis Can Be Enhanced by Inhibiting AKT Signaling

Importantly, this research showed that activating the AKT signaling pathway was necessary for providing resistance to apoptosis induced by GT-7. When exposed to GT-7, each of the three PDAC cell lines that were investigated for this study exhibited a distinctively different susceptibility to apoptosis. I demonstrated that a correlation exists between sustained phosphorylation of AKT and resistance to apoptosis induced by GT-7 (Figure 11). As shown by the quantification of relative cleaved Caspase-3/full-length Caspase-3 and the FCM analysis, the susceptibility of inducing apoptosis is highest in T3M4, followed by MIA-Pa-Ca-2, and then PANC-1. The lowest susceptibility is observed in PANC-1. As was stated previously, resistance to the GT-7-induced apoptosis is correlated with sustained phosphorylation of the AKT protein. The most sustained AKT phosphorylation was seen in PANC-1, followed by MIA-Pa-Ca-2. After a transient increase in AKT phosphorylation 2 hours after being treated with GT-7, T3M4 cells exhibited a marked down-regulation of AKT phosphorylation levels that were significantly lower than the basal levels. Consistently, in our previous research, Sugiura's lab reported that GT-7 and the original compound ACA-28 induced ERK-dependent apoptosis in SK-MEL-28, an ERK-active melanoma cell line **[45]**, wherein AKT phosphorylation levels are low. These observations and the effect of Wortmannin and PTEN silencing suggest that AKT signaling activation plays a key role in determining susceptibility to GT-7.

What is the mechanism of action for GT-7 that results in an increase in both pERK and pAKT? Since numerous compounds with the ability to induce ERK-dependent cell death to elicit ROS signaling, I hypothesize that ROS may play a role in the GT-7 action. Previous research conducted in Sugiura's lab described a mechanism by which ERK signaling is stimulated, and it

involved the inactivation or degradation of DUSP by ROS. **[64]**. In addition, ROS facilitated cell death by activating AKT. Chetram et al. reported that, ROS increases pAKT expression in human prostate cancer cells **[74]**. ROS oxidize PTEN, a catalytically inactive negative regulator of AKT signaling, and this allows AKT to be activated in a manner that bypasses traditional activation mechanisms.Vanadium compounds bis(acetylacetonato)-oxidovanadium(IV) and sodium metavanadate significantly suppress cell proliferation in human pancreatic cancer AsPC-1 cells by downregulating PI3K-AKT and MAPK **[75]**. The antioxidant NAC could be used to counteract the effects of the two compounds. Thus, GT-7 could induce ROS, thereby increasing both pERK and pAKT.

The distinct KRAS mutations in the three PDAC cell lines may provide an explanation for the different AKT signaling activation observed in these cell lines. KRAS Q61H is found in T3M4 cells, whereas KRAS G12D is found in PANC-1 cells and KRAS G12C is found in MIA-Pa-Ca-2 cells. By interacting with RAF and PI3K, wild-type KRAS relays signals down the ERK and AKT signaling pathways. Notably, KRAS Q61H preferentially binds to RAF rather than PI3K, which results in increased MAPK signaling **[48]**. Furthermore, compared to ERK signaling, the KRAS G12D mutant found in PANC-1 preferentially activates AKT signaling **[76,77]**. In MIA-Pa-Ca-2 cells, the KRAS G12C mutant strongly interacts with RAF1, and AKT signaling activation was minimal **[78]**. These previous findings in the literature and my data on the responses of AKT signaling activation upon GT-7 seem to consistently explain the basis of cells' resistance to GT-7. The three PDAC cell lines have similar genomic sequences, including the presence of wild-type PTEN and CDKN1A and the presence of a TP53 mutation, suggesting that these three mutations are the most common potential oncogenic alterations in PDAC. However, because the effect of these KRAS mutations on ERK and AKT

signaling may vary depending on the cell lines and the number of alleles, as well as other mutations in the genome in each cell line, special caution is required.

The susceptibility to cell viability inhibition is stronger in MIA-Pa-Ca-2, intermediate in T3M4, and weaker in PANC-1. Thus, both in terms of the WST-8 assay and the evaluation of cell death, PANC-1 demonstrated the strongest resistance. The reversal of susceptibility between MIA-Pa-Ca-2 and T3M4 may suggest that GT-7 may affect growth inhibitory mechanisms other than apoptosis induction. Cell cycle arrest and induction of autophagy are two potential growth inhibitory mechanisms. Thus, the effect of GT-7 (especially at lower concentrations) on the cell cycle and/or autophagy in MIA-Pa-Ca-2 compared with other PDAC cell lines may reveal a novel mode of action of GT-7.

An additional factor that should be taken into consideration, relevant to cells' responses to GT-7, is the epithelial-mesenchymal phenotype because epithelial-mesenchymal transition (EMT) is closely associated with enhanced migration, invasion, and resistance to anti-cancer chemotherapy **[79]**. It has been extensively studied and characterized over the past few decades that the epithelial-to-mesenchymal transition is a highly plastic and dynamic process. Epithelial cells lose their apical-basal polarity during EMT, separate from the epithelium, and move to new locations **[79]**. EMT has been described as a crucial stage in the progression of diseases like organ fibrosis or malignant epithelial tumors as well as normal growth, wound healing, and disease development **[79]**. Mesenchymal to epithelial transition, which is the opposite of this process, denotes the cessation of migration and the acquisition of apico-basal polarisation as well as other epithelial characteristics **[80]**. EMT is induced in

response to signaling factors that cause the expression of tightly regulated proteins known as EMT-transcription factors as well as microRNAs and epigenetic regulators that support the transition to a mesenchymal state **[80]**. In addition, Epithelial cells undergo a phenotypic change and take on mesenchymal characteristics, such as spindle-like fusiform, motile cells that express mesenchymal markers like vimentin and N-cadherin **[80]**.

A morpho-functional analysis of various PDAC cell lines using 2- and 3-dimensional cultures was conducted by Minami et al., and significant results were reported. PDAC cells create 3-dimensional tumors, and these offer important information on cell morphology and how it affects pathological and functional aspects of the tumor **[81]**. Based on this study, MIA-Pa-Ca-2 and PANC-1 showed spheres with a grape-like morphology and small oval cells, whereas T3M4 cells showed small oval and flat-lining cells on the surface of the spheres. MIA-Pa-Ca-2 cells are considered epithelial cells with mesenchymal features **[82]**. Morphological analysis revealed that PANC-1 and MIA-Pa-Ca-2 cells exhibited mesenchymal characteristics, while T3M4 cells were epithelial in nature. Moreover, in terms of the EMT phenotype, T3M4 had high E-cadherin and low vimentin mRNA levels, whereas PANC-1 and MIA-Pa-Ca-2 had low E-cadherin and high vimentin mRNA levels. The EMT-positive cell lines showed some resistance to the apoptosis induced by GT-7, while the EMT-negative T3M4 cells were more sensitive to the drug's effects.

