A Study of New Therapeutic Approaching RAF Degradation by Using Proteolysis Targeting Chimera

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ABSTRACT

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Pancreatic cancer is one of the most aggressive tumors with extremely poor prognosis and limited options of treatment. *KRAS* is mutated in approximately 90% of pancreatic cancer and is a well-validated driver of cancer growth, proliferation and maintenance. The Raf family, which is central component of the mitogen-activated protein kinase pathway, has emerged in the past few years as an notably promising target for pancreatic cancer treatment. However, selective Raf inhibition therapy has not been revealed to improve clinical outcome. In this study, we evaluated the effect of a new therapeutic approaching RAF degradation by combining proteolysis targeting moiety and Rigosertib, a Ras mimetic, for treating pancreatic cancer. Furthermore, for the first time, we developed Rigosertib resistant pancreatic cancer cell line and gave evidences that by using proteolysis targeting chimera technology, this new combination drug is partially able to overcome the resistance with Rigosertib in the indicated cancer.

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1. Introduction

1.1 Background of the study

1.1.1 Pancreatic cancer

Pancreatic cancer is the most lethal malignant and aggressive tumor across the world with the majority of patient death within one year after diagnosis. More than 50% patients are diagnosed at advanced stages and the five-year survival rate is approximately 6%, ranking pancreatic cancer as the seventh leading cause of cancer death in over the world [1]. Pancreatic ductal adenocarcinoma and pancreatic endocrine tumour are two major types of pancreatic cancer and contribute about 85% and 5% of cases respectively [2].

After more than three decades of exploration and investigation, it is now relatively clear that pancreatic ductal adenocarcinoma is genetic disease with a high complexity of mutations (including germ line and somatic mutations). The development of biogenetic techniques has increased our understanding about this diversity carcinogenesis and reveals the four genetic alterations of pancreatic cancer progression in *KRAS*, *CDKN2a*, *TP53* and *CPC4* genes [3]. Activating mutations in the *KRAS* oncogene are identified in 90% of pancreatic ductal carcinoma and modifications in G12 contribute about 99% of all cases. Importantly, these activations of the *KRAS* oncogene are believed as one of the earliest mutation identified in the progression model of pancreatic cancer upon other mutations [4, 5]. In vivo studies have shown that activation *KRAS* oncogene is necessary event in the early stage of tumour progression, and has different

roles in later stages of tumour. In clinical, pancreatic cancer patients with wild type KRAS are believed to have better response to Gemcitabine base line chemotherapy compared with those have mutation *KRAS*. The second common mutation in pancreatic cancer is inactivation of the *CDKN2a* gene, accounts for approximately 90% of cases, with the resultant loss of the p16 protein, a regulator of the G1-S transition of the cell cycle, and a corresponding increase in cell proliferation [6]. *TP53* mutation is observed in 50%–75% of tumors, allowing cells to bypass DNA damage control checkpoints and apoptotic signals and contributing to genomic instability. Patients wit1h regularly TP53 expression show significant improvement in progression free survival compared to complete loss [7]. Loss of DPC4 expression (deleted in pancreatic carcinoma4*)* is present in approximately 50% of pancreatic cancers, leads to anomalous signaling via the transforming growth factor-β pathway. In comparison between primary and metastatic samples of human pancreatic cancer, mutations in the *DPC4* gene has been associated with higher metastatic potential [8]. This complexity of pancreatic cancer molecular biology is a tremendous issue that causes chemotherapy resistance, but also gives unlimited potential to identify better precision treatment options.

Current treatments for pancreatic cancer are surgery, chemotherapy and radiotherapy [9]. Less than 10% pancreatic cancer population are able to receive Whipple and modified Whipple procedure (as known as pancreaticoduodenectomy) which remove the head of the pancreas, the

duodenum, the gallbladder and the bile duct. Despite of the improvement in surgery techniques, the 5 years survival rate of less than 25% has remained essentially unchanged in this operable group over the past four decades [10]. Since surgery is not an appropriate option for pancreatic at advanced stages, the major clinical practice in pancreatic cancer is chemotherapy. FOLFIRINOX is the first line of treatment for advanced pancreatic cancer with 11.1 months of overall survival in comparison with Gemcitabine (6.8 months) and Nab-Palitaxel (8.5 months) [11-13]. Nevertheless, patients received FOLFOXIRI and FOLFORINOX remedy experienced more severe advents such as stage III of neutropenia, diarrhea, and fatigue [12]. Furthermore, in contrast with other types of cancer, targeted therapies failed to show relevant activity either alone or in combination with chemotherapy and, thus, current clinical practice does not include them. Numerous phase 3 trials of agents such as farnesyltransferase inhibitors and matrix metalloproteinase inhibitors, anti-EGFR monoclonal antibody Cetuximab, anti-VEGF monoclonal antibody Bevacizumab and other targeted therapies have failed to benefit unselected PDA populations, although patients do occasionally respond [14] .

