Dopamine receptor D2 (DRD2) antagonism as a therapeutic approach against pancreatic cancer

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A thesis submitted to McGill University in partial fulfillment of the requirement of the degree of Master of Science

July 2019

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List of Abbreviations

PDAC	Pancreatic ductal adenocarcinoma
KRAS	Kirsten rat sarcoma
PanIN	Intraepithelial Neoplasia
MCN	Mucinous cystic neoplasms
5-FU	5-fluorouracil
EGFR	Epidermal growth factor receptor
VEGF	Vascular endothelial growth factor
DRD2	Dopamine receptors D2
DRD1	Dopamine receptors D1
DRD5	Dopamine receptors D5
DRD3	Dopamine receptors D3
DRD4	Dopamine receptors D4
cAMP	Cyclic adenosine monophosphate
PKA	Protein kinase A
AC	Adenylyl cyclase
MTDs	Maximum tolerated doses
ER	Endoplasmic reticulum
PERK	transmembrane protein kinase
FDA	Food and Drug Administration
SRB	Sulforhodamine B
cDNA	Complementary DNA
CSCs	Cancer stem cells
RNAi	RNA interference

shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SDR	Safe dose recommended
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
TP53	Tumor Protein 53
ASCO	American Society of Clinical Oncology
SRB	Sulforhodamine B
ATP	Adenosine Tri-Phosphate
Na3VO4	Sodium orthovanadate

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer. Chemotherapeutic agents, including gemcitabine, alone or in combination with other anti-cancer drugs, are widely applied for treatment. However, benefits from these treatments are modest due to the therapeutic limitations associated with drug resistance. Therefore, identification of new targetable molecules is urgently needed in order to improve survival and prognosis. Dopamine receptor D2 (DRD2) is a potential target whose inhibition suppresses PDAC proliferation and metastasis upon the functional blockage of the receptor with antagonists *in vitro* and *in vivo*. In this study, we investigated possible therapeutic benefits of a combination therapy using a DRD2 antagonist and gemcitabine. For this purpose, effects of co-treatment with DRD2 antagonist Haloperidol and chemotherapy agent gemcitabine on viability of multiple PDAC cell lines were examined for various combination formulations to understand the interaction between both drugs and to determine the most efficacious combination approach. Furthermore, we examined the ability of co-treatment to induce apoptosis in different PDAC cells. Moreover, we investigated the effects of treatment on metastasis-potential of PDAC cells through analyzing invasion capacity of cells. Our results show that, while a 1:1 constant combination ratio provides the strongest growth inhibitory effect in PDAC cells, different responses to the combination treatment are observed in PDAC cell lines, in line with variable levels of DRD2 expression in these lines. The combination treatment leads to significant induction of apoptosis. Moreover, it reduces cell migration relative to vehicle-treated controls. We further validated a commercially available antibody against DRD2, which can be used for detection of DRD2 protein in PDAC. Taken together, our results indicate that the combination of Haloperidol with gemcitabine has a greater anti-cancer effect in comparison to single therapy with either of them.

Resume

L'adénocarcinome canalaire du pancréas (ADCP) est la forme la plus courante du cancer du pancréas. Les agents chimiothérapeutiques comme la gemcitabine, seuls ou en association avec d'autres médicaments anticancéreux, sont communément utilisés pour traiter ce cancer. Cependant, en raison des limitations thérapeutiques associées à la résistance aux médicaments, les avantages de ces traitements demeurent modestes. Par conséquent, il est primordial d'identifier de nouvelles molécules à cibler afin d'améliorer la survie et le pronostic des patients. Le récepteur dopaminergique D2 (DRD2) constitue une cible potentielle dont le blocage fonctionnel par des antagonistes inhibe la prolifération de l'ADCP et les métastases, *in vitro* et *in vivo*. Dans cette étude, nous avons étudié les possibles avantages thérapeutiques de l'association médicamenteuse entre l'Haloperidol, un antagoniste du DRD2 et la gemcitabine, un agent chimiothérapeutique. À cette fin, les effets du traitement sur la viabilité de plusieurs lignées cellulaires de l'ADCP ont été examinés sous diverses formulations, afin de comprendre l'interaction entre les deux médicaments et de déterminer la formule la plus efficace. De plus, nous avons examiné l'aptitude de l'association de traitements à induire l'apoptose dans les différentes lignées cellulaires. Nous avons également étudié les effets du traitement sur le potentiel métastatique des cellules de l'ADCP en analysant la capacité d'invasion des cellules. Nos résultats démontrent qu'un rapport de concentration constant de 1:1 produit la plus puissante inhibition de la croissance des cellules de l'ADCP. Cependant, nous avons observé des réponses inégales dans les différentes lignées cellulaires, ce qui reflète le niveau d'expression variable du DRD2 dans ces lignées. Le traitement d'association mène à une induction significative de l'apoptose et réduit la migration cellulaire par rapport au contrôle. De plus, nous avons validé un anticorps disponible commercialement contre le DRD2, qui est utilisé pour détecter la protéine DRD2 dans l'ADCP. Dans l'ensemble, nos résultats indiquent que l'association de l'halopéridol avec

la gemcitabine a un effet anticancéreux plus important par rapport à une thérapie unique où l'un ou l'autre est utilisé seul.

Acknowledgment

I would like to express my gratitude to my wonderful and supportive family for their love and support through years. I would also like to thank my supervisor Dr. Yasser Riazalhosseini for his scientific guidance. I thank all the lab members for the help, support, and guidance. I would also like to thank my committee members for all the knowledgeable advice.

Statement of Contribution

The experimental work in this thesis was performed by me, some of the siRNA and shRNA-based knock-down and western blotting experiments were conducted by research assistant Maryam Rajaee under the supervision of Dr. Yasser Riazalhosseini and Dr. Pouria Jandaghi.

Chapter I

Literature Review

Pancreatic ductal adenocarcinoma

Currently, pancreatic ductal adenocarcinoma (PDAC) is considered the fourth leading cause of cancer death worldwide, and expected to be the third most common cause of death after lung cancer by 2030 [1, 2]. Despondently, the median survival of PDAC is less than six months and only 2% of metastatic cases survive for 5 years [3-5]. Several risk factors are associated with PDAC incidence including age, sex, ethnicity, smoking, alcohol, diabetes, obesity, infection, and chronic pancreatitis, which are accounted for one- quarter to one third of all cases [6-9]. About 90% of PDAC patients are over 55 years of age, and PDAC is slightly higher in males than in females, presumably due to the different interactions with environmental exposure and genetic factors [10]. Infection with certain pathogens increases PDAC risk; for instance, the infection with Helicobacter pylori (H-pylori) or hepatitis C is associated with PDAC development that has been extensively investigated [11]. Additionally, family history of pancreatic cancer is counted for 5-10% of PDAC incidence [12-14].

Accumulation of genetic mutations has been shown to be involved in cancer initiation, progression, and metastasis[15]. Aberrations in Kirsten rat sarcoma (*KRAS)* have been shown to be responsible for >90% pancreatic ductal adenocarcinoma initiation; followed by mutations in *SMAD4*, *CDKN2A* and *TP53* [15].

Recent comprehensive genomic analyses, including whole exome and genome sequencing, have revealed previously-unrecognized genetic heterogeneity within pancreatic tumors and between tumors from individual patients [16]. To gain a better understanding of intra-tumoral heterogeneity and clonal composition genetics analyses of multiple regions from a single patient tumor are under consideration [16]. The intra-tumoral heterogeneity has important impacts on tumor evolution and outcomes of drug treatment. This has recently been studied in PDAC using clonal tracking experiments through cell barcoding

technologies, which are promising tools to detect low-representing clonal populations in the tumor. [17]. This study reports on small population of cells with stem cells features like quiescence, drug-detoxifying capability, and tolerance to DNA damage, which help them to exhibit resistance to drug treatments. Thus, treating tumors with gemcitabine depleted 20% of clonal subpopulations, and resulted in the enrichment of some clones, which pre-existed in the tumor [17].

Inter-individual heterogeneity in PDAC tumors have also been linked to different treatment outcomes. For example, Collisson et al. have used gene expression microarray analysis and identified three intrinsic PDAC subtypes, which are classical, quasi-mesenchymal (QM-PDA), and exocrine- like [18]. The authors showed that QM-PDA subtype exhibit high sensitivity to conventional chemotherapy (gemcitabine) as compared to the classical subtype [18].

Early Stages of PDAC

Pancreas physiological function

The pancreas has irregular shape located behind the stomach and surrounded by spleen, liver, and duodenum. The location of the pancreas makes it difficult to visualize by the current imaging methods for tumor detection. It consists of three types of cells with diverse functional properties depending on their location in the exocrine or endocrine gland [22]. The exocrine gland has acini cells that secrete digestive enzymes, and ductal cells that transport fluid from the acinar cells to the stomach, beside bicarbonate secretion for stomach acid neutralization[22]. The endocrine gland comprises of Islets cells, which produce insulin (Figure 1) [23].