In addition, the EMT score was evaluated by Young Kwang Chae's method **[54]**, which was based on the statistical analysis of the gene expression profiling (Figure 14). This analysis revealed that T3M4 displayed a strong epithelial phenotype, whereas MIA-Pa-Ca-2 and PANC-1 displayed a mesenchymal or quasi-mesenchymal phenotype, which was consistent with the results of the 3D analysis. Through the participation of EMT-

associated transcription factors in a variety of human cancers, there is mounting evidence to suggest that EMT has a close connection with chemotherapy resistance. As a result, cells that have an EMT phenotype have a greater chance of being resistant to the induction of apoptosis by GT-7.

Type	Gene Name	T3M4_Zscore	MIA-Pa-Ca-2_Zscore	PANC-1_Zscore
mesenchymal	VIM	-1.414195703	0.71325292	0.700942784
mesenchymal	MMP2	-0.621030773	-0.789821191	1.410851964
mesenchymal	CDH2	-0.635547009	-0.776328867	1.411875876
mesenchymal	FOXC2	-0.698987882	-0.715194725	1.414182607
mesenchymal	SNAI1	-1.183168227	-0.079299417	1.262467643
mesenchymal	MMP9	-0.803870264	-0.605707885	1.409578149
mesenchymal	GSC	-0.909568561	1.392608238	-0.483039676
mesenchymal	TWIST1	1.12997879	-1.301440989	0.171462198
mesenchymal	SOX10	-0.30285882	1.347760218	-1.044901398
mesenchymal	MMP3	0.707106781	0.707106781	-1.414213562
mesenchymal	FN1	1.397817658	-0.884864057	-0.512953601
mesenchymal	ITGB6	1.414206969	-0.710843459	-0.70336351
mesenchymal	SNAI2	1.24282107	-0.036990445	-1.205830625
epithelial	TJP1	0.931879211	-1.38718922	0.455310009
epithelial	CDH1	1.052043807	-1.344497848	0.292454041
epithelial	DSP	1.372522449	-0.981450309	-0.39107214
mesenchymal average (M)		-0.05209969	-0.133827914	0.185927604
epithelial average (E)		1.118815156	-1.237712459	0.118897303
EMT score (M-E)		-1.170914846	1.103884545	0.067030301

Figure 14: EMT score was evaluated by Young Kwang Chae's method

Furthermore, the genome-wide gene expression profile was investigated using the Cancer Cell Line Encyclopedia (CCLE: https://sites.broadinstitute.org/cl/, accessed on 29 August 2021) and qRT-PCR in order to reveal the basis for responses of the three PDAC cell lines to GT-7. My goal was to determine which genes were involved in the responses of the PDAC cell lines. In the CCLE database, I looked for components of the apoptosis pathway that might explain the observed

sensitivity to the agent. Therefore, I found a number of apoptosis-related genes that are strongly expressed in MIA-Pa-Ca-2 and T3M4 but poorly expressed in PANC-1. This could explain the difference in sensitivity to the agent between these three cell lines. These include the EREG gene, which encodes the epiregulin protein, and the ANXA1 gene, which encodes the Annexin 1 protein (Figure 15). Epiregulin is essential for EGFR-mediated growth signaling, and Annexins 1 attracts a lot of attention because of its prognostic significance in PDAC **[83]**. Epiregulin is a member of the family of ligands known as epidermal growth factors (EGF), which play significant roles in the pathology of pancreatic ductal adenocarcinoma (PDAC) by activating the MEK/ERK signaling pathway **[84]**. It has been demonstrated that annexin A1 (ANXA1) can induce programmed cell death (apoptosis) in cancerous cells including PDAC **[85]**. Notably, a lower expression of Annexin A1 has been shown to promote drug resistance in human pancreatic cancer **[86]**. This resistance was observed in response to gemcitabine and 5-fluorouracil treatment. These data are consistent with my data that MIA-Pa-Ca-2 and T3M4 are relatively sensitive to GT-7-mediated apoptosis, whereas PANC-1 showed strong resistance against the agent.

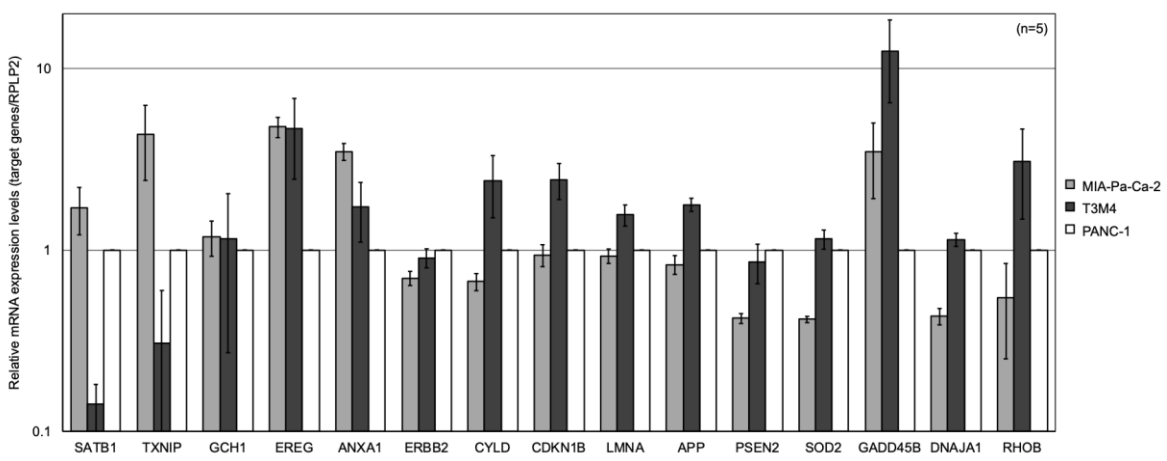


Figure: 15: qRT-PCR analyses on the expression of apoptosis-related genes in PDAC cells. RPLP2 was used as a reference gene. Relative mRNA expression levels in PANC-1 cells were set as 1. The data were averaged from five independent experiments (n = 5). Columns, means; bars, SEM.