The combination between poor prognosis and the lack of efficacy treatment makes pancreatic cancer an indispensable objective for research and treatment. There must be more efforts to find out an appropriate method to improve the overall survival of pancreatic cancer which is almost unchanged since 1960s [15].

1.1.2 Targeting Raf for Pancreatic cancer treatment

KRAS has been found as the most common mutation in pancreatic cancer and responsible for tumor proliferation, transformation, adhesion and survival [5, 16]. The KRAS protein is a small GTPase that acts as a molecular switch coupling cell-membrane growth-factor receptors to intracellular signaling pathways and transcription factors to control various cellular processes. After the stimulation of these pathways, nuclear transcription factors are also activated to stimulate cell proliferation, transformation, adhesion and survival. Several researches give strong evidences of *KRAS* mutations in most early-stage pancreatic intraepithelial neoplasia and the results of research with *KRAS*-driven PDAC mouse models also support an important role for EGFR early during PDAC progression [17, 18]. It has proven that KRAS appears as an undruggable target [19, 20], hence KRAS downstream proteins are validated as high-interest therapeutic target. The Raf proteins are essential components of the RAS-MAPK pathway and their mutations were identified in many types of tumour. There are several methods in which Raf could be approached and targeted, such as directly targeting the Raf activity or indirectly inhibiting Raf through knocking down Raf mRNA, reducing Raf transcription and destabilizing Raf at the protein level [21]. Preclinical studies suggest a role for Raf inhibitors in PDAC with consideration being given to the possibility of combining Raf inhibitors with other targeted therapies (e.g. MEK or AKT inhibitors). In order to have effectiveness, continuous daily dosing of the targeted therapy is required. However, due to the overlapping toxicities of the small molecule inhibitors, patients would not able to

stay on a standard dose, leading to the incapable of sustain target inhibition level.

1.1.3 Rigosertib

Developed by Oncova Therapeutic, Rigosertib recently has been considered as a novel type of anti-cancer agent for myelodysplastic syndromes treatment and investigated for using in another types of cancer [22]. Rigosertib was investigated in in invitro as a non-ATP competitive anticancer agent that has ability to block mitotic progression and induces apoptosis in many types of cancer without affecting normal cells. In various tumor xenograft mouse models, including human liver, breast, and pancreatic cancer models, Rigosertib did not only show promising anti-tumor activity but also showed a low toxicity profile with rare hematotoxicity. One of the mechanisms of Rigosertib is ability to inhibit the PLK1 and Akt-PI3K .This small molecule is derived from a family of novel small molecule kinase inhibitors that are unrelated to ATP or other nucleosides with antitumor activity [23]. It was reported that Rigosertib inhibits growth of 57 human cancer cells including multidrug-resistant (MDR) cell lines in vitro by inducing G2/M arrest, resulting in spindle abnormalities and apoptosis. Rigosertib also inhibits tumor growth in xenograft models of Bel-7402, MCF-7, and MIA-PaCa. This drug was known as multi-kinase inhibitor which targets not only PLK1 with IC50 of 9nM, but also PLK2, PDGFR, Flt1, BCR-ABL, Fyn, Src, and CDK1 with IC50 of 18-260nM. It also exhibits inhibition against PI3K [24-26].

In deeper discovering the mechanism of Rigosertib, several researches reveal that Rigosertib acts as a RAS-mimetic that binds to RAS-binding domains (RBDs) of

RAS effector molecules such as RAF, PI3Ks, and RalGDS. Thus, Rigosertib inhibits RAS-RAF-MEK signaling as well phosphorylation of c-RAF at Ser338, which is essential for the activation of its kinase activity and the association with PLK1 [27].

The efficacy and toxicity of Rigosertib in combination with Gemcitabine were investigated in a phase I trial with the participation of 40 patients with advanced solid malignancies [25]. Patients with pancreatic adenocarcinoma, thymic cancer, and Hodgkin lymphoma have responded partially with this combination therapy. Unfortunately, the combination of Rigosertib and Gemcitabine failed to bring a prolongement in survival or response compared with Gemcitabine alone in metastatic pancreatic adenomacarcinoma patients [28].

1.1.4 PROTAC

Traditional approach of small molecule drug study which focuses on the attack an active site that directly alter protein function fails to target proteins that lack of susceptible sites. The commencing idea of Proteinlysis Targeting Chimeras (PROTACs) is manipulating cellular quality control machinery to selectively degrade proteins of interest, thus does not require a sensitive or active sites of target protein and brings other the advantages over conventional small molecule approach [29].

