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DESIGN OF SF3B1 SUBUNIT MODULATORS OF THE SF3B SPLICEOSOME COMPLEX

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Chapter 1

Introduction and Thesis outline

1. About the pancreas: structure and function

The pancreas is a retroperitoneal glandular organ with both exocrine and endocrine secretory properties. Specifically, the endocrine cells form isolated clusters called the islets of Langerhans containing five different cell types: (1) alpha cells that produce the hormone glucagon with hyperglycemic action; (2) beta cells that produce the hormone insulin with hypoglycemic action; (3) delta cells that secrete somatostatin which inhibits the production and secretion of insulin and glucagon; (4) epsilon cells that secrete ghrelin involved in inhibiting insulin secretion from beta cells and stimulating growth hormone secretion; (5) F cells (or formerly as gamma cells) that release the pancreatic polypeptide (PP) which inhibits the contraction of gallbladder and regulates the production of certain pancreatic enzymes [1–3] (**Figure 1A, B**).

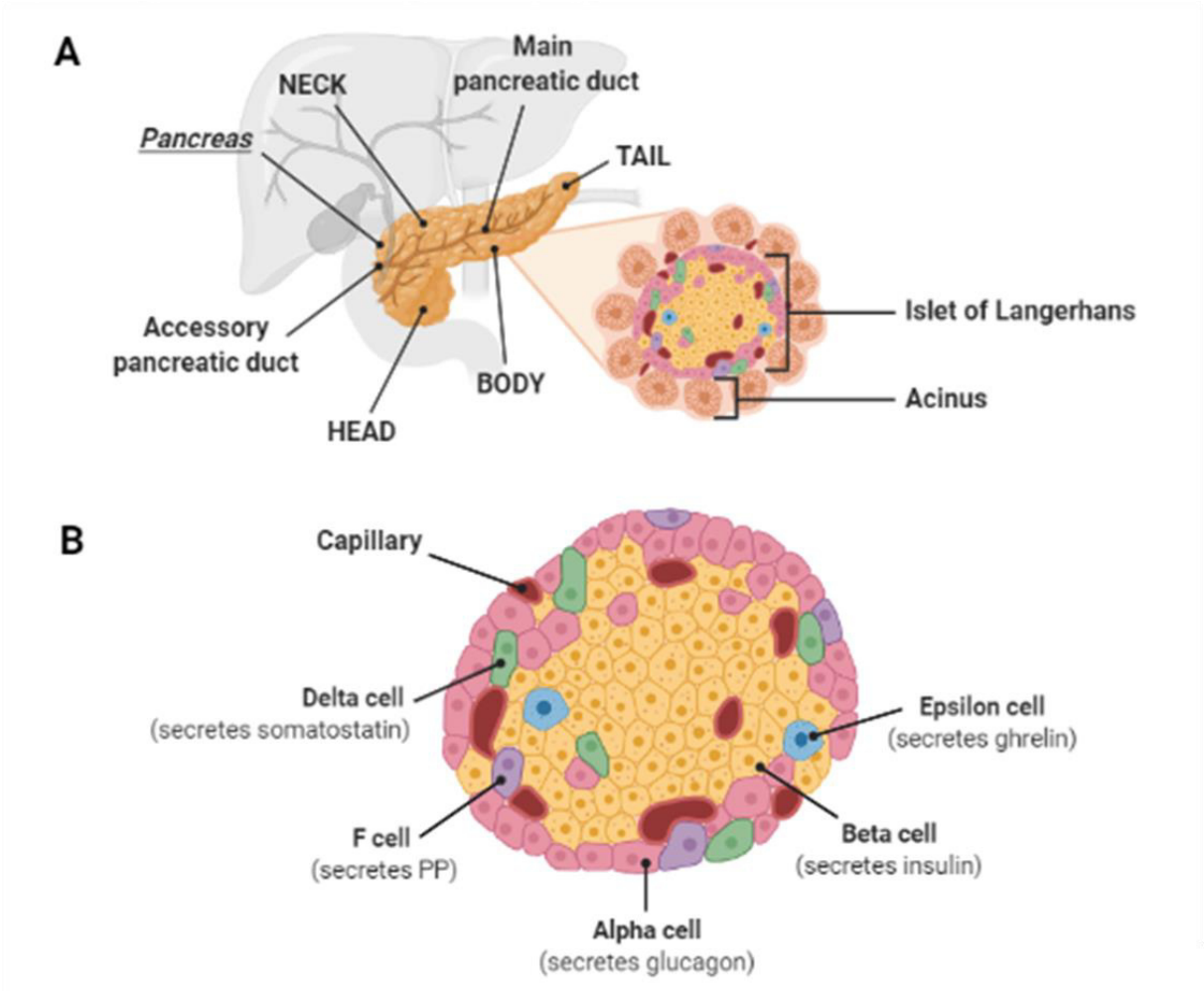

Figure 1. (A) Representative image of the macroscopic anatomy of the pancreas divided into four parts: head, neck, body and tail. The main pancreatic duct (or duct of Wirsung) and the accessory pancreatic duct (or duct of Santorini) are also visible. There is also a focus on the miscroscopic anatomy of the

pancreatic parenchyma in which both the exocrine acini and the cluster of endocrine cells known as islet of Langerhans are visible. **(B)** Focus on the cellular composition of the islet of Langerhans. Created with BioRender.com.

pp, pancreatic polypeptide.

1.1 Overview of pancreatic cancer: types and focus on Pancreatic Ductal Adenocarcinoma (PDAC)

Histologically, pancreatic tumors are classified in epithelial (exocrine or endocrine) and non-epithelial [4]. Pancreatic neuroendocrine tumors (pancreatic NETs or PNETs) are rare tumors that account for less than 2% of all pancreatic tumors. PNETs, also known as islet cell tumors, arise from the endocrine cells in the pancreas called islet cells (or islets of Langerhans). Based on the secreted hormones, PNETs are classified into several types: insulinoma (insulin), gastrinoma (gastrin), glucagonoma (glucagon), somatostatinoma (somatostatin), VIPoma (vasoactive intestinal peptide) [5].