As mentioned earlier, apoptotic responses to GT-7 in these PDAC cell lines were influenced by pAKT levels. However, according to the threshold theory proposed by Park's group, higher cellular ERK phosphorylation levels may be preferable in order to achieve ERK-dependent apoptosis. High levels of EREG and ANXA1, which are expressed in MIA-Pa-Ca-2 and T3M4 but not in PANC-1, may contribute to the more sustained ERK signaling activation preferred to achieve GT-7-mediated apoptosis. The potential roles of epiregulin and annexin A1 in the different responses of the three PDAC cell lines to this agent needs future validation.

5. Conclusion and Perspectives

The PI3K/AKT and ERK MAPK pathways both of which have feedback mechanisms that regulate the activity of the other pathway. For instance, inhibiting MEK with U0126 results in increases in the amount of AKT phosphorylation caused by EGF and FGF **[87,88]**. Furthermore, research has shown that inhibiting both PI3K and mTOR at the same time leads to an overactivation of the MEK/ERK pathway in human pancreatic cancer **[89]**. Therefore, the rapid up-regulation of compensatory alternative pathways and feedback loops within tumor cells may explain the ineffectiveness of single-agent treatments of these promising targeted therapies **[90]**. Several combination therapies involving ERK signaling inhibitors and BRAF or MEK inhibitors, or ERK signaling inhibitors and the PI3-kinase signaling inhibitors (PI3-kinase, mTOR, AKT), have been described as effective ways to overcome resistance in a single treatment. The dual PI3K/mTOR kinase inhibitor BEZ235, the MEK inhibitor PD0325901, and the combined targeting of RAF/MEK/ERK signaling and autophagy survival responses in PDAC are all examples **[9]**. My findings suggest a potentially novel approach for concurrent targeting of ERK and PI3K/AKT signaling pathways, as I show that the combination of GT-7 (ERK stimulation) and Wortmannin (PI3K inhibition) effectively induces apoptosis. Additionally, these results may provide novel information on how to induce apoptosis in PDAC based on the different KRAS mutation alleles.

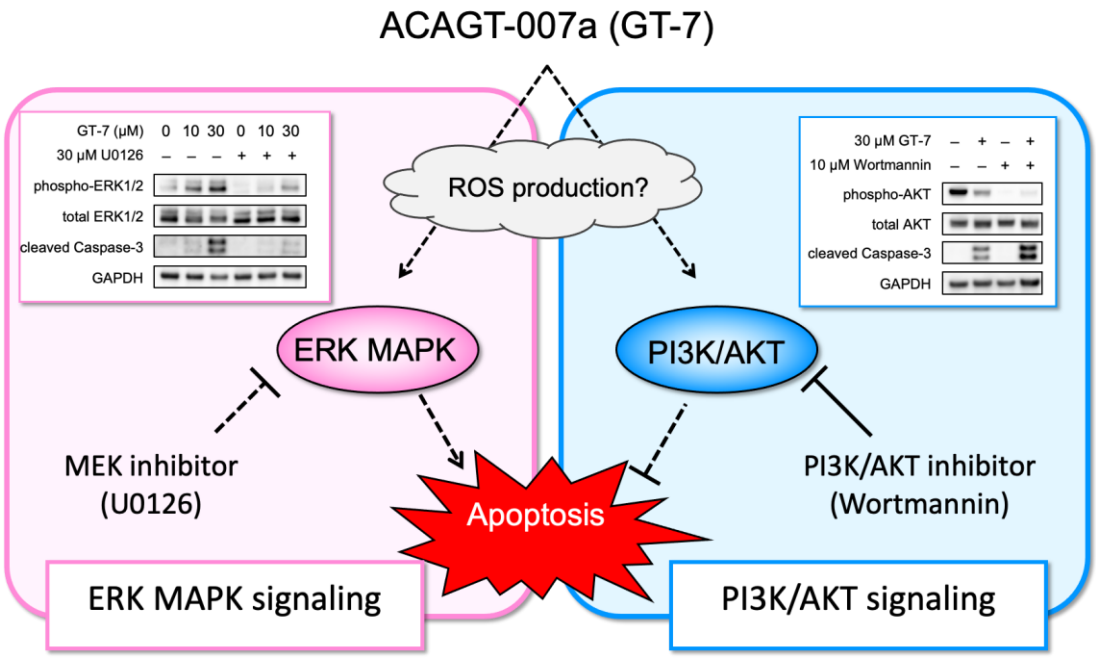


Figure 16: Combination of ACAGT-007a, a novel ERK signaling modulator, with AKT signaling inhibitor effectively induces apoptosis in KRAS mutant pancreatic cancer cells.

6. References

1. Orth, M., Metzger, P., Gerum, S., Mayerle, J., Schneider, G., Belka, C., Schnurr, M., & Lauber, K. (2019). Pancreatic ductal adenocarcinoma: biological hallmarks, current status, and future perspectives of combined modality treatment approaches. *Radiation oncology (London, England)*, *14*(1), 141. https://doi.org/10.1186/s13014-019-1345-6
2. Rahib, L., Smith, B. D., Aizenberg, R., Rosenzweig, A. B., Fleshman, J. M., & 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 research*, *74*(11), 2913–2921. https://doi.org/10.1158/0008-5472.CAN-14-0155
3. Huang, J., Lok, V., Ngai, C. H., Zhang, L., Yuan, J., Lao, X. Q., Ng, K., Chong, C., Zheng, Z. J., & Wong, M. C. S. (2021). Worldwide Burden of, Risk Factors for, and Trends in Pancreatic Cancer. *Gastroenterology*, *160*(3), 744–754. https://doi.org/10.1053/j.gastro.2020.10.007
4. Embuscado, E. E., Laheru, D., Ricci, F., Yun, K. J., de Boom Witzel, S., Seigel, A., Flickinger, K., Hidalgo, M., Bova, G. S., & Iacobuzio-Donahue, C. A. (2005). Immortalizing the complexity of cancer metastasis: genetic features of lethal metastatic pancreatic cancer obtained from rapid autopsy. *Cancer biology & therapy*, *4*(5), 548–554. https://doi.org/10.4161/cbt.4.5.1663
5. Lanfredini, S., Thapa, A., & O'Neill, E. (2019). RAS in pancreatic cancer. *Biochemical Society transactions*, *47*(4), 961–972. https://doi.org/10.1042/BST20170521

6. Castellano, E., & Downward, J. (2011). RAS Interaction with PI3K: More Than Just Another Effector Pathway. *Genes & cancer*, *2*(3), 261–274. https://doi.org/10.1177/1947601911408079