PROTACs give effect through hijacking E3 ubiquitin ligase for degradation. Bifunctional PROTAC molecules have two important binding sites: one bind to the protein of interest and the other end binds to an E3 enzyme to form a ternary complex. Upon binding, a surface lysine on the substrate attacks the thioester

functionality between the ubiquitin molecule and the E2 component of the ligase. PROTACs mediate the association of an E3 ligase with a non-natural substrate protein, thus tagging it for degradation. The ternary complex disengages, the ubiquitylated target protein is degraded by the proteasome and finally the PROTACs can return to another protein of interest[30].Consequently, PROTACs overcome the need of high systemic drug exposure to ensure the effect of inhibition which is a big challenge in small molecule drug study. In addition, by using degraders instead of protein ligands, PROTACs is able to target protein with or without active sites or functional ligands, thus transient interactions between drugs and target result in inhibition of the degradation process and give a more durable loss of protein activity[31]. Moreover, the issue that which E3 ligase is occupied to the targets offers an added layer of the ability to increase specific activity of PROTACs. Numerous studies have shown that the selective degradation of PROTACs not only preserve, but can also outreach to bind to off-target protein, targets with scaffold function or highly mutated targets [32].

The idea of induced protein degradation is not a relatively new. Inhibitor of chaperone heat shock protein 90 (HSP90) first entered clinical trials in 1999and later became the most well-understanding molecule member in HSPs family. HSP90 is ubiquitously expressed chaperone and performs an important role in the folding, stabilization, activation, maturation, function and proteolytic degradation of several client proteins that are considered as oncoproteins involved in multiple tumor types [33]. The recent PROTACs technology has recruited Thalidomide, which was administered as a sedative to pregnant

women and led to the birth of thousands of children with multiple defects; thus no longer used as hypnotic or morning sickness, but used for treating certain cancers (multiple myeloma) and of a complication of leprosy. Interestingly, it has been shown recently in several researches that Thalidomide has ability to bind to E3 ligase cereblon (CRBN) and then inactivated it. This activity was the used for recruiting E3 in PROTAC technology [34].

By the indicated mechanism of action, PROTAC technology gives an extremely promising solution for the question of treating cancer in low dose with a robust drug efficacy.

1.2 Objective

In this study, we evaluated the effect of a series of Rigosertib-PROTAC based drug in pancreatic cancer with hypothesis that this novel drug might not only have more effective than Rigosertib but also overcome the resistance with Rigosertib in the indicated cancer. Furthermore, for the first time, we developed a Rigosertib resistant pancreatic cancer cell lines to identify the specific mechanism for the lack of improved efficiency with the combination of Rigosertib and Gemcitabine.

2. Materials and methods

2.1 Cell line and culture

Immortalized cancer cell (CFPAC1) were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (HyClone). Cell cultures were maintained in 100mm culture dishes and incubated at 37℃ with 5% CO_2 until they achieved 80% confluency. The cells were then trypsinized using 0.25% trypsin (HyClone) and passaged into 100mm culture dishes at a density of 1 X 10^6 cells.

2.2 Drugs

Rigosertib was supplied by Oncova Therapeutic Korea (Seoul, Korea),Rigosertib-PROTAC drugs (TDH001~019) were purchased from Korea Research Institute of Chemical Technology, dissolved in DMSO as a stock and stored at -80ºC. The Rigosertib and Rigosertib-PROTAC solution were diluted in culture medium immediately before use.

2.3 Drugs screening

Promega'sCellTiter-Glo® Luminescent Cell Viability Assay was used to determine the number of viable cells in a culture by quantification of ATP (Promega. Madison, Wis.). CFPAC1 cells were seeded in a 96 well plate with 125-1,000 cells per well and were allowed to attach for 24 hours. After then, the cells were treated with Rigosertib or Rigosertib-PROTAC (TDH-001~019) at different concentrations for 72 hours. The detection reagent was prepared per manufactures protocol and equal volume of CellTiter-Glo® reagent was added to

each well of cell culture for 10 min and luminescence was measured by Perkin Elmer's Wallac Victor Light 1420 luminescence counter. The Glo titer Viability Assay has a linear range is from 0 to 50,000 cells per well. Each concentration was done in triplicate.

2.4 Antibodies and Western blot

Protein was extracted with RIPA buffer with complete protease inhibitors (Roche), separated by electrophoresis, transferred to PVDF Membrane (Millipore), blocked with 5% bovine serum albumin. The primary antibodies, ARAF, CRAF, BRAF (Erk1/2) (Cell signaling), phospho-Akt (Ser473) (Cell signaling), phosphor-Akt (Thr308) (Cell signaling), were incubated overnight at 4°C. Immunoreactive bands were visualized using peroxidase-labeled affinity purified secondary antibodies (KPL) and the detection reagent Amersham ECL prime western blotting detection reagent (GE Healthcare).

2.5 Rigosertib resistance invitro model

Rigosertib-resistant pancreatic cancer cells were established by escalating doses of Rigosertibserially in CFPAC-1 cells. Initially cells were cultured for 72 hours with EC50 of Rigosertib with defined drug free interval. As the cells adapted to the drug dose, the Rigosertibconcentration was increased serially with different methods of treatment. The Rigosertib-resistant cell lines were established after 19 weeks.