Conversely, pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic exocrine cancer accounting for more than 85% of all pancreatic malignancies [6]. Approximately 60-70% of PDAC cases arise in the head of the pancreas, while the remainder are placed in the body (15%) or tail (15%) and are associated with a worse prognosis [7]. The main morphological variants of PDAC include adenosquamous, colloid/mucinous, undifferentiated, signet-ring cell, medullary and hepatoid carcinoma that differ in biology and prognosis [7,8]. Notably, up to 20% of PDAC patients are eligible for potentially curative surgery, while most patients present unresectable tumors at the time of diagnosis due to the metastatic spread of the disease [9].

Several studies have suggested a relationship between tumor location and survival. Data from the Surveillance, Epidemiology and End Results (SEER) database reported higher survival rates for patients with PDAC located in the pancreatic head compared to those with PDAC of the pancreatic body or tail. The late diagnosis and therefore the poor prognosis in pancreatic cancer of the body/tail is usually related to the lack of early clinical symptoms due to obstruction of the bile duct [10,11].

Recently, Dreyer *et al.* showed that PDAC in the body/tail may have a more aggressive tumor biology than PDAC of the head; this may in part explain the worse prognosis [12]. Furthermore, PDAC in the body/tail is generally larger and more metastasized than PDAC in the head. Thus, the survival is slightly lower for PDAC in the body/tail [10].

Lee *et al.* found that pancreatic cancer location is not an independent risk factor (head vs. body/tail: HR 1.174, CI 0.932-1.478, p=0.173). However, there are controversial findings regarding tumor location as an independent prognostic factor for pancreatic cancer [13].