7. Waters, A. M., & Der, C. J. (2018). KRAS: The Critical Driver and Therapeutic Target for Pancreatic Cancer. *Cold Spring Harbor perspectives in medicine*, *8*(9), a031435. https://doi.org/10.1101/cshperspect.a031435

8. Swayden, M., Iovanna, J., & Soubeyran, P. (2018). Pancreatic cancer chemo-resistance is driven by tumor phenotype rather than tumor genotype. *Heliyon*, *4*(12),e01055. https://doi.org/10.1016/j.heliyon.2018.e01055

9. Mollinedo, F., & Gajate, C. (2019). Novel therapeutic approaches for pancreatic cancer by combined targeting of RAF→MEK→ERK signaling and autophagy survival response. *Annals of translational medicine*, *7*(Suppl 3), S153. https://doi.org/10.21037/atm.2019.06.40

10. Zavoral, M., Minarikova, P., Zavada, F., Salek, C., & Minarik, M. (2011). Molecular biology of pancreatic cancer. *World journal of gastroenterology*, *17*(24),2897–2908. https://doi.org/10.3748/wjg.v17.i24.2897

11. Makohon-Moore, A., & Iacobuzio-Donahue, C. A. (2016). Pancreatic cancer biology and genetics from an evolutionary perspective. *Nature reviews. Cancer*, *16*(9), 553–565. https://doi.org/10.1038/nrc.2016.66

12. Yachida, S., & Iacobuzio-Donahue, C. A. (2013). Evolution and dynamics of pancreatic cancer progression. *Oncogene*, *32*(45), 5253–5260. https://doi.org/10.1038/onc.2013.29

13. Eser, S., Schnieke, A., Schneider, G., & Saur, D. (2014). Oncogenic KRAS signalling in pancreatic cancer. *British journal of cancer*, *111*(5), 817–822. https://doi.org/10.1038/bjc.2014.215

14. Hezel, A. F., Kimmelman, A. C., Stanger, B. Z., Bardeesy, N., & Depinho, R. A. (2006). Genetics and biology of pancreatic ductal adenocarcinoma. *Genes & development*, *20*(10), 1218–1249. https://doi.org/10.1101/gad.1415606

15. Chen, P. Y., Muzumdar, M. D., Dorans, K. J., Robbins, R., Bhutkar, A., Del Rosario, A., Mertins, P., Qiao, J., Schafer, A. C., Gertler, F., Carr, S., & Jacks, T. (2018). Adaptive and Reversible Resistance to Kras Inhibition in Pancreatic Cancer Cells. *Cancer research*, *78*(4), 985–1002. https://doi.org/10.1158/0008-5472.CAN-17-2129

16. Caldas, C., & Kern, S. E. (1995). K-ras mutation and pancreatic adenocarcinoma. *International journal of pancreatology : official journal of the International Association of Pancreatology*, *18*(1), 1–6. https://doi.org/10.1007/BF02825415

17. Satoh, K., Shimosegawa, T., Moriizumi, S., Koizumi, M., & Toyota, T. (1996). K-ras mutation and p53 protein accumulation in intraductal mucin-hypersecreting neoplasms of the pancreas. *Pancreas*, *12*(4), 362–368. https://doi.org/10.1097/00006676-199605000-00007

18. Cui, Y., & Guo, G. (2016). Immunomodulatory Function of the Tumor Suppressor p53 in Host Immune Response and the Tumor Microenvironment. *International journal of molecular sciences*, *17*(11), 1942. https://doi.org/10.3390/ijms17111942

19. Gasparini, C., Tommasini, A., & Zauli, G. (2012). The MDM2 inhibitor Nutlin-3 modulates dendritic cell-induced T cell proliferation. *Human immunology*, *73*(4), 342–345. https://doi.org/10.1016/j.humimm.2012.01.018

20. Taniguchi, K., & Karin, M. (2018). NF-κB, inflammation, immunity and cancer: coming of age. *Nature reviews. Immunology*, *18*(5), 309–324. https://doi.org/10.1038/nri.2017.142

21. Cooks, T., Pateras, I. S., Tarcic, O., Solomon, H., Schetter, A. J., Wilder, S., Lozano, G., Pikarsky, E., Forshew, T., Rosenfeld, N., Harpaz, N., Itzkowitz, S., Harris, C. C., Rotter, V., Gorgoulis, V. G., & Oren, M. (2013). Mutant p53 prolongs NF-κB activation and promotes chronic inflammation and inflammation-associated colorectal cancer. *Cancer cell*, *23*(5), 634–646. https://doi.org/10.1016/j.ccr.2013.03.022

22. McCubrey, J. A., Yang, L. V., Abrams, S. L., Steelman, L. S., Follo, M. Y., Cocco, L., Ratti, S., Martelli, A. M., Augello, G., & Cervello, M. (2022). Effects of TP53 Mutations and miRs on Immune Responses in the Tumor Microenvironment Important in Pancreatic Cancer Progression. *Cells*, *11*(14), 2155. https://doi.org/10.3390/cells11142155

23. Kamisawa, T., Wood, L. D., Itoi, T., & Takaori, K. (2016). Pancreatic cancer. *Lancet (London, England)*, *388*(10039), 73–85. https://doi.org/10.1016/S0140-6736(16)00141-0

24. Dardare, J., Witz, A., Merlin, J. L., Gilson, P., & Harlé, A. (2020). SMAD4 and the TGFβ Pathway in Patients with Pancreatic Ductal Adenocarcinoma. *International journal of molecular sciences*, *21*(10), 3534. https://doi.org/10.3390/ijms2110353

25. Bos J. L. (1989). ras oncogenes in human cancer: a review. *Cancer research*, *49*(17), 4682–4689.

26. Simanshu, D. K., Nissley, D. V., & McCormick, F. (2017). RAS Proteins and Their Regulators in Human Disease. *Cell*, *170*(1), 17–33. https://doi.org/10.1016/j.cell.2017.06.009

27. Guo, Y. J., Pan, W. W., Liu, S. B., Shen, Z. F., Xu, Y., & Hu, L. L. (2020). ERK/MAPK signalling pathway and tumorigenesis. *Experimental and therapeutic medicine*, *19*(3), 1997–2007. https://doi.org/10.3892/etm.2020.8454

28. Barbosa, R., Acevedo, L. A., & Marmorstein, R. (2021). The MEK/ERK Network as a Therapeutic Target in Human Cancer. *Molecular cancer research : MCR*, *19*(3), 361–374. https://doi.org/10.1158/1541-7786.MCR-20-0687