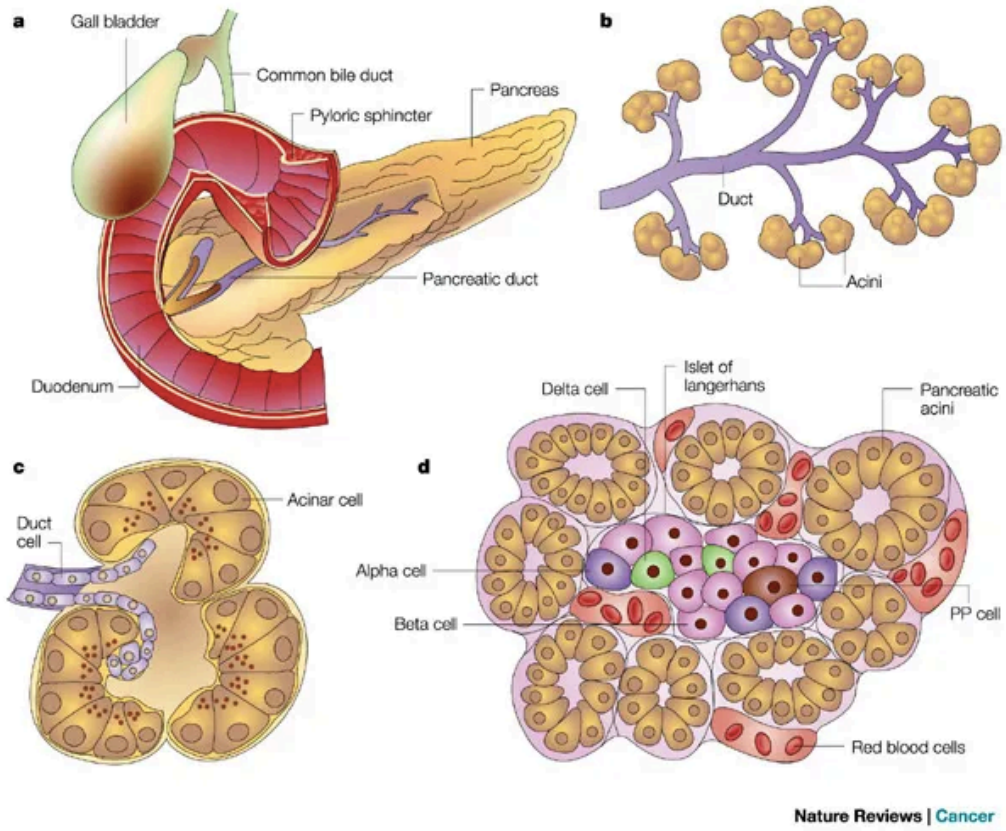


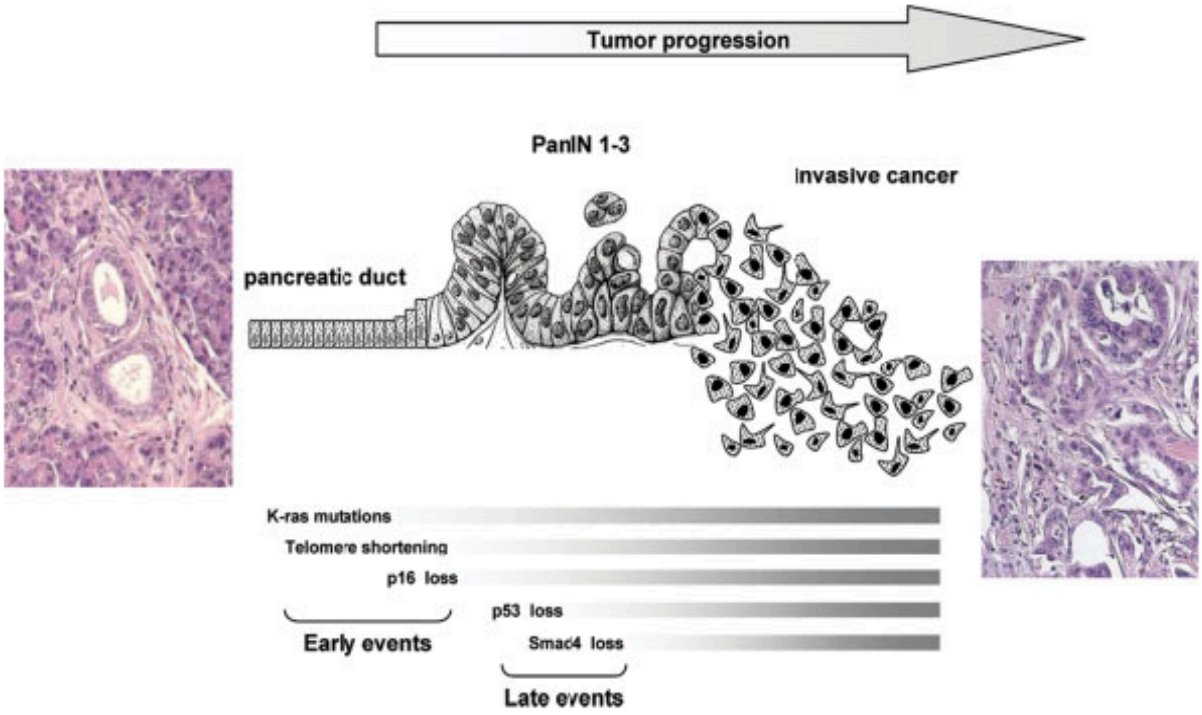
Figure 1. Schematic representation of the pancreas physiological location and its cell types [23].

Pancreatic Intraepithelial Neoplasia

Approximately, 60-70 % of PDAC arise in the head of the pancreas, followed by 11% in the body, and another 11% in pancreas tail. PDAC develops following a series of mutations from normal mucosa to precursor lesions and invasive malignancy [24, 25]. It is histologically characterized by intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMN), and mucinous cystic neoplasms (MCN) [25]. PanIN is sub-classified according to the disease degree into PanIN-1 (A and B), PanIN-2 and PanIN-3[26]. Genetics analyses have indicated many significant genetic alternations in PanINs that are also found in invasive PDAC [24, 27]. The molecular genetics abnormalities in lower grade of PDAC include mutations in KRAS gene and telomere shortening that are associated with developing invasive malignancy [28]. In the higher grades of PanIN 2-3, the prevalence of

KRAS mutations is higher, and other mutations in p16, CDNK27, p53, and SMAD4 appear to take a place (Figure 2) [29].

Figure 2. PDAC progression from PanIN formation to invasive PDAC**.** Different stages of



disease development from normal epithelium to the formation of PanIN-1 to PanIN 3 lesions, and eventually to invasive PDAC with various driver mutations throughout disease development are shown [29].

Clinical presentation of pancreatic ductal adenocarcinoma

The non- specific and asymptomatic manifestations, lack of biomarkers, and difficulties in imaging at an early stage of PDAC lead to diagnosis at an advanced stage with limited treatment options. Pancreatic cancer can occur in the head, body, and tail of the pancreases. When tumors locate in the head of the pancreas symptoms present early due to biliary obstruction [30]. But tumors that develop in the body and tail remain undetected until a late stage. Abdominal pain, jaundice, pruritus, weight loss, new-onset diabetes mellitus, and depression are the common PDAC manifestations [31, 32].

Later symptoms are related to metastasis in the liver, stomach, and colon, that cause gastric obstruction or delay gastric emptying, with nausea and satiety. Moreover, patients present with acute pancreatitis, thrombophlebitis, hypoglycemia or hypercalcemia (Figure 3) [25, 33]. .

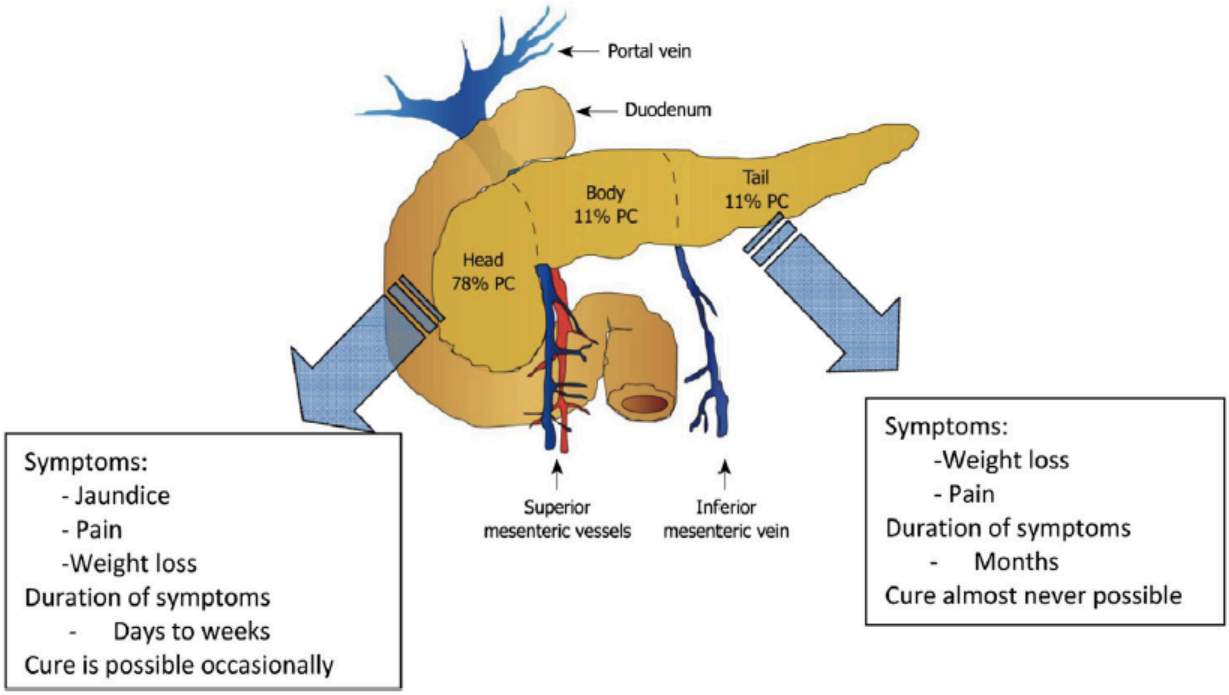


Figure 3. Pancreatic cancer location and clinical presentation [25].

In consequence, surgery treatment is possible only for 15-20% of patients [10]. Systemic therapy with gemcitabine is clinically administrated to advanced and metastatic cases with a median survival of four to six months. Clinical efficacy using chemotherapies based on gemcitabine fails to achieve greater survival due to the emergence of drug resistance [34]. Advances in therapy are therefore urgently needed.

Pancreatic ductal adenocarcinoma current treatment strategies

Surgery

Surgical resection provides significant hope for only 15- 20% of patients with resectable or borderline-resectable tumors [35]. Majority of patients 80% with a tumor in the pancreas

head with jaundice symptoms due to biliary obstruction [36, 37]. Due to complications associated with preoperative biliary drainage, early surgery without preoperative biliary resection is performed for patients who exhibit jaundice [38]. Poor predictors for successful resection include lymph node involvement [39], high tumor grade [40], large tumor size [41], and positive margins of tumor pro-resection [4, 42]. Surgery treatment is not considered without additional therapy, since more than 90% of patients relapse and die after a potentially curative operation. Accordingly, chemotherapy treatment strategies in adjuvant setting have been used over the past decades.

Chemotherapy

Generally, treatment of locally advanced pancreatic ductal adenocarcinoma is complicated, and initially systemic chemotherapy is administered, but if the tumor metastasizes and no metastatic reduction after treatment is observed, therapies for tumor control and/or symptoms relief are administrated. Borderline resectable tumors are usually treated with chemotherapy to improve the chance of curative tumor resection and to increase patient's survival rates [43, 44]. Patients with resectable and locally advanced unresectable tumors can also benefit from neoadjuvant or perioperative chemotherapy. The initiation of the neoadjuvant therapy process is evaluated by histological confirmation of PDAC. 5-fluorouracil (5-FU) based chemotherapy was the first chemotherapy for PDAC with a mean survival of 3 months. Combination of 5-FU with other chemotherapies such as doxorubicin, methotrexate, and cisplatin did not improve overall patient's survival[45-47].

The chemotherapeutic agent gemcitabine (or 2', 2'- difluoro 2'-deoxycytidine, dFdC) is a nucleoside cytidine analog, which is widely used for treatment of PDAC [48]. It is the first monotherapy drug that surpassed 5-FU at increasing overall survival to 6 months [46]. The biological uptake of gemcitabine into the cell is mediated by three nucleoside transporters SLC28A1, SLC28A3, and SLC29A1 [49]. It metabolizes into its active form by a series of

phosphorylation events that result in binding to DNA strands during replication, inhibiting DNA synthesis and causing cell death [34]. There are several mechanisms associated with gemcitabine resistance phenomena such as the dysregulation of proteins involved in the gemcitabine metabolism pathways, and high expression of ATP-binding cassette transporters [50]. Therefore, combination of gemcitabine with other drugs has been evaluated in PDAC treatment[51]. A recent trial has shown that adjuvant therapy with a gemcitabine-capecitabine combination in patients with resected disease increased median survival up to 28 months compared to 25.5 months with gemcitabine alone [47, 52]. The combination therapy of gemcitabine- capecitabine toxicity was tolerable and manageable to be considered as a new standard adjuvant combination therapy in the 2017 American Society of Clinical Oncology (ASCO) Clinical Practice Guideline update for potentially curable pancreatic cancer [52]. Likewise, combination of gemcitabine with nab-paclitaxel (nanoparticle albumin-bound paclitaxel) prolonged overall survival for two months as compared to gemcitabine treatment alone [53].

A revolution that has marked the history of PDAC treatment is the application of FOLFIRINOX that shows significant survival prolongation of one year compared to gemcitabine [54]. FOLFIRINOX is composed of four chemo-agents: 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin. However, diverse toxicity and side effects including severe hematologic toxic effects and diarrhea make this treatment less favorable [55]. A modified FOLFIRINOX with less toxicity as an adjuvant therapy in patients with resected PDAC tumors has considerably improved median disease- free survival up to 21.6 months compared to 12.8 months in the gemcitabine- treated group [55, 56]. Despite the significant achievements in the development of PDAC therapeutics, the modest efficacy of the currently used chemotherapies highlights the need for developing novel targeted therapies, particularly for patients who cannot be offered curative surgery [57].

Targeted therapy in pancreatic ductal adenocarcinoma

Recent studies have revealed significant pathologic molecular processes that are involved in PDAC development or progression [57]. This knowledge has led to the development of therapeutic strategies targeting key disease-driving molecules[57]. For example, aberrant activation of the epidermal growth factor receptor (EGFR) pathway in PDAC results in tumor progression and metastasis[57, 58]. Thus, erlotinib and cetuximab, which inhibit the EGFR pathway, have been used to treat PDAC, and has exhibited tumor-growth inhibitory effects, although some molecular aspects of the response are not yet fully understood [57, 59, 60]. Furthermore, the combination of erlotinib with gemcitabine improves median survival by two weeks compared to gemcitabine treatment alone [61]. Also, several other proteins have been examined as potential therapeutic targets [57], including vascular endothelial growth factor (VEGF) receptor, which is associated with poor prognosis of PDAC, [62] and is inhibited by bevacizumab; the drug is also used in combination with gemcitabine and improved the overall survival to 8.8 months in phase II trial [63]. Unfortunately, phase III trial of gemcitabine and VEGF inhibitors (Placebo or bevacizumab) combinations has failed to improve overall survival [63]. Moreover, trametinib, which inhibits MEK1/2, downstream of KRAS, is also used in combination with gemcitabine [57]. Although preclinical or clinical trial studies of these targeted therapies showed promising outcomes, the effects are overall marginal and no further targeted concept exists [47, 57]. As such, despite the latest improvements, the identification of valid and effective therapeutic targets for PDAC has remained an ongoing challenge, which requires further insights into the biology of the disease in order to pinpoint novel targetable pathways.