3. Results

3.1. PROTAC- Rigosertib based drugs enhance the effect of Rigosertib on Pancreatic cancer through RAF degradation.

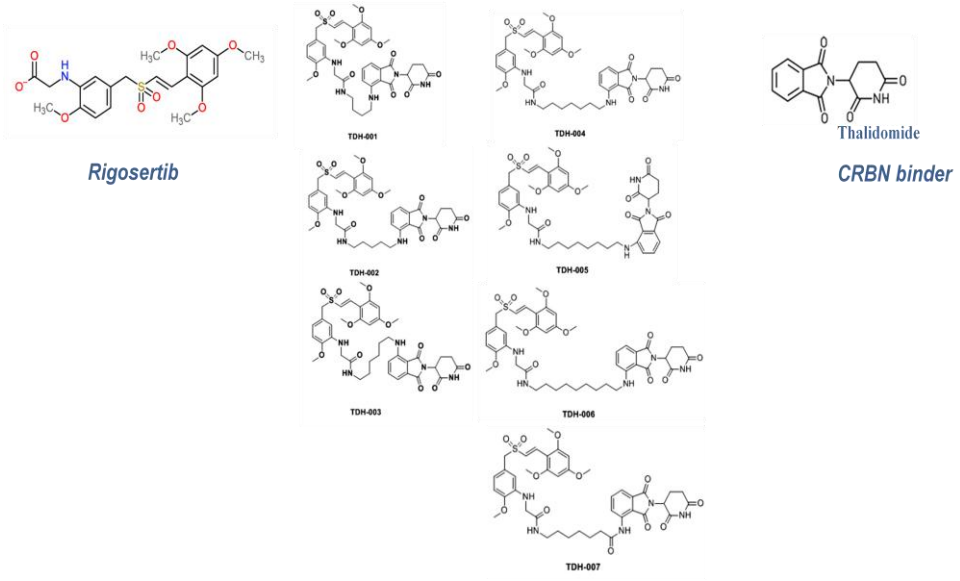
To determine the most appropriate PROTAC- Rigosertib chemical structure for treating Pancreatic cancer, we performed drug screening assay by measuring viability of CFPAC1 (pancreatic cancer cell) treated with Rigosertib and 19 types of PROTAC-Rigosertib based drugs (TDH 001 ~ 019) . These drugs were synthesized by Professor Heejung Jung in Korea Research Institute of Chemical Technology (KRICT), combining Rigosertib with Thalidomide moiety, which is a CRBN E3 ligase recruiting motif, using different length of linker between Rigosertib and Thalidomide moiety (Figure 1). After treating 100nM of drugs for 48 hours, PROTAC drug named TDH 004 showed significant effective compared to Rigosertib with survival rate are 0.25 and 0.55 respectively (Figure 2A). In addition, it was seen that ARAF signaling was downregulated along with cell viability (Figure 2B) .We chose TDH 004 for further steps of our research.

Numerous researches reveal that the Rigosertib acts as a RAS mimetic, thus suppress activities of RAF and other downstream signaling of RAS. We therefore compared these mechanisms between Rigosertib and PROTAC TDH 004. CFPAC naive cells were treated with multiple concentrations of Rigosertib and TDH 004, cell signalings were examined with Western blotting. It was interesting to note that TDH 004 inhibited ARAF, CRAF signals significantly at the concentration of 100nM while Rigosertib did not show any similar effect

until reaching 1000nM concentration (Figure 3).

For the following steps, we used Pomalidomide as a control to see whether the effect of TDH 004 was given by Pomalidomide toxicity. After 18 hours being treated with Rigosertib, Polmalidomide, TDH 004 at 50nM and 100nM, cell imagines under Micro scope were taken (Figure 4). The effect of TDH 004 at low concentration were confirmed once again with Wesrern Blot analysis, giving evidence that the combination between Rigosertib and Thalidomide robust the effect of TDH 004 but not Rigosertib or Thalidomide itself. Similarly, ARAF was knocked down by TDH 004 convincingly at 50nM whereas Rigosertib and Pomalidomide not actually gave any decrease in ARAF signal at the same concentration.

The mechanism of Rigosertib- PROTAC based drug was confirmed in our preliminary data by using proteasome inhibitor. We treated HeLa cells with Epoxomicin which is a selective, irreversible proteome inhibitor 1 hour before treating with TDH 004 to see whether PROTAC utilizes Ubiquitin-proteasome degradation system. Obviously, the Epoxomicin treatment blocked Rigosertib-PROTAC based drug effect on Hela cells and not gave any change in the RAF degradation event (Supplement figure 1).



A **B** **C**

Figure 1: The chemical structure of Rigosertib andRigosertib-PROTAC drug series.

(A) The 2D structure of Rigosertib, a synthetic benzyl styrylsulfone.

(B) Rigosertib-PROTAC based drugs were developed by combining Rigosertib and Thalidomide.

(C) The 2D structure of Thalidomide, a piperidinylisoindole, which has potential to recruit E3 ligase.