29. Fernández-Medarde, A., & Santos, E. (2011). Ras in cancer and developmental diseases. *Genes & cancer*, *2*(3), 344–358. https://doi.org/10.1177/1947601911411084

30. Cantrell D. A. (2001). Phosphoinositide 3-kinase signalling pathways. *Journal of cell science*, *114*(Pt 8), 1439–1445. https://doi.org/10.1242/jcs.114.8.1439

31. Czech M. P. (2000). PIP2 and PIP3: complex roles at the cell surface. *Cell*, *100*(6), 603–606. https://doi.org/10.1016/s0092-8674(00)80696-0

32. Mao, Y., Xi, L., Li, Q., Cai, Z., Lai, Y., Zhang, X., & Yu, C. (2016). Regulation of cell apoptosis and proliferation in pancreatic cancer through PI3K/Akt pathway via Polo-like kinase 1. *Oncology reports*, *36*(1), 49–56. https://doi.org/10.3892/or.2016.4820

33. Conway, J. R., Herrmann, D., Evans, T. J., Morton, J. P., & Timpson, P. (2019). Combating pancreatic cancer with PI3K pathway inhibitors in the era of personalised medicine. *Gut*, *68*(4), 742–758. https://doi.org/10.1136/gutjnl-2018-316822

34. McCubrey, J. A., Steelman, L. S., Chappell, W. H., Abrams, S. L., Wong, E. W., Chang, F., Lehmann, B., Terrian, D. M., Milella, M., Tafuri, A., Stivala, F., Libra, M., Basecke, J., Evangelisti, C., Martelli, A. M., & Franklin, R. A. (2007). Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochimica et biophysica acta*, *1773*(8),1263–1284. https://doi.org/10.1016/j.bbamcr.2006.10.001

35. Yang, S., & Liu, G. (2017). Targeting the Ras/Raf/MEK/ERK pathway in hepatocellular carcinoma. *Oncology letters*, *13*(3), 1041–1047. https://doi.org/10.3892/ol.2017.5557

36. Knight, T., & Irving, J. A. (2014). Ras/Raf/MEK/ERK Pathway Activation in Childhood Acute Lymphoblastic Leukemia and Its Therapeutic Targeting. *Frontiers in oncology*, *4*, 160. https://doi.org/10.3389/fonc.2014.00160

37. Cao, Z., Liao, Q., Su, M., Huang, K., Jin, J., & Cao, D. (2019). AKT and ERK dual inhibitors: The way forward?. *Cancer letters*, *459*, 30–40. https://doi.org/10.1016/j.canlet.2019.05.025

38. Hall, B. R., Cannon, A., Atri, P., Wichman, C. S., Smith, L. M., Ganti, A. K., Are, C., Sasson, A. R., Kumar, S., & Batra, S. K. (2018). Advanced pancreatic cancer: a meta-analysis of clinical trials over thirty years. *Oncotarget*, *9*(27),19396–19405. https://doi.org/10.18632/oncotarget.25036

39. Klein-Brill, A., Amar-Farkash, S., Lawrence, G., Collisson, E. A., & Aran, D. (2022). Comparison of FOLFIRINOX vs Gemcitabine Plus Nab-Paclitaxel as First-Line Chemotherapy for Metastatic Pancreatic Ductal Adenocarcinoma. *JAMA network open*, *5*(6), e2216199. https://doi.org/10.1001/jamanetworkopen.2022.16199

40. Thummuri, D., Khan, S., Underwood, P. W., Zhang, P., Wiegand, J., Zhang, X., Budamagunta, V., Sobh, A., Tagmount, A., Loguinov, A., Riner, A. N., Akki, A. S., Williamson, E., Hromas, R., Vulpe, C. D., Zheng, G., Trevino, J. G., & Zhou, D. (2022). Overcoming Gemcitabine Resistance in Pancreatic Cancer Using the BCL-X_L-Specific Degrader DT2216. *Molecular cancer therapeutics*, *21*(1), 184–192. https://doi.org/10.1158/1535-7163.MCT-21-0474

41. Braicu, C., Buse, M., Busuioc, C., Drula, R., Gulei, D., Raduly, L., Rusu, A., Irimie, A., Atanasov, A. G., Slaby, O., Ionescu, C., & Berindan-Neagoe, I.

(2019). A Comprehensive Review on MAPK: A Promising Therapeutic Target in Cancer. *Cancers*, *11*(10), 1618. https://doi.org/10.3390/cancers11101618

42. Qian, Y., Gong, Y., Fan, Z., Luo, G., Huang, Q., Deng, S., Cheng, H., Jin, K., Ni, Q., Yu, X., & Liu, C. (2020). Molecular alterations and targeted therapy in pancreatic ductal adenocarcinoma. *Journal of hematology & oncology*, *13*(1), 130. https://doi.org/10.1186/s13045-020-00958-3

43. Joseph, E. W., Pratilas, C. A., Poulikakos, P. I., Tadi, M., Wang, W., Taylor, B. S., Halilovic, E., Persaud, Y., Xing, F., Viale, A., Tsai, J., Chapman, P. B., Bollag, G., Solit, D. B., & Rosen, N. (2010). The RAF inhibitor PLX4032 inhibits ERK signaling and tumor cell proliferation in a V600E BRAF-selective manner. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(33), 14903–14908. https://doi.org/10.1073/pnas.1008990107

44. Satoh, R.; Hagihara, K.; Matsuura, K.; Manse, Y.; Kita, A.; Kunoh, T.; Masuko, T.; Moriyama, M.; Moriyama, H.; Tanabe, G.; et al. Identification of ACA-28, a 1′-acetoxychavicol acetate analogue compound, as a novel modulator of ERK MAPK signaling, which preferentially kills human melanoma cells. *Genes Cells* **2017**, *22*, 608–618.

45. Satoh, R.; Hamada, N.; Yamada, A.; Kanda, Y.; Ishikawa, F.; Takasaki, T.; Tanabe, G.; Sugiura, R. Discovery of new benzhydrol biscarbonate esters as potent and selective apoptosis inducers of human melanomas bearing the activated ERK pathway: SAR studies on an ERK MAPK signaling modulator, ACA-28. *Bioorg. Chem.* **2020**, *103*, 104137.