The existence of genomic transcriptomic data of PDAC tumors has provided an opportunity to identify molecular subtypes of PDAC that benefit from particular treatments. For instance, recent research has shown that PDAC patients with deficient DNA repair

mechanisms may benefit from platinum-based chemotherapy [47, 57, 64]. Moreover, the use of such genome dataset can help to advance our understanding of the molecular mechanisms of PDAC formation and progression, and thereby to identify new potentially targetable molecules. Specifically, the development of computational modelling and quantitative methods that integrate large-scale genomic data of cancers with knowledge of molecular networks (e.g. protein-protein interaction or gene-regulatory networks) have facilitated the identification of molecules that are critical for growth or survival of cancer cells. [65, 66]. Applying such computational approaches to transcriptome data of PDAC, our lab identified dopamine receptor D2 (DRD2) as a key factor involved in pancreatic cancer cells survival, and as a potentially novel target for PDAC treatment [67].

Dopamine receptors, and their emerging roles in cancer

Dopamine receptors belong to the G protein-coupled receptors family, which are encoded by genes localized on different chromosomal loci, with homology in protein structure and function [68]. They consist of two groups: D1- like receptors, including DRD1 and DRD5 receptors, and D2- like receptor (DRD2, DRD3, and DRD4), which generally have seven transmembrane domains and COOH and NH2 terminals (Figure.4). They are associated with stimulatory and inhibitory function of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) [69]. Dopamine receptors are predominantly expressed in the brain, cardiovascular system, retina, and kidney [70-72].

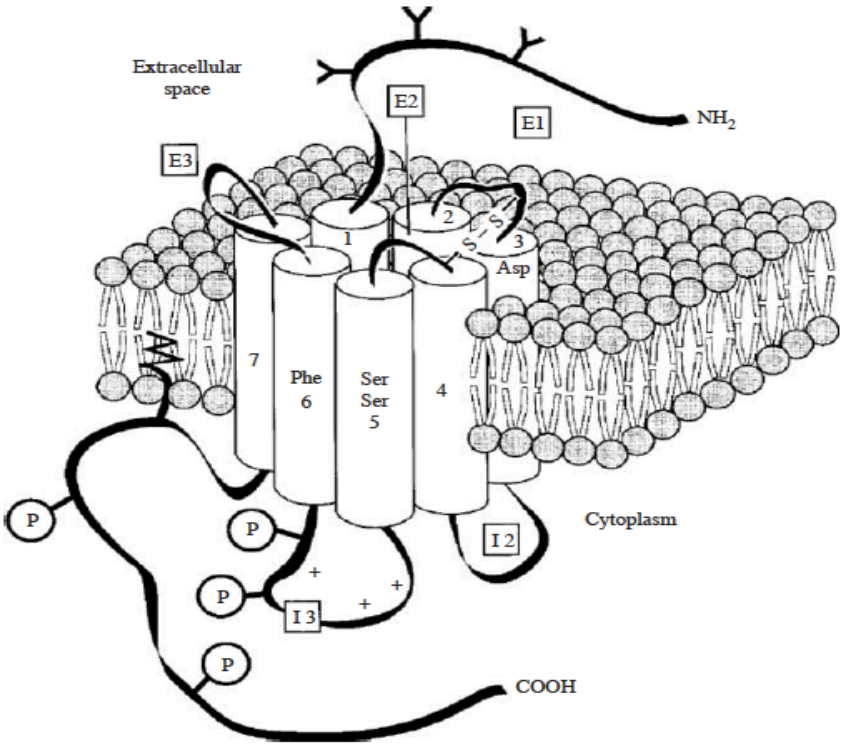


Figure 4. Dopamine receptor structure. D2-like receptors have seven transmembrane domains with short COOH- terminal tail and bigger third intracellular loop than D1-like receptor. Phosphorylation sites are found on the third loop as well on the COOH- terminal. The glycosylation sites are present on the NH_2-termnial [68]

There are two main variants of DRD2, long and short isoforms $D2_L$ and $D2_S$, which are produced by alternative splicing of exon 6, which is 87 bp long [73]. They differ by 29 amino acid residues in the protein structure but share similar pharmacological characteristics [74]. However, DRD2 isoforms showed to have different physiological functions, since $D2_L$ acts at the postsynaptic sites and $D2_S$ acts at the presynaptic autoreceptor functions (Figure 5) [75]. Several studies have described crucial roles for DRD2 in modulating the cardiovascular and renal function and in gastrointestinal motility[69]. In the brain, dopamine receptors have a significant role in mediating the effect of dopamine neurotransmitter, as well as motor, cognitive, and neuroendocrine functions[76, 77]. The dysfunction of DRD2 has been

implicated in a variety of neurological disorders including Parkinson's disease and schizophrenia [77, 78].

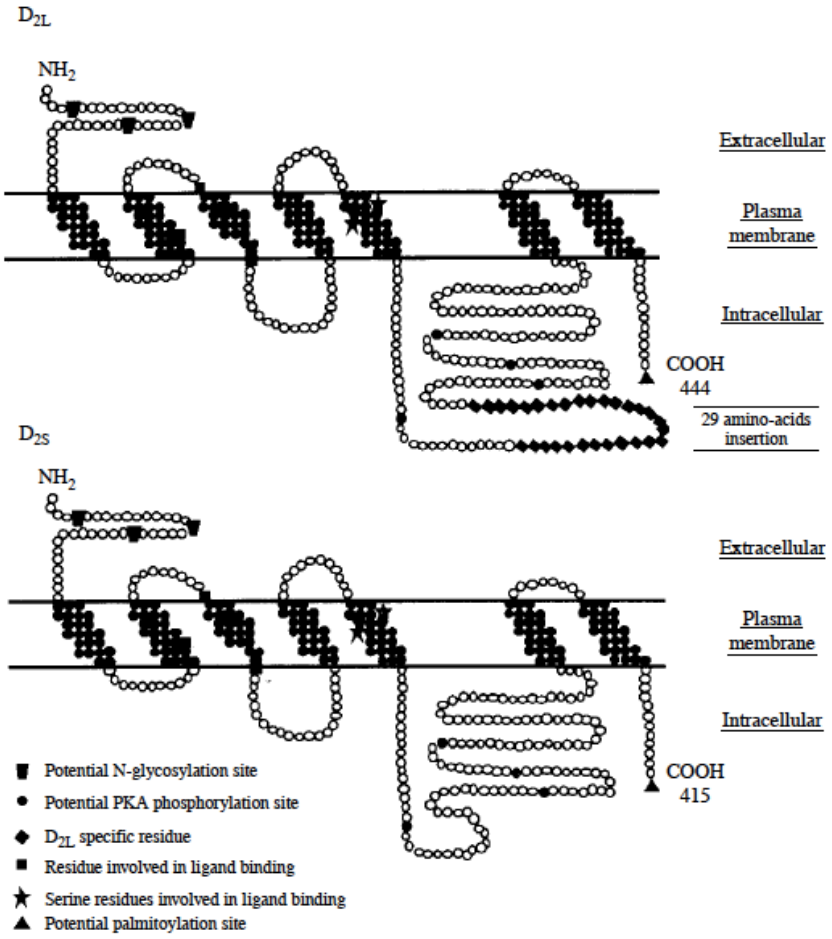


Figure 5. DRD2 receptors isoforms structure representation. The specific 29 amino acids differences represent in $D2_L$ and absent in $D2_S$ that is located in the third cytoplasmic loop. The ligand, phosphorylation, and glycosylation sites are shown in both DRD2 isoforms[68].

Interestingly, in Parkinson's disease, the defective dopaminergic signaling has been observed to be associated with lower cancer incidence rate [79]. Recent studies have shed light on the contribution of dopamine receptors in cancer progression and metastases in different types of cancer including glioblastoma, colorectal, breast, gastric, and PDAC [80-83]. It has been shown that the overexpression of DRD2 alters signaling pathways that are implicated in tumor growth, angiogenesis, and metastasis [84]. Specifically in PDAC, the

inhibition of DRD2 function through inhibitory RNAi or pharmacological approaches using DRD2 antagonists haloperidol and pimozide reduced pancreatic tumor growth and metastases *in vitro* and *in vivo* by increasing cAMP/PKA activation [67], leading to a rapid increase in the cytosolic Ca2+ level and causing endoplasmic reticulum (ER) stress[67]. Consequently, it induces cell cycle arrest at the G1 phase with a higher number of apoptotic cells [67]. These observations indicate the potential of DRD2 inhibition as a novel targeted therapy in PDAC. Given that DRD2 antagonists are already used in the clinic to manage psychiatric disorders (e.g. schizophrenia), the use of DRD2 inhibition approach for PDAC treatment may be facilitated through a drug repositioning strategy.

Haloperidol is a standard antipsychotic drug that is used to treat many psychiatric diseases such as schizophrenia with high clinical efficacy in reducing schizophrenia symptoms. It binds to dopamine and 1-adrenergic receptors and with modest affinity to histamine H_1, serotonin 5-HT_{2c}, and muscarinic M_1 receptors[85, 86]. It highly metabolizes in the liver. Since haloperidol is approved by the Food and Drug Administration (FDA) for the treatment of psychiatric disorders, data is already available on tolerability of its side effects, making it very attractive candidate for drug repositioning for PDAC treatment.

Approaches for drug combination in cancer

The concept of drug combination is commonly employed in treating fatal diseases such as cancer and AIDS. The purpose of combination therapy is to achieve synergistic effect, dose and toxicity reduction, and to minimize or delay the emergence of drug resistance [87]. There are two combination strategies; vertical combinations, where two or more drugs target the same pathway at different points, and horizontal combination, in which, drugs have different intercellular signaling pathways targets. To determine if drug combinations are synergistically affecting tumor growth, preclinical studies (in *vitro* and in *vivo*) allow for more rational design of clinical setting protocols, which is usually administrating each drug at

their maximum tolerated doses (MTDs) [88, 89]. MTDs may result in high toxicity with less beneficial effects of the combination due to concentration- dependent drug interactions [90]. The in *vitro* beneficial combination treatment effect is dependent on drug: drug ratio. Therefore, constant and non-constant combination ratios are used to define the optimal drug-combination interaction effects. In the constant ratio method, fixed dilution factors (e.g. 2-fold dilution) are applied to the IC50 values of both drugs to create a series of drug combination doses (above or below IC50 values), in which a constant ratio exists between the two drug doses. These fixed dilution factors can be equal, for example 1:1 for drug 1: drug 2 combinations, or they can be different, for example 2:1 or 1:2 for drug 1: drug 2 combinations. Non-equal constant-ratio methods can be used to have a wide view on the optimal drug combination ratio that can be affected by limitations in solubility or toxicity of one of the two drugs [87]. Another method to explore the effect of the combination treatment is non-constant ratio, which is maintaining fixed concentrations of one drug and increasing concentrations of the other drug [87].

Rational and Objectives

Obstacles in pancreatic cancer early diagnosis, disease heterogeneity, and emergence of resistance to current therapies highlight an urgent need to identify novel and effective therapeutic strategies for PDAC. A promising target in PDAC treatment is dopamine receptor D2 whose expression has been found in 80% of PDAC tissues. Our laboratory showed the contribution of DRD2 to PDAC growth and metastasis, marking DRD2 as a promising potential target for therapy. The commonly used chemotherapy agent gemcitabine exert its function by binding to DNA strands during replication and consequently inhibiting DNA synthesis process, whereas DRD2 antagonists induce excessive ER stress in cancer cells. Therefore, we **hypothesize** that a combination of DRD2 antagonism with gemcitabine may result in improved anti-tumor effects of either drug individually, and may serve as a novel therapeutic approach for PDAC treatment. The **objective** of this study is to evaluate potential beneficial effects of a combination therapy involving DRD2 inhibition and gemcitabine in PDAC. For this purpose, we have examined the effects of co-treatments with DRD2 antagonist haloperidol and gemcitabine on viability, apoptosis induction and migration capacity of multiple PDAC cells lines, as described below.