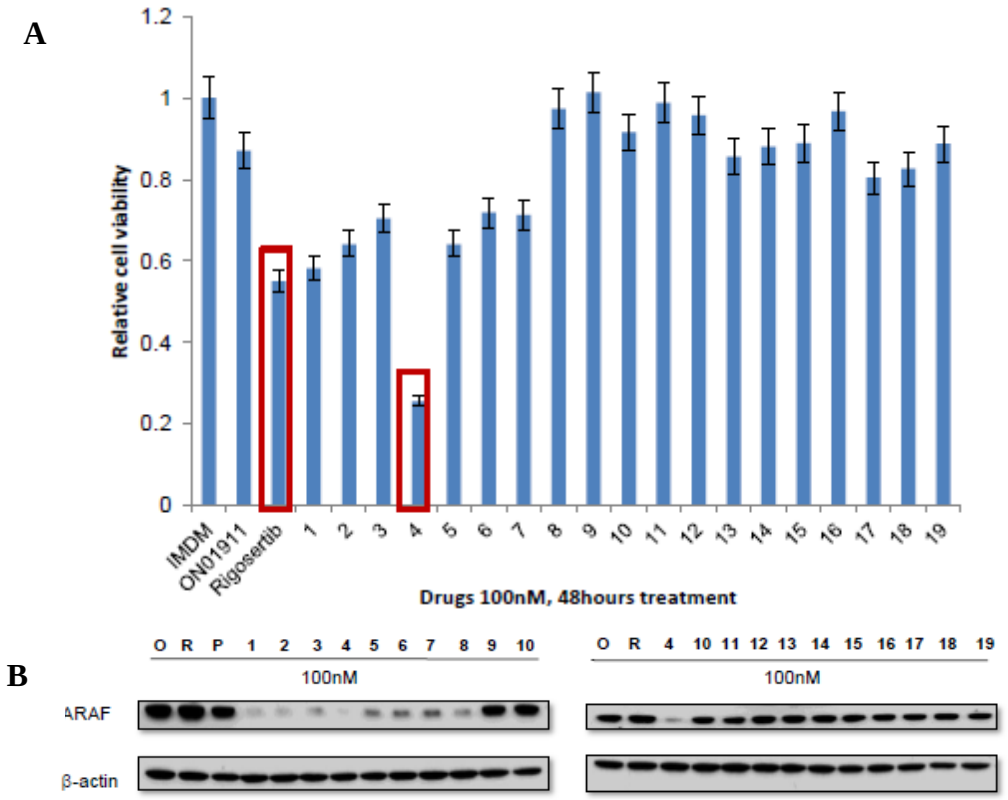


Figure 2: PROTAC- Rigosertib based drugs enhance the effect of Rigosertib on Pancreatic cancer through RAF degradation.

(A) Cell viability in response with 100nM of Rigosertib and a series of Rigosertib-PROTAC based drugs was evaluated. PROTAC drug named TDH 004 showed significant effective compared to Rigosertib with survival rate are 0.25 and 0.55 respectively.

(B) Western blot analysis of CFPAC1 for ARAF after treatment with Rigosertive at 100nM concentration with O stands for ON0911Na, R: Rigosertib, P: Pomalidomide, 01~09: Rigosertib-PROTAC based drug series.

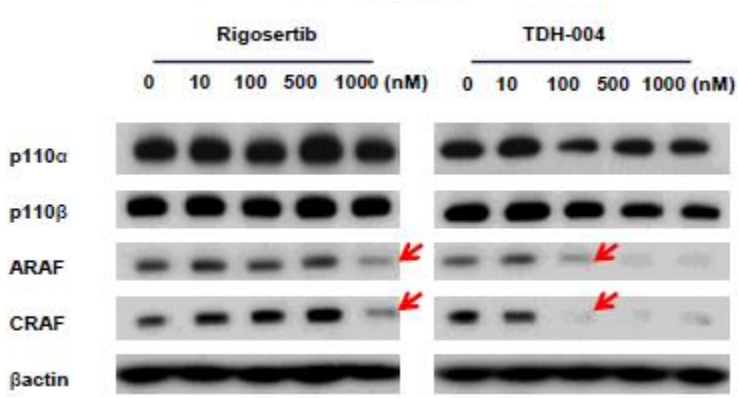


Figure 3: Drug using PROTAC technology has reduced RAF protein levels at lower concentration compared to origin Rigosertib.

Western blot analysis of CFPAC1 cell for p110α, p110β, ARAF, CRAF after treatment with Rigosertib and TDH 004 at concentration of 0nM, 10nM, 100nM, 500nM, 1000nM serially. TDH and Rigosertib started to inhibit RAF signaling significantly at 100nM and 1000nM concentration respectively.