46. Muñoz-Maldonado, C., Zimmer, Y., & Medová, M. (2019). A Comparative Analysis of Individual RAS Mutations in Cancer Biology. *Frontiers in oncology*, *9*, 1088. https://doi.org/10.3389/fonc.2019.01088

47. Gebregiworgis, T., Kano, Y., St-Germain, J., Radulovich, N., Udaskin, M. L., Mentes, A., Huang, R., Poon, B. P. K., He, W., Valencia-Sama, I., Robinson, C. M., Huestis, M., Miao, J., Yeh, J. J., Zhang, Z. Y., Irwin, M. S., Lee, J. E., Tsao, M. S., Raught, B., Marshall, C. B., … Ikura, M. (2021). The Q61H mutation decouples KRAS from upstream regulation and renders cancer cells resistant to SHP2 inhibitors. *Nature communications*, *12*(1), 6274. https://doi.org/10.1038/s41467-021-26526-y

48. Zhou, Z. W., Ambrogio, C., Bera, A. K., Li, Q., Li, X. X., Li, L., Son, J., Gondi, S., Li, J., Campbell, E., Jin, H., Okoro, J. J., Xu, C. X., Janne, P. A., & Westover, K. D. (2020). KRAS^{Q61H} Preferentially Signals through MAPK in a RAF Dimer-Dependent Manner in Non-Small Cell Lung Cancer. *Cancer research*, *80*(17), 3719–3731. https://doi.org/10.1158/0008-5472.CAN-20-0448

49. Janes, M. R., Zhang, J., Li, L. S., Hansen, R., Peters, U., Guo, X., Chen, Y., Babbar, A., Firdaus, S. J., Darjania, L., Feng, J., Chen, J. H., Li, S., Li, S., Long, Y. O., Thach, C., Liu, Y., Zarieh, A., Ely, T., Kucharski, J. M., … Liu, Y. (2018). Targeting KRAS Mutant Cancers with a Covalent G12C-Specific Inhibitor. *Cell*, *172*(3), 578–589.e17. https://doi.org/10.1016/j.cell.2018.01.006

50. Arora, S., Bhardwaj, A., Srivastava, S. K., Singh, S., McClellan, S., Wang, B., & Singh, A. P. (2011). Honokiol arrests cell cycle, induces apoptosis, and potentiates the cytotoxic effect of gemcitabine in human pancreatic cancer cells. *PloS one*, *6*(6), e21573. https://doi.org/10.1371/journal.pone.0021573

51. Gradiz, R., Silva, H. C., Carvalho, L., Botelho, M. F., & Mota-Pinto, A. (2016). MIA PaCa-2 and PANC-1 - pancreas ductal adenocarcinoma cell lines with neuroendocrine differentiation and somatostatin receptors. *Scientific reports*, *6*, 21648. https://doi.org/10.1038/srep21648

52. Ryan, M. B., Finn, A. J., Pedone, K. H., Thomas, N. E., Der, C. J., & Cox, A. D. (2016). ERK/MAPK Signaling Drives Overexpression of the Rac-GEF, PREX1, in BRAF- and NRAS-Mutant Melanoma. *Molecular cancer research : MCR*, *14*(10), 1009–1018. https://doi.org/10.1158/1541-7786.MCR-16-0184

53. Bondar, V. M., Sweeney-Gotsch, B., Andreeff, M., Mills, G. B., & McConkey, D. J. (2002). Inhibition of the phosphatidylinositol 3'-kinase-AKT pathway induces apoptosis in pancreatic carcinoma cells in vitro and in vivo. *Molecular cancer therapeutics*, *1*(12), 989–997.

54. Chae, Y. K., Chang, S., Ko, T., Anker, J., Agte, S., Iams, W., Choi, W. M., Lee, K., & Cruz, M. (2018). Epithelial-mesenchymal transition (EMT) signature is inversely associated with T-cell infiltration in non-small cell lung cancer (NSCLC). *Scientific reports*, *8*(1), 2918. https://doi.org/10.1038/s41598-018-21061-1

55. Haddadi, N., Lin, Y., Travis, G., Simpson, A. M., Nassif, N. T., & McGowan, E. M. (2018). PTEN/PTENP1: 'Regulating the regulator of RTK-dependent PI3K/Akt signalling', new targets for cancer therapy. *Molecular cancer*, *17*(1), 37. https://doi.org/10.1186/s12943-018-0803-3

56. Sugiura, R., Satoh, R., & Takasaki, T. (2021). ERK: A Double-Edged Sword in Cancer. ERK-Dependent Apoptosis as a Potential Therapeutic Strategy for Cancer. *Cells*, *10*(10), 2509. https://doi.org/10.3390/cells10102509

57. Cagnol, S., & Chambard, J. C. (2010). ERK and cell death: mechanisms of ERK-induced cell death--apoptosis, autophagy and senescence. *The FEBS journal*, *277*(1), 2–21. https://doi.org/10.1111/j.1742-4658.2009.07366.x

58. Principe, D. R., Underwood, P. W., Kumar, S., Timbers, K. E., Koch, R. M., Trevino, J. G., Munshi, H. G., & Rana, A. (2022). Loss of SMAD4 Is Associated With Poor Tumor Immunogenicity and Reduced PD-L1

Expression in Pancreatic Cancer. *Frontiers in oncology*, *12*, 806963. https://doi.org/10.3389/fonc.2022.806963

59. Racu, M. L., Lebrun, L., Schiavo, A. A., Van Campenhout, C., De Clercq, S., Absil, L., Minguijon Perez, E., Maris, C., Decaestecker, C., Salmon, I., & D'Haene, N. (2022). The Role of *SMAD4* Inactivation in Epithelial-Mesenchymal Plasticity of Pancreatic Ductal Adenocarcinoma: The Missing Link?. *Cancers*, *14*(4), 973. https://doi.org/10.3390/cancers14040973

60. Wang, Z., Li, Y., Zhan, S., Zhang, L., Zhang, S., Tang, Q., Li, M., Tan, Z., Liu, S., & Xing, X. (2019). SMAD4 Y353C promotes the progression of PDAC. *BMC cancer*, *19*(1), 1037. https://doi.org/10.1186/s12885-019-6251-7

61. Liu, X., Hao, J., Wei, P., Zhao, X., Lan, Q., Ni, L., Chen, Y., Bai, X., Ni, L., & Dong, C. (2022). SMAD4, activated by the TCR-triggered MEK/ERK signaling pathway, critically regulates $CD8^+$ T cell cytotoxic function. *Science advances*, *8*(30), eabo4577. https://doi.org/10.1126/sciadv.abo4577