Chapter II

Material and Methods

Assessment of drug concentration- effect on 2D models of PDAC cell lines

The Sulforhodamine B (SRB) assay, a cell viability determination assay that measures cellular protein content, was performed to examine the effect of the combination treatment on cell viability in PDAC cell lines Panc-1, MiaPaCa-2, and BxPC-3. These cell lines differently express DRD2 with highest expression in BxPC-3 to lowest in Panc-1. Cells were plated in 96-well plates (11×10^2- 5×10^2 cells/well) and 24h after were treated with various concentrations of gemcitabine. 24h after treatment with gemcitabine, cells were treated with different doses of haloperidol. Assays were developed after 96h post gemcitabine treatment. Initially, the concentration of a single drug that inhibits 50% of cell proliferation (IC50) was determined by fitting the slope to an inhibitory response curve utilizing GraphPad 7 software.

Assessment of drug concentration-effect on 3D spheroid models of PDAC cell lines

Cell Titer-Glo, is a cell viability determination assay that measures cellular ATP, which is an indicator of metabolically active cells. The assay was used to examine the effect of the combination treatment on the cell viability in 3D models of PDAC cell lines Panc-1, MiaPaCa-2, and BxPC-3. To generate 3D spheroids, cells were plated in 96- well U-shaped plates (5×10^2- 2×10^3 cells/ well) and on the fourth day after plating, spheroids were treated with gemcitabine for 24h, then haloperidol was added. Assays were developed after 12 days from plating cells. The concentration of single drugs that inhibits 50% of cell proliferation (IC50%) was determined, and the combination treatment was tested.

Knockdown experiments using siRNAs and shRNAs

DRD2 siRNAs were purchased from Dharmacon Company. siRNA transfection was performed with 25 nM final concentration of siRNA using Lipofectamine RNAiMAX protocol. Lentiviral-delivery of shRNAs was performed as described previously [67].

DRD2 siRNA	Sequence
siRNA-2	CCUGAGGGCUCCACUAAAG
siRNA-4	GAUGGUGAGUGGAAAUUCA
siRNA-17	CUGUCAUCGUCUUCGGCAA
siRNA-18	CCAUGCACCAAACUAAUAA

Quantitative real-time PCR

The miRNAeasy kit (Qiagen) was used to extract total RNA. 1ug RNA was reversed transcribed into complementary DNA (cDNA) using Transcriptor First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific Rockford, USA). Real-time PCR reactions were prepared using the Lightcycler 480 SYBR green I master kit (Roche), and were run on a LightCycler 480 instrument (Roche) according to the manufacturer's instructions. Relative DRD2 mRNA levels were normalized to the expression of the housekeeping gene 18S ribosomal RNA. Quantitative values were calculated according to a delta ct method.

Following primers were used for DRD2 and 18S:

DRD2:

Forward: AGCCACCACCAGCTGACTCT

Reverse: GGGCATGGTCTGGATCTCAA

18S:

Forward: AACCCGTTGAACCCC ATT
Reverse: CCATCCAATCGGTAGTAGCG

Western blotting

Cells were washed with cold PBS, and protein isolation was performed using a scraper in the presence of cold M-PER lysis buffer (Thermo Scientific) containing 1mM Na3VO4, 10 mM NaF, and anti-phosphatase and protease inhibitor. Total protein was quantified using BCA protein assay and was mixed with Laemmli Sample Buffer (Bio-Rad). After denaturing the lysate at 98 ^{o}C for 5 min, 10 ug of each protein lysate was separated by 10% SDS-PAGE and transferred into nitrocellulose membranes. After blocking for 1 hr with 5% (w/v) BSA or non-fat milk in TBST (50mM Tris-HCL pH 7.4, 150 mM NaCl, 0.1 % Tween20),

membranes were incubated with primary antibodies DRD2 Millipore) 1: 3000 dilution, GAPDH (Thermo Scientific) 1:10000 overnight at 4^{o}C followed by secondary antibodies (HRP-conjugated anti-rabbit) for 1 hr at RT. Signals were detected using prime ECL plus (Sigma) and exposing the membrane to an X-ray film (Thermo Scientific).

Apoptosis Assay -Caspase 3/7 activity

Apoptosis induction upon treatment with the DRD2 antagonist, gemcitabine, and combinations treatment was determined using a Caspase-Glo 3/7 Assay kit (Promega, Madison, USA) according to the manufacturer's instructions. Cells were incubated with gemcitabine for 24h, before treatment with haloperidol for another 24h, then the Caspase-Glo 3/7 reagent in a volume equal to that of the existing medium in each well was added to the well. Plates were shook for 30 sec and incubated at RT for 30 min before luminescent signals were quantified.

Transwell migration assay (Boyden chamber assay)

Cell culture Transwell inserts of 8.0 um pore polycarbonate membranes were placed into 96 well plates. The bottom chambers were filled with 325ul medium containing 20% FBS. Then, cells were suspended in 75ul 0.1% serum medium supplemented with DMSO (solvent control) or drugs and were added to the upper part of the chamber. Following 4 h incubation at 37^{o}C medium was removed and the chambers were washed with PBS. Penetrated cells were detached with accutase, and the cell number was determined by Cell Titer- Glo Luminescent cell viability assay.

Statistics

All graphs were prepared using GraphPad prism version 8. Data are expressed as mean ±SD. Student *t*-test was used to examine the differences between treatment groups, and results were considered significant at p-values<0.05.

Chapter III

Results

Combination treatment effects on PDAC cell growth

In this study, we investigated a potential treatment using a combination of haloperidol with gemcitabine against various PDAC cells lines to further boost gemcitabine therapeutic effects. Various combination approaches, including constant and non-constant combination ratios [91] were applied to identify the most-effective combination strategy *in vitro*. First, single drug treatment of gemcitabine (nM) and haloperidol (uM) were applied separately to identify IC50 values for each compound in Panc-1, MiaPaCa-2, and BxPC-3 cell lines. Figure 6 shows response curves to treatments with the drugs and the IC50 values corresponding to each cell line. The results in figure 1 show that Panc1 is more resistant to both drugs (with highest IC50s) than MiaPaca-2 and BxPC-3. Moreover, MiaPaca-2 and BxPC-3 show greater sensitivity to haloperidol treatment than Panc1 cells. These results are consistent with results from our earlier study demonstrating a correlation between expression levels of DRD2 and the sensitivity to haloperidol in MiaPaca-2 and BxPC-3 cell lines [67]. Next, constant-ratio drug combination approaches of 1:1, 1:2 or 2:1 was applied based on $IC50_{Gemcitabine}$:$IC50_{Haloperidol}$ concentrations to investigate effects of combination of drugs on cell viability. The 1:1 combination strategy resulted in significantly stronger effects on reducing cell viability when compared to individual single treatments in BxPC-3 cells. We also observed a similar trend in MiaPaca-2 cells, though the differences were not significant. However, this combination strategy did not show any beneficial effects on reducing viability of Panc-1 cells. (Figure 7A). Other constant combination ratio approaches of haloperidol and gemcitabine (1:2, 2:1) did not show beneficial effects in PDAC cell lines examined (Figure 7B-C).

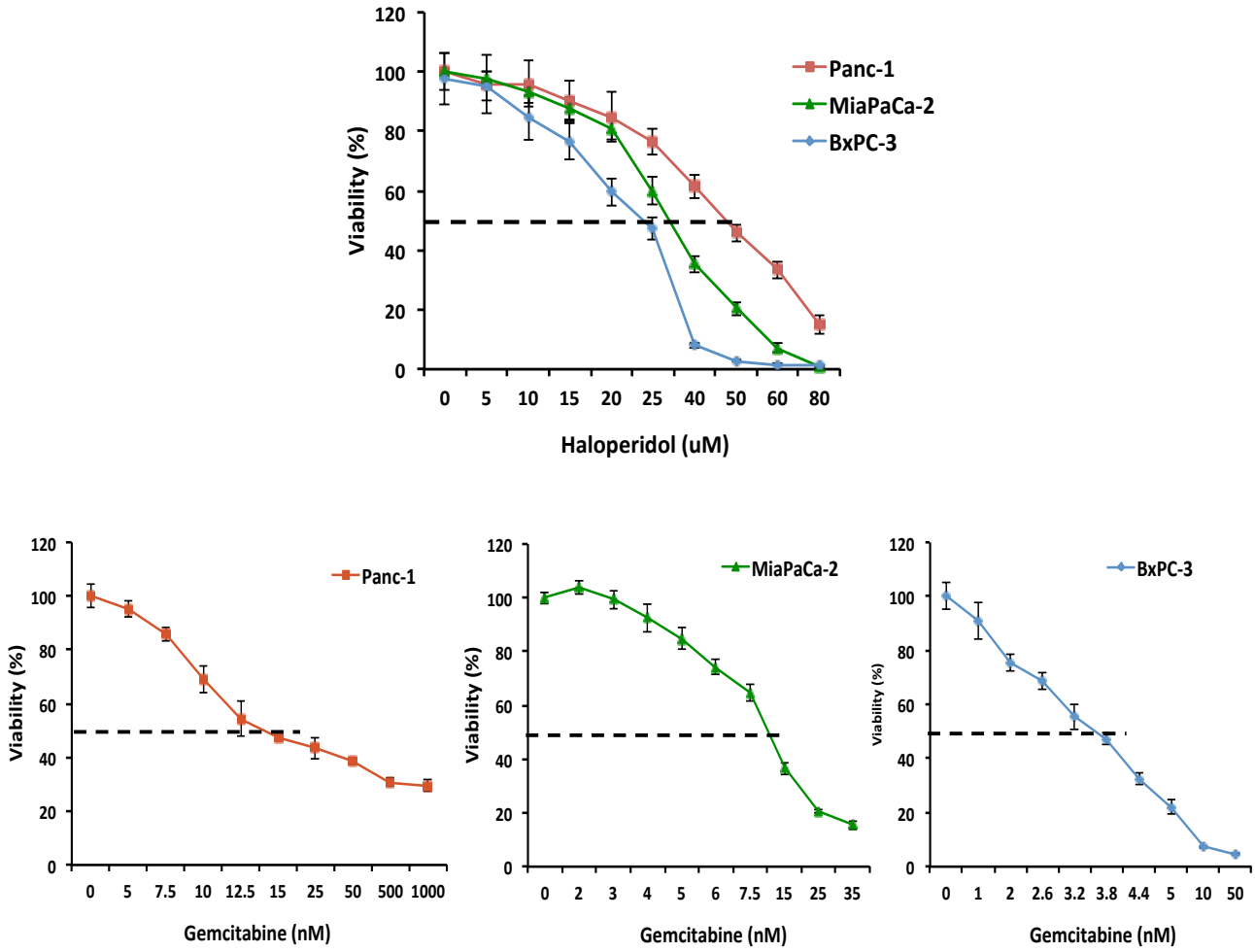


Figure 6. Response curves showing the effects of treatment with Haloperidol or Gemcitabine on the viability of PDAC cell lines. Cell viability was measured by SRB assay 96h post-treatment. The effect of treatment on cell growth was plotted as the fraction of live cells in each treatment condition relative to that of cells treated with carrier solvent. Error bars represent standard deviation (SD) of three experiments done in quadruplicate.

To determine the potential beneficial effects of combination treatment using non-constant ratio approach we treated cells with different fixed doses of haloperidol that were independently combined with increasing concentrations of gemcitabine. As such, this strategy provides an opportunity to explore the combination effects in a wide range of drug combination scenarios.