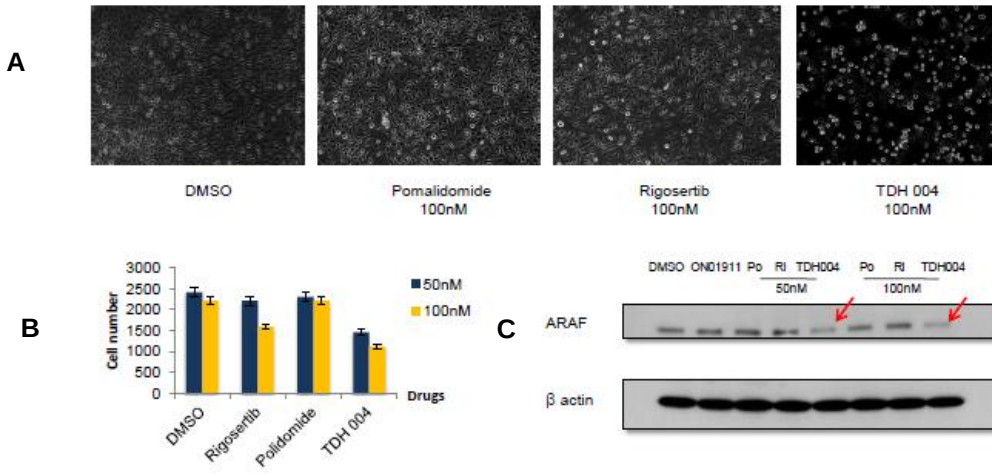


Figure 4: RAF degradation is not caused by Pomalidomide (Thalidomide) at same concentrations.

(A) Cell images were observed by using fluorescence inverted microscopes with digital. Axiovert 200M (Carl Zeiss). While Pomalidomide and Rigosertib have not give significant effect to CFPAC cells, those treated with TDH 004 detached from the bottom of plates and died.

(B) Cell numbers from microscope images were counted by using ImageJ software.

(C) Western blot analysis for RAF protein level after treatment with Pomalidomide, Rigosertib and TDH 004 at 50 and 100 mM concentration.

3.2. Direct degradation of target proteins can be the solution for overcoming the drug resistance

The addition of Rigosertib failed to demonstrate an improvement in survival or response compared with Gemcitabine alone in patients with metastatic pancreatic adenocarcinoma. Hence, for the first time, we developed an invitro model for Rigosertib resistance and evaluate the efficacy of PROTAC upon the Rigosertib resistant CFPAC1 cells.

CFPAC1 parental cell were exposed to different Rigosertib doses in different methods (Table 1). Cells were harvested after forming resistance colonies (Figure 5) and checked for resistance fold under microscope and confirm by viability assay. After comparing the effect of Rigosertib upon series of resistance sublines, cells being treated with incremental Rigosertib dose were chose to test for the effect of PROTAC –Rigosertib. Undoubtedly, these Rigosertib resistance subline shows sensitive with TDH004 (Figure 6). After long-term Rigosertib exposure, the resistant cells have undergone distinct morphologic changes. Compared with parental cells, resistant cells showed spindle-shaped morphology, abundant pseudopodia, and loss of adhesion characteristics, which are hallmark of epithelial-to-mesenchymal transition (EMT). Shah et al. reported that these morphological changes after drug exposure were related to increase of vimentin and β-catenin nuclear translocation, and decrease of E-cadherin in resistant cells.

Table 1: Treatment schedule for the development of Rigosertib resistance in pancreatic cancer CFPAC1.

Treatment number	1	2	3	4	5
Control	0nm	0 nm	0 nm	0 nm	0 nm
Pulse	200 nm	200 nm	200 nm	200 nm	200 nm
	1000 nm	1000 nm	1000 nm	1000 nm	1000 nm
Continuous	200 nm	200 nm	200 nm	200 nm	200 nm
	1000 nm	1000 nm	1000 nm	1000 nm	1000 nm
Incremental	100 nm	200 nm	500 nm	1000 nm	1000 nm