62. Phuah, N. H., Azmi, M. N., Awang, K., & Nagoor, N. H. (2017). Down-Regulation of MicroRNA-210 Confers Sensitivity towards 1'S-1'-Acetoxychavicol Acetate (ACA) in Cervical Cancer Cells by Targeting SMAD4. *Molecules and cells*, *40*(4), 291–298. https://doi.org/10.14348/molcells.2017.2285

63. Wang, X., Martindale, J. L., & Holbrook, N. J. (2000). Requirement for ERK activation in cisplatin-induced apoptosis. *The Journal of biological chemistry*, *275*(50), 39435–39443. https://doi.org/10.1074/jbc.M004583200

64. Kanda, Y., Mizuno, A., Takasaki, T., Satoh, R., Hagihara, K., Masuko, T., Endo, Y., Tanabe, G., & Sugiura, R. (2021). Down-regulation of dual-specificity phosphatase 6, a negative regulator of oncogenic ERK

signaling, by ACA-28 induces apoptosis in NIH/3T3 cells overexpressing HER2/ErbB2. *Genes to cells: devoted to molecular & cellular mechanisms*, *26*(2), 109–116. https://doi.org/10.1111/gtc.12823

65. Song, Y., Sun, X., Duan, F., He, C., Wu, J., Huang, X., Xing, K., Sun, S., Wang, R., Xie, F., Mao, Y., Wang, J., & Li, S. (2020). SYPL1 Inhibits Apoptosis in Pancreatic Ductal Adenocarcinoma *via* Suppression of ROS-Induced ERK Activation. *Frontiersinoncology*, *10*,1482. https://doi.org/10.3389/fonc.2020.01482

66. Chen, C. H., Wang, W. J., Kuo, J. C., Tsai, H. C., Lin, J. R., Chang, Z. F., & Chen, R. H. (2005). Bidirectional signals transduced by DAPK-ERK interaction promote the apoptotic effect of DAPK. *The EMBO journal*, *24*(2), 294–304. https://doi.org/10.1038/sj.emboj.7600510

67. Formstecher, E., Ramos, J. W., Fauquet, M., Calderwood, D. A., Hsieh, J. C., Canton, B., Nguyen, X. T., Barnier, J. V., Camonis, J., Ginsberg, M. H., & Chneiweiss, H. (2001). PEA-15 mediates cytoplasmic sequestration of ERK MAP kinase. *Developmental cell*, *1*(2), 239–250. https://doi.org/10.1016/s1534-5807(01)00035-1

68. Bermudez, O., Pagès, G., & Gimond, C. (2010). The dual-specificity MAP kinase phosphatases: critical roles in development and cancer. *American journal of physiology. Cell physiology*, *299*(2), C189–C202. https://doi.org/10.1152/ajpcell.00347.2009

69. Kim, H. S., Song, M. C., Kwak, I. H., Park, T. J., & Lim, I. K. (2003). Constitutive induction of p-Erk1/2 accompanied by reduced activities of protein phosphatases 1 and 2A and MKP3 due to reactive oxygen species during cellular senescence. *The Journal of biological chemistry*, *278*(39), 37497–37510. https://doi.org/10.1074/jbc.M211739200

70. Tamura, S., Shiomi, A., Kaneko, M., Ye, Y., Yoshida, M., Yoshikawa, M., Kimura, T., Kobayashi, M., & Murakami, N. (2009). New Rev-export inhibitor from Alpinia galanga and structure-activity

relationship. *Bioorganic & medicinal chemistry letters*, *19*(9), 2555–2557. https://doi.org/10.1016/j.bmcl.2009.03.047

71. Tamura, S., Shiomi, A., Kimura, T., & Murakami, N. (2010). Halogenated analogs of 1'-acetoxychavicol acetate, Rev-export inhibitor from Alpinia galanga, designed from mechanism of action. *Bioorganic & medicinal chemistry letters*, *20*(7), 2082–2085. https://doi.org/10.1016/j.bmcl.2010.02.070

72. Kato, R., Matsui-Yuasa, I., Azuma, H., & Kojima-Yuasa, A. (2014). The synergistic effect of 1'-acetoxychavicol acetate and sodium butyrate on the death of human hepatocellular carcinoma cells. *Chemico-biological interactions*, *212*, 1–10. https://doi.org/10.1016/j.cbi.2014.01.010

73. Ito, K., Nakazato, T., Murakami, A., Yamato, K., Miyakawa, Y., Yamada, T., Hozumi, N., Ohigashi, H., Ikeda, Y., & Kizaki, M. (2004). Induction of apoptosis in human myeloid leukemic cells by 1'-acetoxychavicol acetate through a mitochondrial- and Fas-mediated dual mechanism. *Clinical cancer research : an official journal of the American Association for Cancer Research*, *10*(6), 2120–2130. https://doi.org/10.1158/1078-0432.ccr-1142-03

74. Chetram, M. A., Bethea, D. A., Odero-Marah, V. A., Don-Salu-Hewage, A. S., Jones, K. J., & Hinton, C. V. (2013). ROS-mediated activation of AKT induces apoptosis via pVHL in prostate cancer cells. *Molecular and cellular biochemistry*, *376*(1-2), 63–71. https://doi.org/10.1007/s11010-012-1549-7

75. Wu, J. X., Hong, Y. H., & Yang, X. G. (2016). Bis(acetylacetonato)-oxidovanadium(IV) and sodium metavanadate inhibit cell proliferation via ROS-induced sustained MAPK/ERK activation but with elevated AKT activity in human pancreatic cancer AsPC-1 cells. *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological*

Inorganic Chemistry, *21*(8), 919–929. https://doi.org/10.1007/s00775-016-1389-0

76. Céspedes, M. V., Sancho, F. J., Guerrero, S., Parreño, M., Casanova, I., Pavón, M. A., Marcuello, E., Trias, M., Cascante, M., Capellà, G., & Mangues, R. (2006). K-ras Asp12 mutant neither interacts with Raf, nor signals through Erk and is less tumorigenic than K-ras Val12. *Carcinogenesis*, *27*(11), 2190–2200. https://doi.org/10.1093/carcin/bgl063

77. Ihle, N. T., Byers, L. A., Kim, E. S., Saintigny, P., Lee, J. J., Blumenschein, G. R., Tsao, A., Liu, S., Larsen, J. E., Wang, J., Diao, L., Coombes, K. R., Chen, L., Zhang, S., Abdelmelek, M. F., Tang, X., Papadimitrakopoulou, V., Minna, J. D., Lippman, S. M., Hong, W. K., … Powis, G. (2012). Effect of KRAS oncogene substitutions on protein behavior: implications for signaling and clinical outcome. *Journal of the National Cancer Institute*, *104*(3), 228–239. https://doi.org/10.1093/jnci/djr523