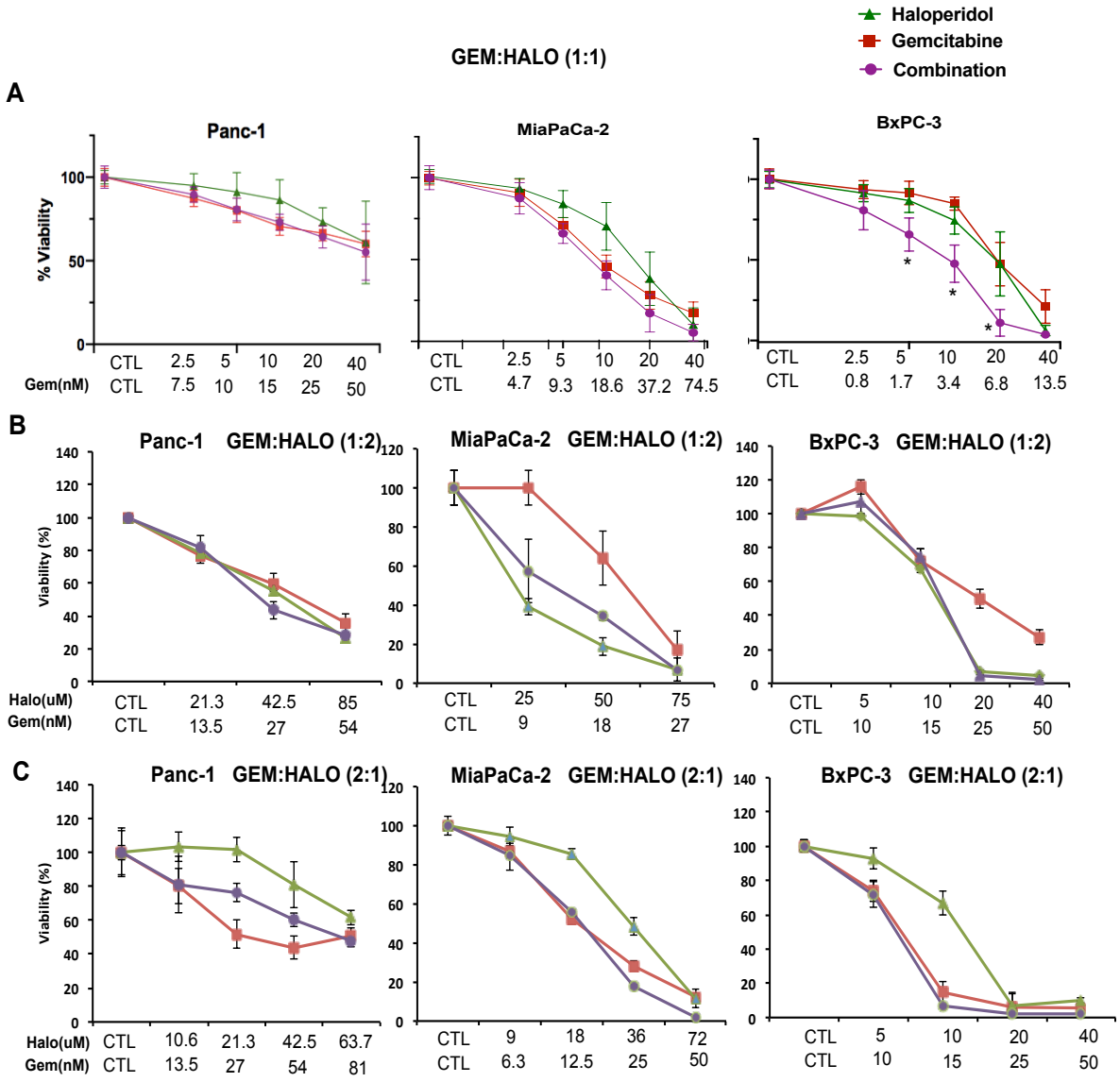


Figure 7. The combination treatment effects using 1:1 (A), 1:2 (B) and 2:1 (C) constant drug ratio method for gemcitabine and haloperidol on the growth of PDAC cell. Cell lines were treated with gemcitabine for 24h, and then different concentrations of haloperidol were added. Cell viability was measured by SRB assay 96h post-treatment with gemcitabine. The effect of drug treatments on cell growth was shown as the average fraction of live cells in each treatment condition relative to that of control cells treated with carrier solvent. Error bars represent SD of three experiments done in quadruplicate. * $P < 0.05$.

In total, in each cell line 20-30 combinations were examined, of which some showed a significant beneficial effect on cell viability (Figure 8). Although we found conditions with potential beneficial combination effects in all examined cell lines, BxPC-3 showed more conditions in which combinations had stronger effects compared to single treatments (see dashed boxes in Figure 8). Notably, these conditions involved lower concentrations of both drugs in BxPC-3 cells in comparison to other cell lines. In other words, beneficial effects were observed with higher doses of drugs in MiaPaca-2 and Panc-1 cells.

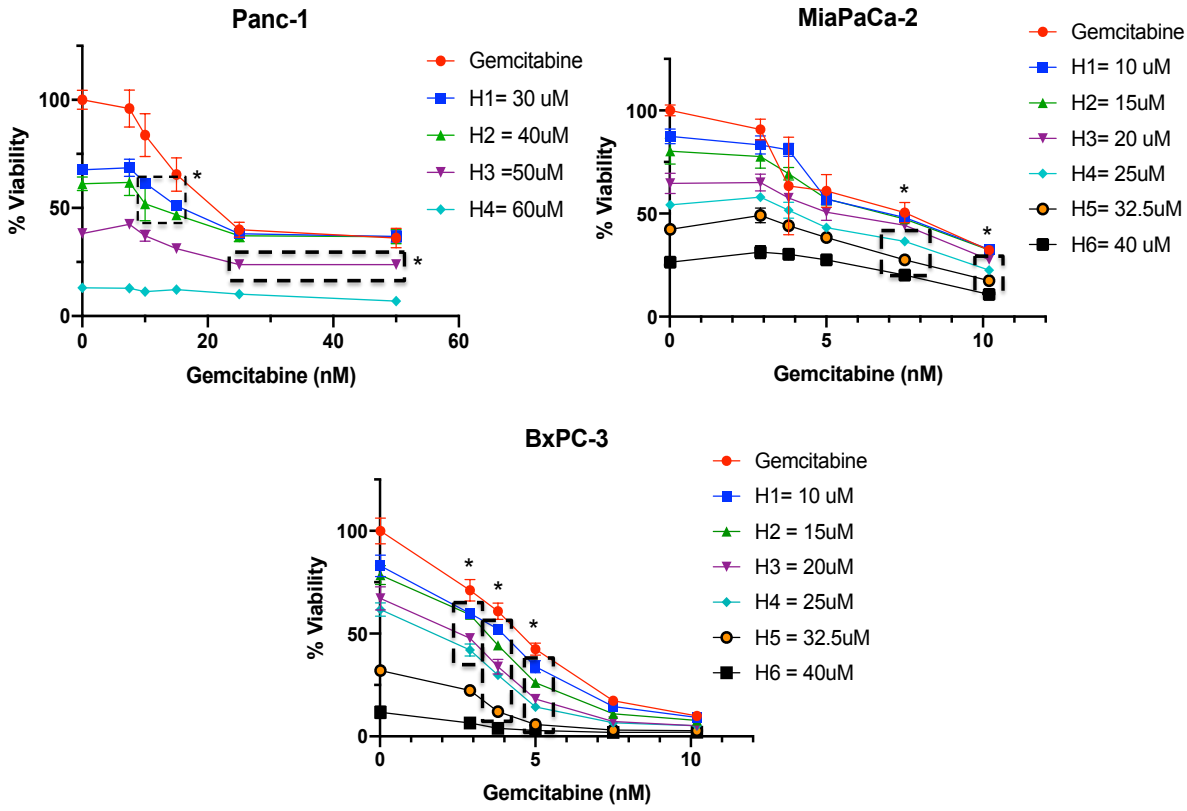


Figure 8. The combination treatment effects on the viability of PDAC cells following non-constant ratio method. PDAC cells were treated with increasing gemcitabine concentration for 24h then multiples fixed concentrations of haloperidol were added. Cell viability was measured 96h post-treatment with gemcitabine. The effect of drug treatments on cell growth was shown as the average fraction of live cells in each treatment condition relative to that of control cells treated with carrier solvent. Error bars represent the SD of three experiments done in quadruplicate. Boxes indicated by dashed lines mark combination conditions that

showed significantly stronger effects on reducing cell viability when compared to their corresponding single treatment conditions with gemcitabine and haloperidol separately. * $P < 0.05$.

Combination treatment induces apoptosis

Given beneficial results on reducing cell viability from 1:1 constant ratio combination therapy, we sought to examine if this combination treatment can induce apoptosis in PDAC cells. To investigate apoptosis induction active caspase 3/7 was measured in PDAC cell lysates after treatment with haloperidol and gemcitabine or either of them. We observed a significant increase in Caspase 3/7 activity upon combination therapy in BxPC-3 and MiaPaca-2 cells but not in Panc1 cells, in comparison to treatments with single drugs (Figure 9). These data are in line with results from cell viability analysis of these cell lines, and indicate that induction of apoptosis by the combination treatment may underlie reduced viability of cells following the combination treatment.

Combination treatment inhibits cancer cell migration

DRD2 regulates cellular cAMP levels, which are known to modulate cancer cell adhesion and invasion potential in pancreatic cancer [92]. Therefore, we tested whether the combination treatment has an effect on cancer cell migration by assessing the motility of PDAC cells using quantitative three-dimensional Boyden chamber assays. To avoid potential confounding results that could originate from cytotoxic effects of combination therapy, cell migration and invasion potential was examined in cells exposed to combination therapy for 4h only, when the combination therapy did not change the viability of cells upon combination therapy (Supplementary Figure 1). The combination treatment reduces cell migration in a larger extent than those observed in single treatments with either drugs in all cell lines (Figure 10). This effect was observed with a range of drug dosage (low and high) in BxPC-3, whereas similar results were obtained only with higher doses of drugs in MiaPaca2 and Panc-1 cells (Figure 10).

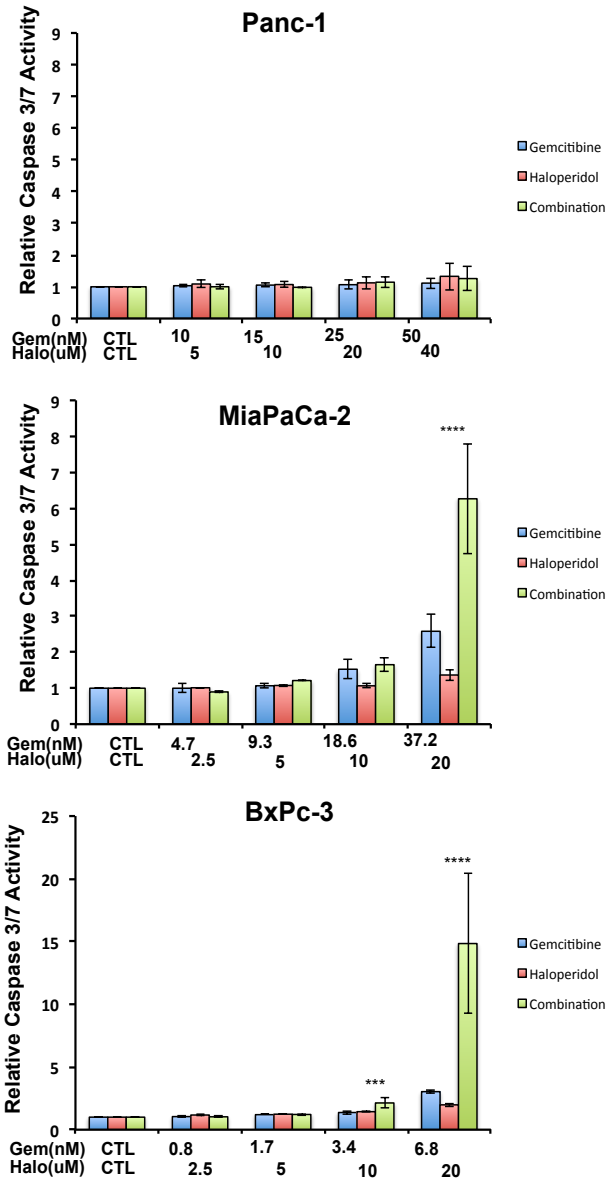


Figure 9. Combination treatment with haloperidol and gemcitabine induces apoptosis. Cells were exposed to gemcitabine for 24h and then haloperidol for 24h when the abundance of active caspase 3/7 was measured in each treatment condition using Caspase 3/7 Glo assay. Graphs represent relative average abundance of active caspase 3/7 in treatment conditions as compared to controls. Error bars represent SD of two independent experiments performed in four replicates. ***$p<0.001$ and ****$p<0.0001$ when compared to both gemcitabine and haloperidol treated conditions.