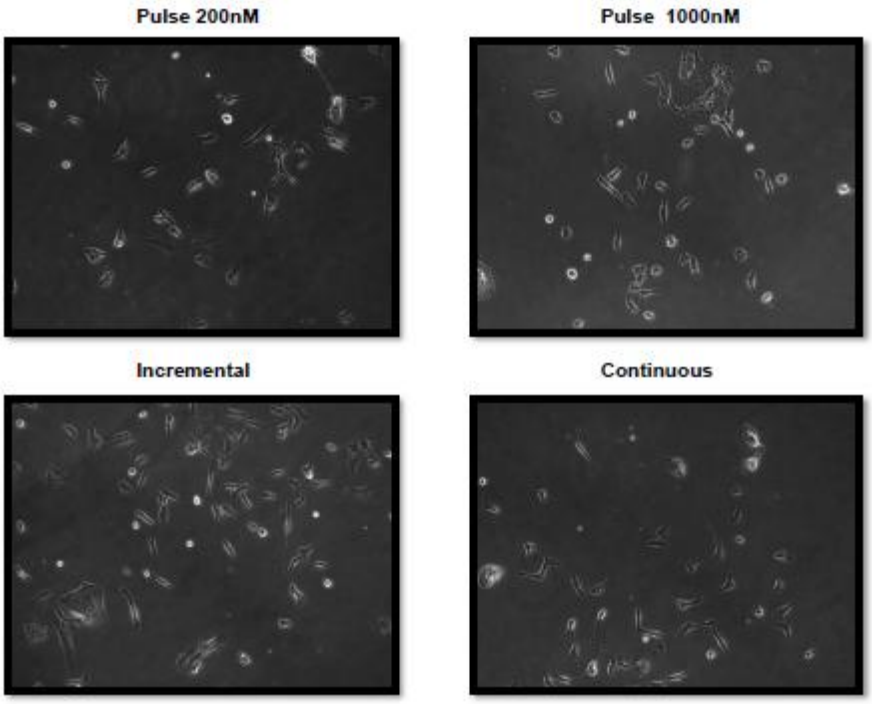


Figure 5: Resistant colonies forming from different treatment methods.

Resistant cells showed spindle-shaped morphology, abundant pseudopodia, and loss of adhesion characteristics, which are hallmark of epithelial-to-mesenchymal transition.

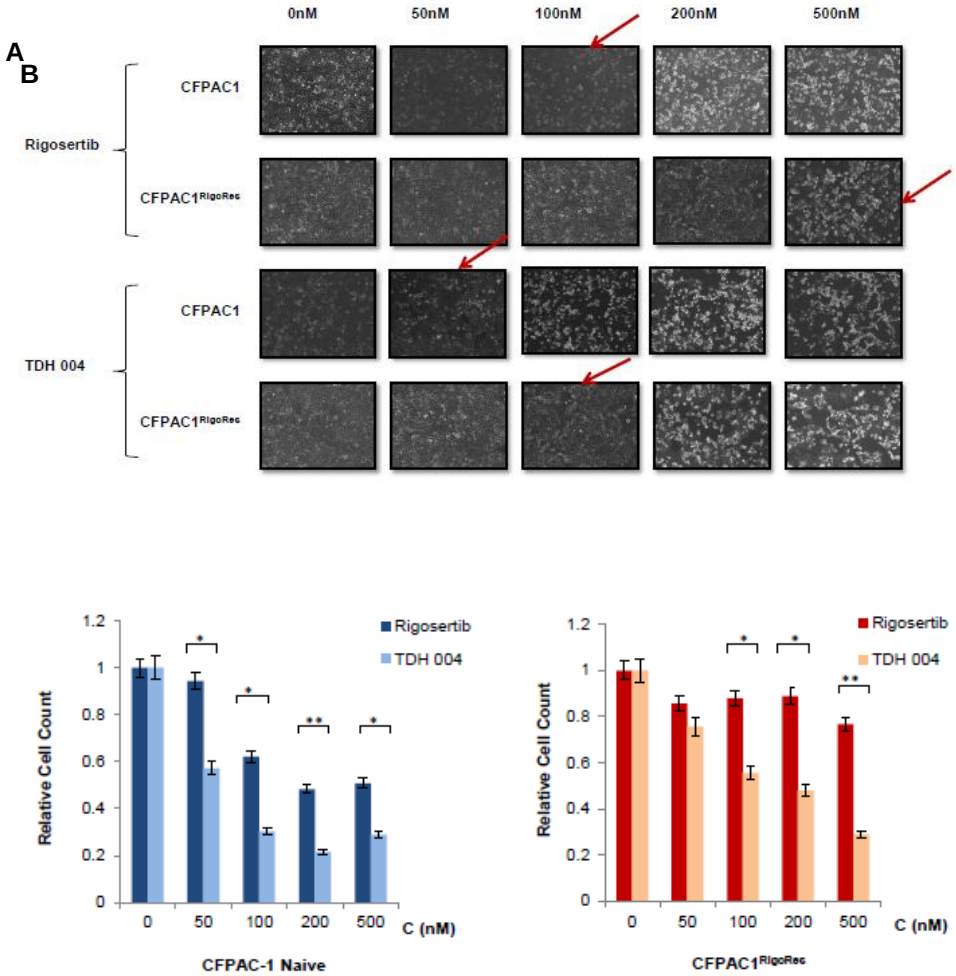


Figure 6: TDH004 gives Rigosertibresistence cell line to be sensitive.

(A)Parental and Rigosertib resistance cell lines were treated with Rigosertib and TDH 004.Rigosertib has effect in naive cell at 100nM and required 500nM of concentration in CFPAC1^{RigoRes} . TDH 004 showed effective to naive and resistant cell lines at 50 and100nM concentration respectively.

(B) Quantity data made by using ImageJ software.*p< 0.05, **p<0.01

4. Discussion

Pancreatic cancer is a challenging malignancy as the overall survival has not improved over the last several decades. The complexity of its biology profile is one of the major issues in finding the most appropriate treatment method. However, it is interesting to note that pancreatic harbors over 90% of RAS mutations and this is one of the key signatures in the development of targeted therapies. Rigosertib, a small molecule inhibitor of multiple signaling pathways, is recently reported that actually binds to RAS effectors. Unfortunately, the fact that a clinical trial of combination therapy (Rigosertib plus Gemcitabine) failed to improve the survival suggests the need of overcoming actual clinical challenge of Rigosertib in pancreatic cancer patients.