78. Cheng, D. K., Oni, T. E., Thalappillil, J. S., Park, Y., Ting, H. C., Alagesan, B., Prasad, N. V., Addison, K., Rivera, K. D., Pappin, D. J., Van Aelst, L., & Tuveson, D. A. (2021). Oncogenic KRAS engages an RSK1/NF1 pathway to inhibit wild-type RAS signaling in pancreatic cancer. *Proceedings of the National Academy of Sciences of the United States of America*, *118*(21), e2016904118. https://doi.org/10.1073/pnas.2016904118

79. Cano, A., Pérez-Moreno, M. A., Rodrigo, I., Locascio, A., Blanco, M. J., del Barrio, M. G., Portillo, F., & Nieto, M. A. (2000). The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nature cell biology*, *2*(2), 76–83. https://doi.org/10.1038/35000025

80. Nieto, M. A., Huang, R. Y., Jackson, R. A., & Thiery, J. P. (2016). EMT: 2016. *Cell*, *166*(1), 21–45. https://doi.org/10.1016/j.cell.2016.06.028

81. Minami, F., Sasaki, N., Shichi, Y., Gomi, F., Michishita, M., Ohkusu-Tsukada, K., Toyoda, M., Takahashi, K., & Ishiwata, T. (2021). Morphofunctional analysis of human pancreatic cancer cell lines in 2- and 3-dimensional cultures. *Scientific reports*, *11*(1), 6775. https://doi.org/10.1038/s41598-021-86028-1

82. Gradiz, R., Silva, H. C., Carvalho, L., Botelho, M. F., & Mota-Pinto, A. (2016). MIA PaCa-2 and PANC-1 - pancreas ductal adenocarcinoma cell lines with neuroendocrine differentiation and somatostatin receptors. *Scientific reports*, 6, 21648. https://doi.org/10.1038/srep21648

83. Zhu, Z., Kleeff, J., Friess, H., Wang, L., Zimmermann, A., Yarden, Y., Büchler, M. W., & Korc, M. (2000). Epiregulin is Up-regulated in pancreatic cancer and stimulates pancreatic cancer cell growth. *Biochemical and biophysical research communications*, *273*(3), 1019–1024. https://doi.org/10.1006/bbrc.2000.3033

84. Cho, M. C., Choi, H. S., Lee, S., Kim, B. Y., Jung, M., Park, S. N., & Yoon, D. Y. (2008). Epiregulin expression by Ets-1 and ERK signaling pathway in Ki-ras-transformed cells. *Biochemical and biophysical research communications*, *377*(3), 832–837. https://doi.org/10.1016/j.bbrc.2008.10.053

85. Oshi, M., Tokumaru, Y., Mukhopadhyay, S., Yan, L., Matsuyama, R., Endo, I., & Takabe, K. (2021). Annexin A1 Expression Is Associated with Epithelial-Mesenchymal Transition (EMT), Cell Proliferation, Prognosis, and Drug Response in Pancreatic Cancer. *Cells*, *10*(3), 653. https://doi.org/10.3390/cells10030653

86. Liu, Q. H., Yong, H. M., Zhuang, Q. X., Zhang, X. P., Hou, P. F., Chen, Y. S., Zhu, M. H., & Bai, J. (2020). Reduced expression of annexin A1 promotes gemcitabine and 5-fluorouracil drug resistance of human pancreatic cancer. *Investigational new drugs*, *38*(2), 350–359. https://doi.org/10.1007/s10637-019-00785-5

87. Moore, M. J., Goldstein, D., Hamm, J., Figer, A., Hecht, J. R., Gallinger, S., Au, H. J., Murawa, P., Walde, D., Wolff, R. A., Campos, D., Lim, R., Ding, K., Clark, G., Voskoglou-Nomikos, T., Ptasynski, M., Parulekar, W., & National Cancer Institute of Canada Clinical Trials Group (2007). Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, *25*(15), 1960–1966. https://doi.org/10.1200/JCO.2006.07.9525

88. Javle, M. M., Shroff, R. T., Xiong, H., Varadhachary, G. A., Fogelman, D., Reddy, S. A., Davis, D., Zhang, Y., Wolff, R. A., & Abbruzzese, J. L. (2010). Inhibition of the mammalian target of rapamycin (mTOR) in advanced pancreatic cancer: results of two phase II studies. *BMC cancer*, *10*, 368. https://doi.org/10.1186/1471-2407-10-368

89. Turke, A. B., Song, Y., Costa, C., Cook, R., Arteaga, C. L., Asara, J. M., & Engelman, J. A. (2012). MEK inhibition leads to PI3K/AKT activation by relieving a negative feedback on ERBB receptors. *Cancer research*, *72*(13), 3228–3237. https://doi.org/10.1158/0008-5472.CAN-11-3747

90. Hayashi, H., Tsuchiya, Y., Nakayama, K., Satoh, T., & Nishida, E. (2008). Down-regulation of the PI3-kinase/Akt pathway by ERK MAP kinase in growth factor signaling. *Genes to cells : devoted to molecular & cellular mechanisms*, *13*(9), 941–947. https://doi.org/10.1111/j.1365-2443.2008.01218.x

Acknowledgments

Firstly, I would like to express my gratitude to Prof. Dr. Reiko Sugiura, my esteemed advisor, for all the guidance, support, and instruction she provided me throughout my doctoral studies. Without the help and encouragement of my supervisor, I never would have been able to finish my Ph.D. Secondly, I'd also like to give special thanks to Professor Dr. Takashi Nakayama, who supported me as the thesis's chief reviewer and provided invaluable feedback and direction. I'd also like to express my gratitude to Professor Dr. Atsufumi Kawabata, whose review comments and overall support were extremely helpful. My deepest appreciation goes to Professor Genzoh Tanabe, who synthesized and generously supplied the compound (ACAGT-007a). I would like to express my deep gratitude to Dr. Ryosuke Satoh and Dr. Teruaki Takasaki, lecturers at the Faculty of Pharmaceutical Sciences, Kindai University, for their kind guidance and cooperation in carrying out this research. I would also like to express my appreciation to all the members of the Laboratory of Molecular Pharmacogenomics, who have shared the joys and sorrows of my research life. My experience in Japan has been invaluable because of their support and encouragement during my studies. In addition, I'd like to give thanks to my parents, my wife, and my son. Without their unwavering support over the past few years, I never would have been able to complete my education. Finally, I would like to thank the MEXT authority for supporting this program.