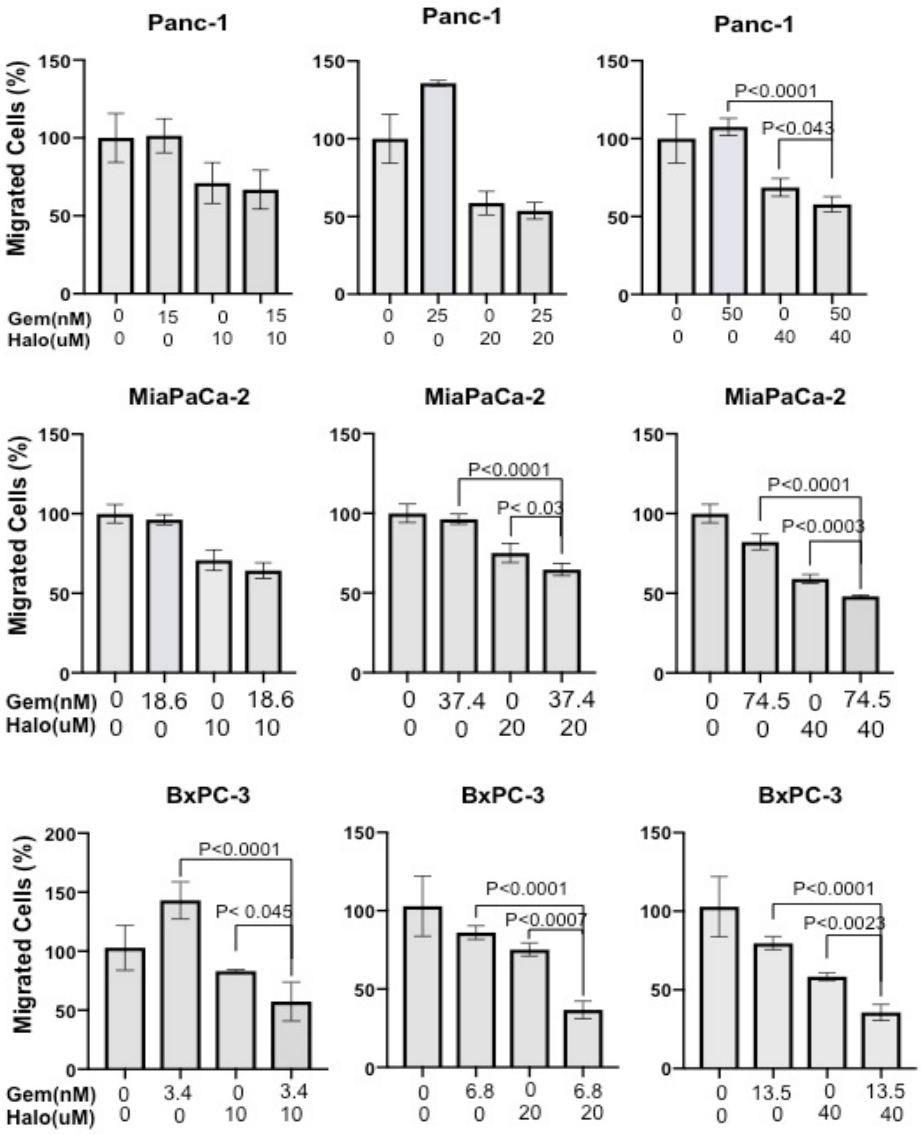


Figure 10. Inhibitory effects of combination therapy with gemcitabine and haloperidol on the migration capacity of Panc-1, MiaPaCa-2 and BxPC-3 cells. Cells were treated with various doses of gemcitabine for 24h and then plated on transwells, migrated cells were counted in each treatment condition and normalized to corresponding number of cells in control condition after exposure to different doses of haloperidol for 4h. Graphs represent results from experiments performed with four replicates as average number of migrated cells relative to control condition. Error bars represent SD of four replicates. P-values are shown when combination treatments have stronger effects in comparison to both single treatments.

Combination treatment effects on 3D spheroid models generated from PDAC cell lines

The above-mentioned observations in 2D monolayer cell culture, prompted us to explore effects of the combination treatments in 3D spheroid cultures. For this purpose, we first performed multiple initial experiments to rule out possibility of changes in expression patterns of DRD2 in 2D and 3D cultures, and to identify the optimized experimental conditions, including appropriate number of initial cells to generate spheroids from each cell line, and appropriate time-points to measure cell viability in spheroid cultures.

To verify similar expression patterns of DRD2 protein in 2D and 3D culture, we examined DRD2 expression by western blotting in both conditions. To identify a reliable antibody that accurately detect DRD2, a set of commercially available antibodies were purchased and were subjected to verification by western blotting in cells in which DRD2 was suppressed by independent siRNA molecules or shRNA constructs. Complementing WB assays, DRD2 mRNA was also measured in the same cells by qRT-PCR. These experiments showed that among the tested antibodies, the Merck Millipore (Cat AB5084P) antibody is reliable for the detection of DRD2 protein. As shown in Figure 11A, out of four individual tested siRNAs, siRNA-18 showed knockdown for DRD2 at protein level. This observation is in line with results from qRT-PCR analysis of the same cells that show knock-down of DRD2 at mRNA levels only for siRNA-18 (Figure 11B). To have an independent validation, we also performed similar tests in cells infected by two different shRNA-expressing constructs against DRD2, which we had previously validated and used in our previous study[67]. As shown in Figure 11C-D, the same antibody showed DRD2 knockdown in cells expressing DRD2-shRNA as compared to the control condition (pLKO), in line with results from qRT-PCR analysis of the same cells. Notably, the same antibody was also validated by another study, in which authors had tested different antibodies against DRD2 using western blots and

immunohistochemistry on mouse striatum in wild type and DRD2-null mice [93]. Therefore, we used this antibody to compare expression levels of DRD2 between 2D and 3D culture. To examine if the 3D model would change DRD2 protein expression, as it is scholarly mentioned for some proteins [94-96], compared to 2D model, we conducted a western blot to compare the protein level of DRD2 in both conditions. This experiment showed that the expression levels of DRD2 in the tested cells remain similar in both models (Figure 12).

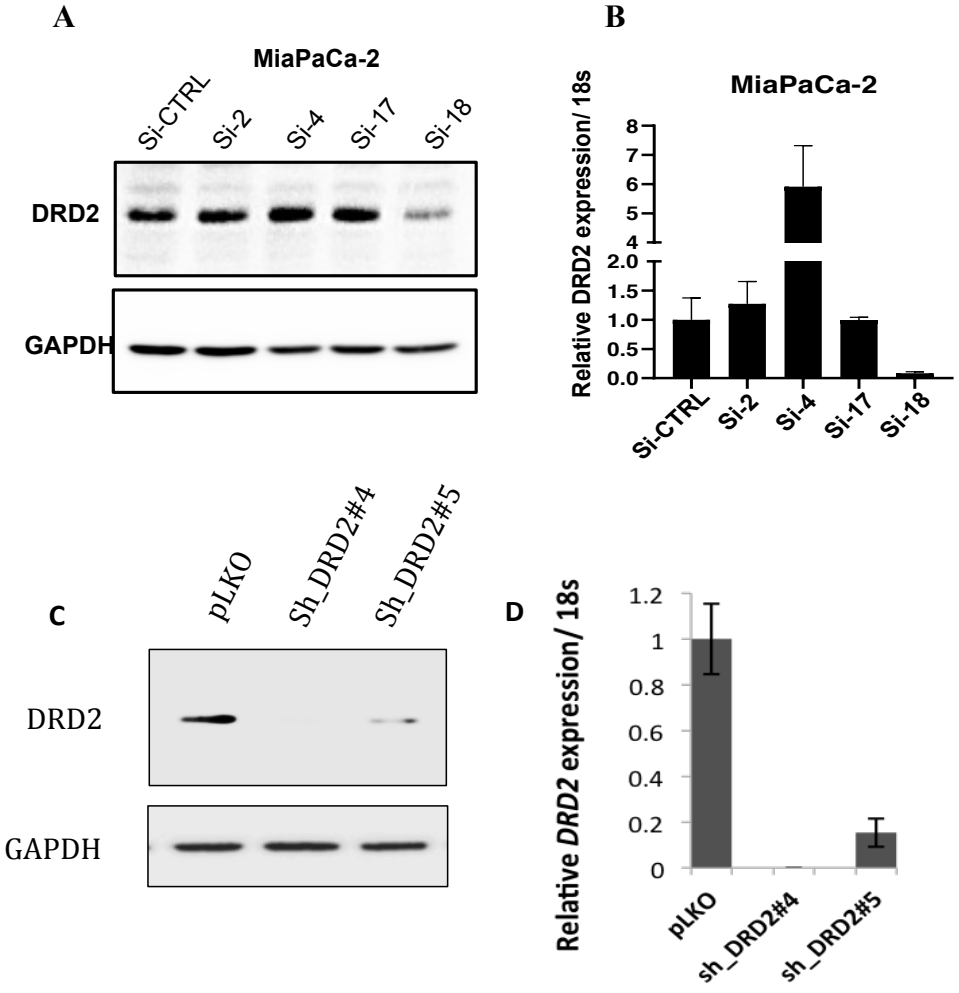


Figure 11. DRD2 knockdown in MiaPaCa-2 cells. Panel A shows western blot analysis for DRD2 using DRD2 Millipore polyclonal antibody in cells transfected with individual siRNAs against DRD2 or control siRNA. Total protein was extracted after 72h treatment with siRNAs and equal amounts of cell lysates were analyzed by Western blot. Panel B represents qTR-PCR results for DRD2 mRNA in the same cells described in A. Expression

values in each condition were normalized to that of 18S and shown as fold-change relative to that of si-control condition. Panel C shows western blot analysis for DRD2 using DRD2 Millipore polyclonal antibody in cells infected through lentiviral delivery of vectors expressing shRNA targeting DRD2 (shDRD2#4 and shDRD2#5) or control pLKO vector. Total protein was extracted 72h post-infection and equal amounts of cell lysates were analyzed by Western blot. Panel D represents qTR-PCR results for DRD2 mRNA in the same cell lines described in C. GAPDH served as a loading control in western blots.

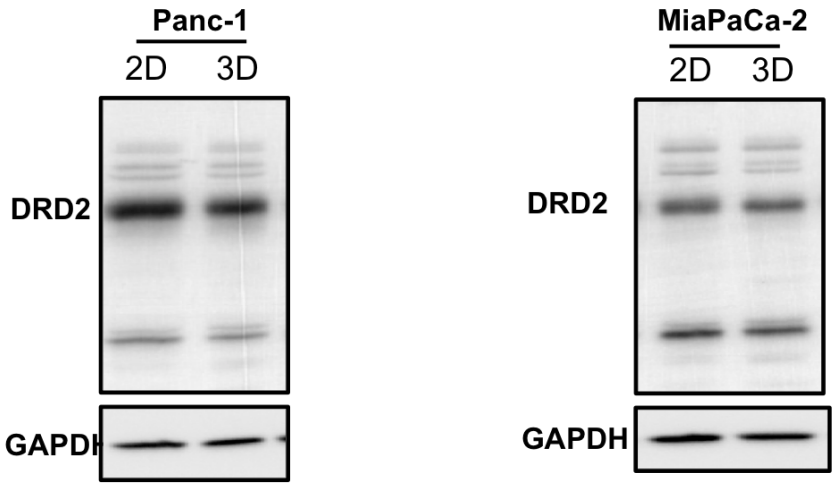


Figure 12. DRD2 expression in 2D and 3D model of PDAC. Total protein was extracted from 2D and 3D models of PDAC cells and equal amounts of cell lysates in each condition were analyzed by Western blot using DRD2-Millipore polyclonal antibody. GAPDH served as a loading control.

Next, we examined different number of cells to generate spheroids, and investigated the reliability of cell viability measurements after 12 days of cultivation. Due to higher sensitivity and technical advantage for application to 3D models we decided to use Cell Titer Glo viability assay to monitor cell viability. As such, to ensure that results generated by Cell Titer Glo assay are comparable to those generated by SRB assay, which was used in

abovementioned experiments, we conduct a side-by-side comparison between readouts of both methods following drug treatments. Our analysis confirmed that results from these methods are similar (Supplementary Figure 2). Therefore, we used Cell titer Glo assay in our next experiments to find the optimal number of cells to generate 3D models and then for drug sensitivity experiments. As shown in Figure 13, a cell number up to 5000 used for initial seeding of cells resulted in reliable quantitative measurements of cell viability at day 12 post-seeding for all cell lines. According to these observations and considering the proliferation of each cell line we chose to use 500, 1000, and 2000 cells for Panc-1, MiapaCa-2, and BxPC-3, respectively to generate spheroid cultures for drug treatment experiments.