Our research has revealed that the effective of Rigosertib, which is a non-ATP competitive and a protein-protein interaction (PPI) disturbing drug, could be improved by using proteolysis targeting chimera (PROTAC) technology. Traditionally, most small molecule drugs target enzymes and receptors, so that proteins which act in protein-protein interaction (PPI) have been deemed “undruggable”. One of advantage of PROTAC is a broadening spectrum of “druggable” target from enzymes and receptors to PPI. By screening a series of PROTACs drug, I was able to notice that the different length of linker between Rigosertib and Thalidomide in PROTACs molecule gave different effect in CFPAC-1 cell line. Unfortunately, I did not find any correlation between chemical structure of drugs and their effectiveness. As shown our data, TDH-004,

one of Rigosertib-PROTACs, gives more efficiently compared to origin Rigosertib even in low concentration. This phenomenon is very important in pancreatic cancer in which the combination between different treatment methods is mostly required and lower dose of drug may give less toxicity. For this observation I suggest that there is the need of testing more types of Rigosertib-PROTACs to find the most appropriate molecule of drug for treating pancreatic cancer.

In other hand, by exposing CFPAC-1 with Rigosertib at different doses and methods, I was able to develop a partial stable Rigosertib resistant cell line. This led to another important finding which proved the effective of Rigosertib-PROTAC based drug in Rigosertib resistant cells. One of the possible explanations is that PROTACs do not require the active protein binding site, and therefore are not affected by certain acquired mutations under Rigosertib exposure. Although many things should be done in the way of making a stable resistance cell line, this brings a hope for surmounting the targeted therapy resistance of pancreatic cancer. Moreover, the effect of Rigosertib-PROTAC should be investigated further in vivo study.

Altogether, I hope that chemical knockdown strategy using PROTAC would be the solution for overcoming limitations of Rigosertib and could be used for treating acquired resistance with targeted therapies or in the combination with other therapies.

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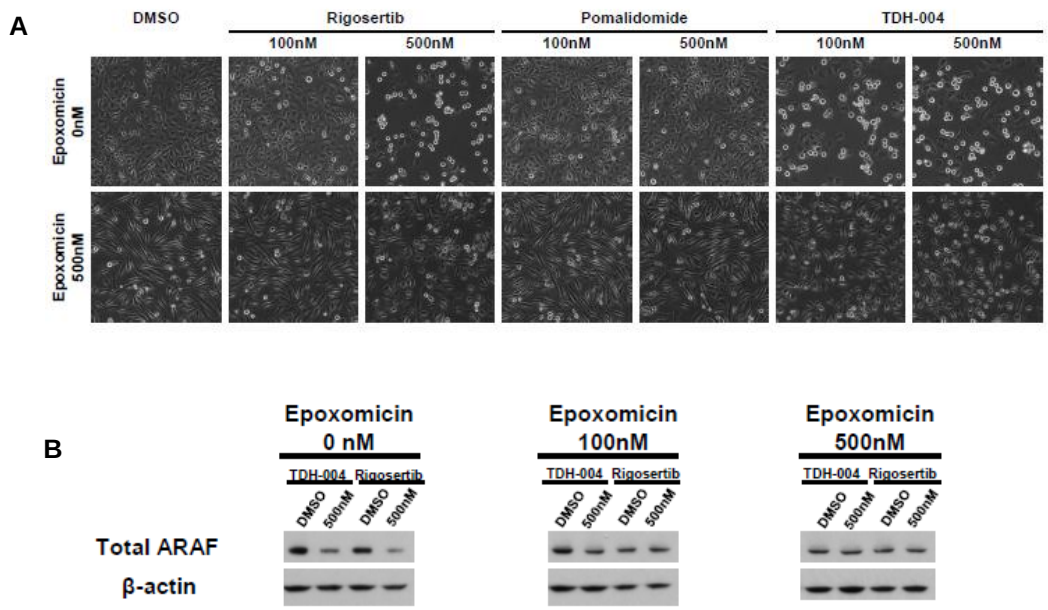

Supplement Figure 1: The irreversible proteasome inhibitor Epoxomicin completely inhibits the RAF degradation effect of Rigosertib- PROTAC based drug.

(A) Hela cells were treated with Epoxomicin 1 hour before treating with Rigosetib, Pomalidomide,TDH 004 separately. Cell images were observed by using fluorescence inverted microscopes with digital. Axiovert 200M (Carl Zeiss). Effect of TDH 004 was inhibited.

(B) Western blot analysis for RAF protein level after treatment with Epoxomicin 1 hour followed by Rigosertib and TDH 004 at 0 and 500mM concentration.Epoxomicin completely blocked the RAF degradation caused by TDH 004.

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