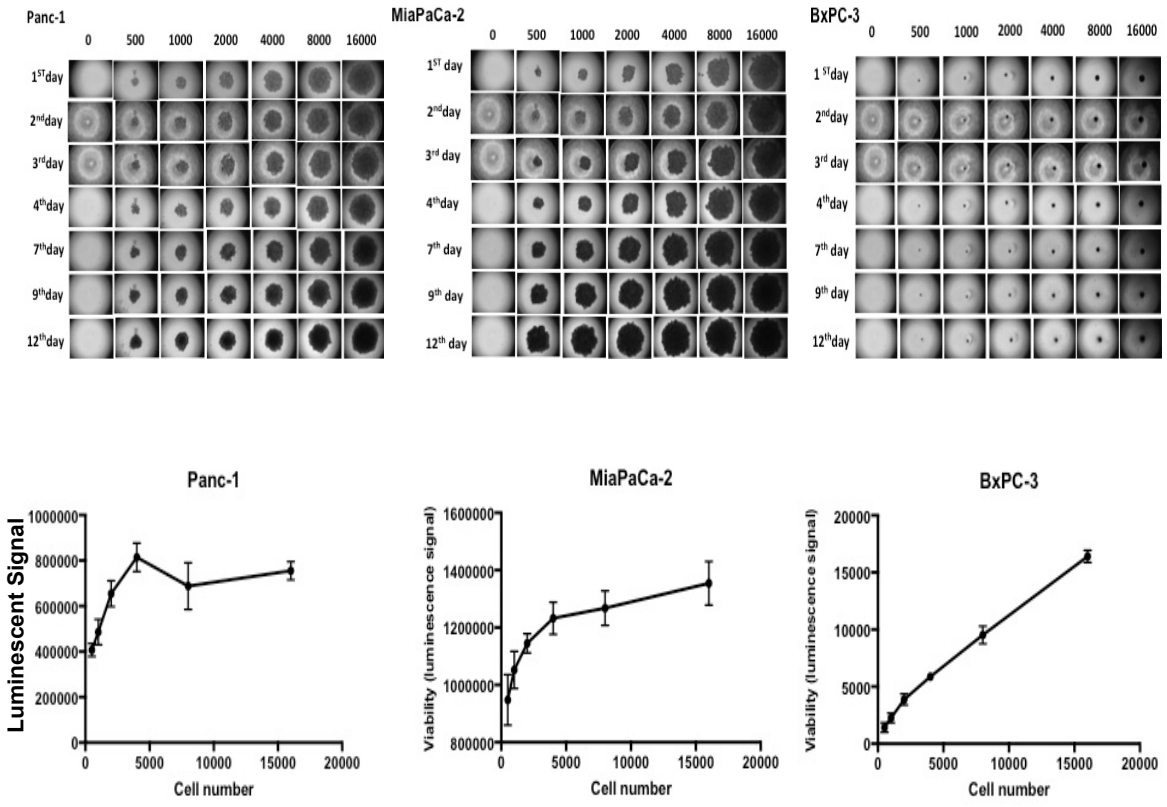


Figure 13. Optimization of the 3D spheroid culture of PDAC cell lines for drug treatment experiments. (A) Phase light images of spheroids generated from different initial number of cells (mentioned on top of images) for each PDAC cell line over a period of a 12-day

cultivation. (B) Cell viability was measured at day 12 post-seeding using cell titer glo assay, and was visualized as luminescent signals vs. number of initial cells seeded. Error bars represent the SD of representative experiments done in replicates.

Then, we compare the treatment results from 3D spheroid to 2D monolayer culture systems; we applied the same drug dosage that had been tested in 2D culture in our 3D spheroid assays. As shown in Figure 14, haloperidol reduced cell viability in 3D spheroid culture of all cell lines examined. Interestingly, haloperidol effects on viability of BxPC-3 cells, which had shown the highest sensitivity among cell lines in 2D culture, was even stronger in 3D culture. That is the IC50 value of haloperidol was lower in 3D culture compared to 2D culture (4uM and 20uM in 3D and 2D cultures, respectively). On the other hand, we observed resistance to gemcitabine treatment in 3D spheroid generated from BxPC-3 cells (Figure. 14). A trend toward resistance to gemcitabine treatment was also observed for low concentration of the drug in spheroid of Panc-1 cells, but not for those of MiaPaca-2. (Figure.14). Finally, combination of haloperidol and gemcitabine did not result in a beneficial effect on reducing viability of cells as compared to those from treatment with either of these drugs. (Figure.14).

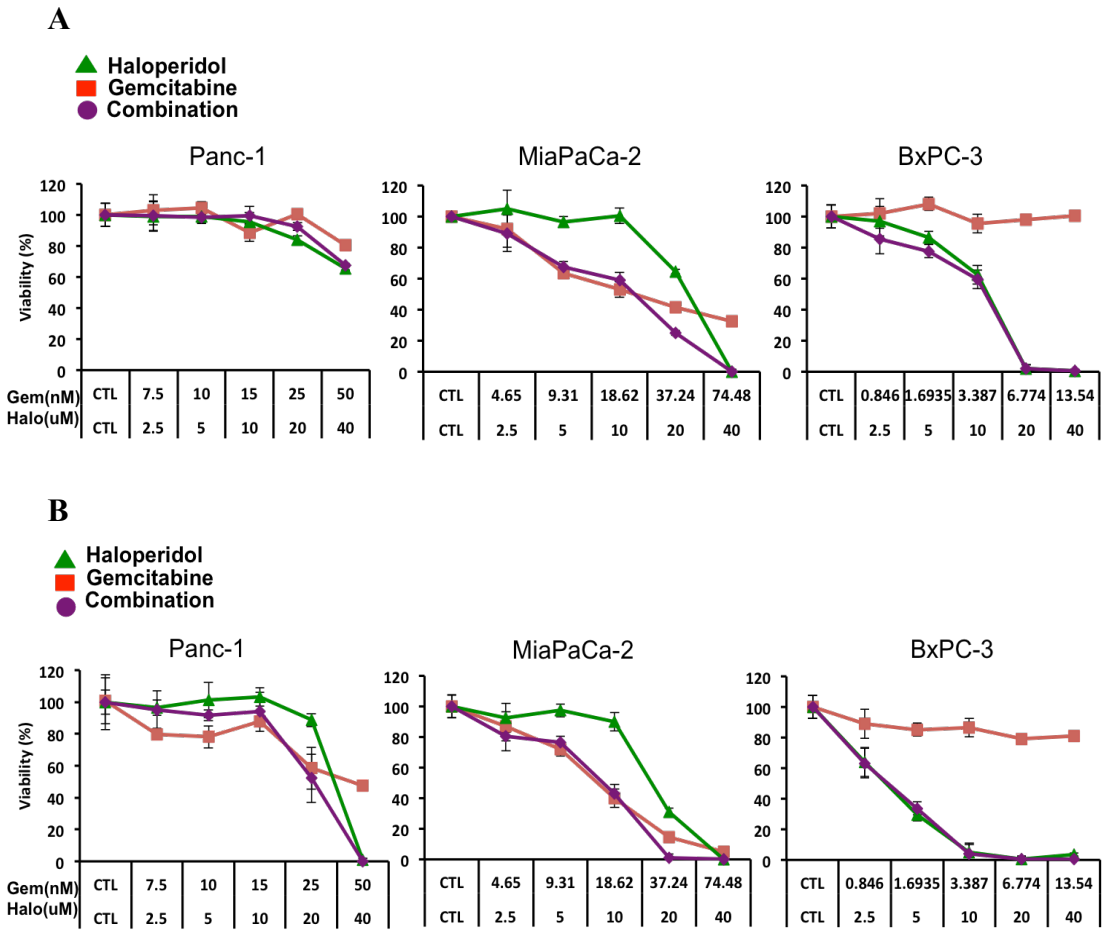


Figure 14. Effects of haloperidol and gemcitabine individually or in combination on viability of 3D spheroid models generated from different PDAC cell lines. Spheroid models were treated with increasing gemcitabine concentration for 24h then multiple concentrations of haloperidol were added following a 1:1 constant ratio strategy. Cell viability was measured for all conditions 4 days (A) and 8 days (B) after treatment with gemcitabine. The effects of drug treatments are shown as average of cell viability measures in each treatment conditions relative to those of control conditions treated with carrier solvent. Error bars represent SD of three experiments done in quadruplicate.

Chapter IV

Discussion

Conventional chemotherapies, including gemcitabine, in pancreatic ductal adenocarcinoma are the standard treatment procedures for the metastatic disease. Despite improvements made by chemotherapy in clinical management of the disease, the emergence of the resistance to chemotherapies remains a major issue. Combination treatment approaches using targeted therapies may help to overcome this obstacle and to improve patient survival.

In this study we examined the potential benefits of a combination treatment with DRD2 antagonist haloperidol and chemotherapeutic agent gemcitabine in inhibiting cell growth and invasion potential of pancreatic cancer cells *in vitro*. Our data suggest that this combination treatment has potential benefits for reducing viability and migration of PDAC cells, when compared to individual drug treatments. However, the extent of these effects varied between different PDAC cell models examined. A similar pattern was observed in our previous study when we observed stronger anti-growth effects for DRD2 inhibitors in PDAC cell lines that have higher endogenous levels of DRD2. As such, we anticipate that higher expression of DRD2 may be indicative of more sensitivity to the treatment. Although, we were not able to examine this possibility thoroughly in this study, we identified and validated a reliable DRD2 antibody, which can serve for screening purposes in the future. Our results are also in line with previous studies reporting that the deficiency of DRD2 promotes ER stress, which eventually triggers cell cycle arrest and apoptosis. Taken together our results are promising in a way that a combination therapy using DRD2 inhibitors and gemcitabine may have better anti-cancer effects than treatment with gemcitabine alone. However, this needs to be examined and validated using better models of the disease. We attempted to test our observations in 3D models generated from PDAC cells lines *in vitro*. However, we realized that these models need further optimization and eventually do not recapitulate important features of tumor microenvironment, which are crucial in PDAC. Therefore, we believe that

the effects of these drugs should be examined in appropriate *in vivo* models in future studies.

ONC201 and ONC212, which are small- molecule inhibitors against DRD2 activity, show growth-inhibitory effects in PDAC *in vivo* as a single agent or in combination with 5-fluorouracil, irinotecan, and oxaliplatin [57, 97, 98]. Notably, the inhibitory effect of ONC201 and ONC212 is also through suppressing Akt and ERK pathways, while DRD2 inhibitors such as haloperidol and pimozide did not affect these pathways in PDAC [67]. Nevertheless, all these agents show a common effect in suppressing PDAC growth, further supporting the potential of DRD2 as a therapeutic target.

Interestingly, the incidence rate of multiple cancers including colon and prostate cancers among schizophrenic patients who received DRD2 inhibitors is lower than the general population [99]. DRD2 overexpression has been detected in 80% of PDAC cases, as well as in leukaemia and glioblastoma. It has also been shown to have significant impacts on critical signalling pathways involved in tumor survival, angiogenesis, migration and metastases [100]. Accordingly, DRD2 inhibition therapy has been examined for multiple cancers (Table 1). In PDAC, DRD2 inhibition has resulted in an anti-cancer activity by inducing cell death through activating the cAMP/PKA pathway, regulating Ca^{2+} levels and subsequently increasing endoplasmic reticulum (ER) stress, thus inducing apoptosis (Figure 15) [67]. In glioblastoma, however, an integrated mitogenic signalling between DRD2 and EGFR seems to mediate the anti-cancer effects of DRD2 inhibition [100]. These observations suggest that the mechanisms by which DRD2 signalling contributes to cancer appear to be cell-type dependent.

DRD2 involvement in cancer stem cells (CSCs) was noted when an anti-CSC activity of Thioridazine, which is DRD2 antagonist, impacted the leukemic stem cell function in forming leukaemia *in vivo* [101]. Given the involvement of CSCs in cancer initiation, development and resistance to treatment, DRD2 inhibition may have a broad clinical

significance for cancer therapy. PDAC stem cells are resistant to all type of treatment including chemotherapy and radiotherapy, leading to relapse and metastasis of untreatable tumor. Indeed, poor prognosis and metastasis in PDAC are associated with the enrichment of CD133+ and CXCR4+ stem cell populations in primary pancreatic cancer [102, 103]. Taken together, the combination of DRD2 antagonists and gemcitabine may represent a promising treatment outcome by targeting both tumor bulk and CSCs. The refinement therapy of DRD2 antagonists and gemcitabine can benefit other malignancies, which are influenced by DRD2 overexpression and treated with gemcitabine.

Table 1. Pre-Clinical studies on DRD2 inhibitors in various cancers, adapted from [57].

Cancer Type	DRD2 Antagonist	PubMed Reference
Pancreatic cancer	Haloperidol and Pimozide	PMID: 27578530
Glioblastoma	Haloperidol	PMID: 24658464
Glioblastoma	ONC201	PMID: 26474387
Colorectal cancr	ONC201	PMID: 29357916
Pancreatic cancer	ONC201	PMID: 27233611

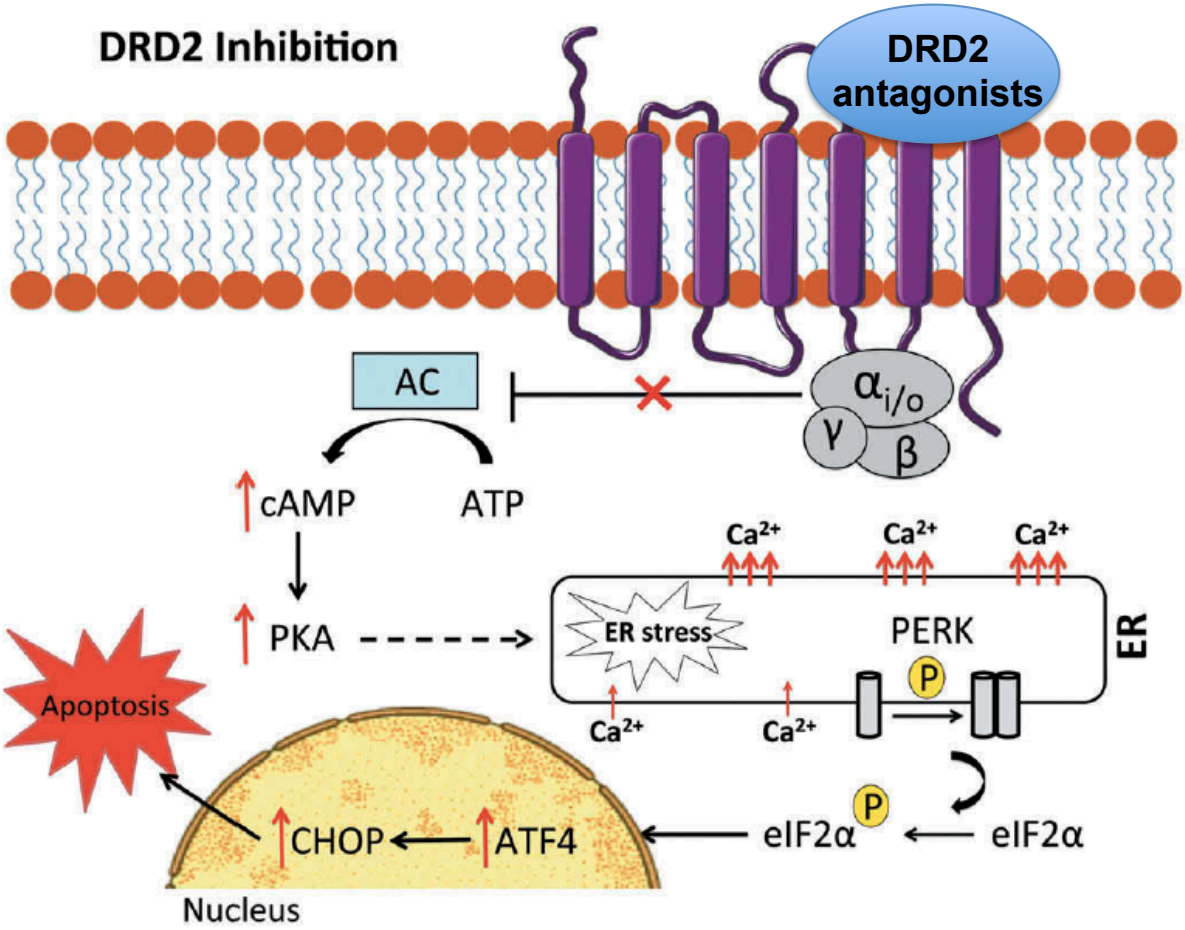


Figure 15. Proposed mechanism of DRD2 inhibition pathway: DRD2 inhibitors increase adenylyl cyclase (AC) that elevate cAMP/PKA level and cause high cytosolic calcium release from ER. Eventually, ER stress activates the transmembrane protein kinase (PERK), leading to activation of series apoptotic proteins, adapted from [57]. cAMP/PKA: cyclic adenosine monophosphate and protein kinase A. ER: endoplasmic reticulum.

Chapter V

Conclusion

The high mortality and short survival rates of PDAC patients make the small treatment advances to have an immediate and significant clinical impact. The improving chemotherapeutic approaches that could be applied to PDAC patients would benefit the majority of patients who cannot be offered surgery and second line treatment alternative. Therefore, DRD2 inhibitors provide an opportunity because of their known and manageable side effects. As these inhibitors have been in clinic for treatment of psychiatric disorders, a drug repositioning can shorten the time needed to perfume clinical trials to prove their efficacy in cancer patients. Preclinical studies of DRD2 antagonists alone or in combination with conventional chemotherapy could determine effective drug dosage with minimum side effect. The first-in man- studies using dosage approved to treat schizophrenia could provide an evaluation on the tolerability and beneficial effects of the combination therapy of DRD2 antagonists and standard chemotherapy in patients with metastatic PDAC. The recommended dosage by the Schizophrenia Patient Outcome Research Team guideline is ranging from 6 to 20 mg/day of haloperidol in adults with acute schizophrenia, and 6 to 12 mg/day for maintenance therapy. To determine the safety and tolerability of haloperidol with standard chemotherapy in patients, an intra-patient-dose-escalating scheme could be applied and maximum tolerated dose (MTD) could be determined and overall safe dose recommended (SDR) for routine application. Although PDAC is a heterogeneous disease, DRD2 is found in about 80% of patients tumors, suggesting that the majority of patients would benefit from a combination therapy of DRD2 inhibitors and chemotherapy.

Chapter VI

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workcited

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Appendix

Supplementary Figures

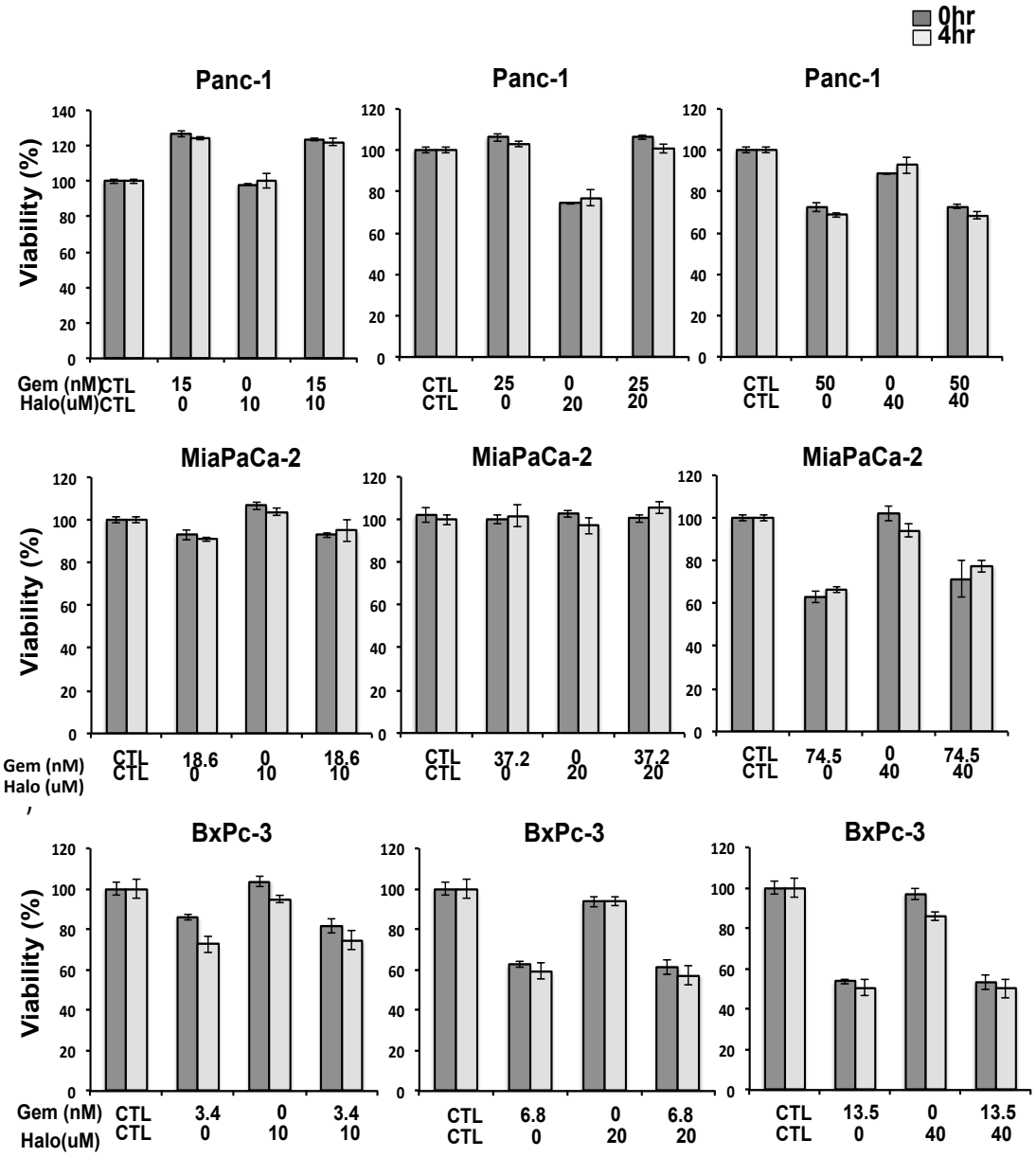

Figure S1. Investigating potential effects of short-time combination therapy with gemcitabine and haloperidol on the survival of PDAC cell lines corresponding to migration assay experiments. Cells were patted in 96 well plates and treated with various doses of gemcitabine for 24h. Then cells were treated different doses of haloperidol for 4h, when migration assay was performed. Cell viability was measured by Cell- titer glo before (time

0h) and after (time 4h) treatment with haloperidol. The effect of treatment on cell growth was plotted as the fraction of live cells in each treatment condition relative to that of cells treated with carrier solvent. Viability of cells at time 4h was compared to the corresponding cells at time 0h for each condition, to evaluate the possible effect of combination therapy on viability of cells within the 4h co-treatment period. Error bars represent standard deviation (SD) of three experiments done in quadruplicate.

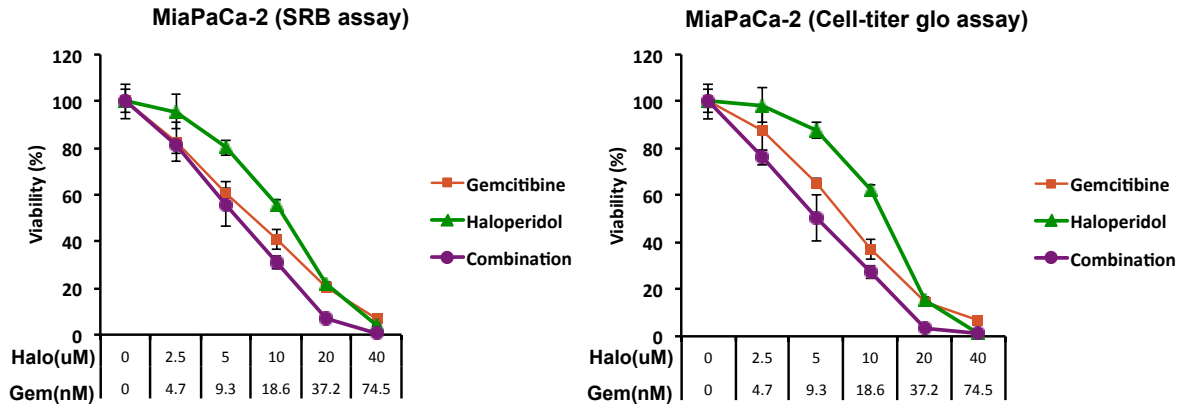

Figure S2. Comparison of results of cell viability measurements between two methods of SRB and cell titer-Glo assay. Response curves show effects of treatment with Haloperidol, Gemcitabine, and combination on the viability of MiaPaCa-2 cell line. Cell viability was measured by SRB (left) and Cell- titer glo (right) assays 96h post- treatment. The effect of treatment on cell growth was plotted as the fraction of live cells in each treatment condition relative to that of cells treated with carrier solvent. Error bars represent standard deviation (SD) of three experiments done in quadruplicate..

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