Currently, there are no screening tests for early detection in the general population due to the low lifetime risk of pancreatic cancer compared to other cancers worldwide and also due to the late stage at which the disease is usually diagnosed [14]. Improvements in diagnosis, prognosis and treatment of pancreatic cancer have been achieved through the identification of novel biomarkers. The serum carbohydrate antigen 19-9 (CA 19-9) is the only FDA (Food and Drug Administration) approved diagnostic marker in the management of pancreatic cancer. However, CA 19-9 is not very reliable as a screening tool due to its low sensitivity and specificity, rather it plays a role as a prognostic biomarker and for monitoring relapses after resection. Greater prediction accuracy is achieved when CA 19-9 is combined with carcinoembryonic (CEA) or serum macrophage inhibitory cytokine 1 (MIC-1) [15,16]. The latter is a member of the transforming growth factor beta (TGF-β) superfamily and appeared to be significantly better than CA 19-9 in discriminating patients with early-stage resectable PDAC from healthy controls. On the contrary, Osteopontin (OPN) which is upregulated in PDAC and is involved in the metastatic growth of the disease, did not show a diagnostic accuracy higher than CA 19-9 [17,18].

Recently, particular attention has been paid to the role of microRNAs (miRNAs) in the development and progression of different types of cancers, including pancreatic cancer. MiRNAs are small non-coding RNA molecules (approximately 19-24 nucleotides) involved in several biological processes such as cell proliferation, differentiation and apoptosis [19]. A plethora of different miRNAs has been investigated in PDAC providing encouraging results. In particular, miRNA-21 and miRNA-155 are overexpressed in precursor lesions as well as miRNA-205 and miRNA-200 [20]. Furthermore, the levels of miRNA-148a, miRNA-217, miRNA-196 and miRNA-10b change among different pancreatic intraepithelial neoplasia (PanIN) stages, while the expression of miRNA-320c, miRNA-200c, miRNA-let-7 family is related to gemcitabine responsiveness [20,21]. Thus, miRNA targeting represents a promising new tool for the early PDAC diagnosis, prognosis and metastasis prediction.

Markers to predict the efficacy of gemcitabine should also be considered in order to optimize the treatment of PDAC. Since intracellular uptake of gemcitabine is highly dependent on nucleoside transporters, several membrane transporters ENTs (equilibrative nucleoside transporters) and CNTs (concentrative nucleoside transporters) have been proposed as potential

predictive and prognostic biomarkers of response to gemcitabine treatment. Notably, human ENT1 (hENT1) is a transmembrane protein abundantly expressed in PDAC tumors [17]. Giovannetti *et al.* established an overall survival of 25.7 months and 8.5 months in PDAC patients with high and low levels of hENT1, respectively [22]. Earlier, it was demonstrated with *in vitro* experiments and specific models that increased sensitivity to gemcitabine was related to a high expression of hENT1 [23,24].

Furthermore, it is worth mentioning that the median overall survival (94.8 months) for patients with high expression of both hCNT3 and hENT1 appears significantly improved compared to that of patients with high expression of a single biomarker (18.7 months) suggesting that the combined use of biomarkers is a promising diagnostic tool [25].

Recently, Wrona *et al.* proposed the cytokine leukemia inhibitory factor (LIF) as a potential diagnostic biomarker and therapeutic target for PDAC. Although LIF is involved in key cancer progression processes, clinical trials are still needed for its validation as a new biomarker for this still incurable disease [26].

In conclusion, despite the countless efforts to provide curative therapy and personalized treatment to PDAC patients, to date there is no ideal diagnostic biomarker for early detection of the disease. Therefore, greater awareness and deeper understanding are needed to properly treat PDAC which unfortunately still remains an ongoing medical concern.

1.2 Worldwide incidence of pancreatic cancer

Pancreatic cancer is a highly lethal gastrointestinal malignancy worldwide. According to GLOBOCAN 2020 estimates of the increasing incidence and mortality of cancer, pancreatic cancer is responsible for 495,773 (2.6%) new cases and 466,003 (4.7%) new deaths worldwide. Actually, it is ranked as the seventh most common cancer in the world and it is projected to become the third leading cause of cancer-related deaths by 2025 surpassing breast cancer [27]. The highest incidence (age-standardized rates by sex) for pancreatic cancer is found in Europe, Northern America, Australia and New Zealand. The worldwide distribution of cases and deaths is quite stable although pancreatic cancer is sligthly more common in males (5.7% per 100,000 with 262,865 cases) compared to females (4.1% per 100,000 with 232,908 cases), but the reasons are still insufficiently known [27]. However, the risk of pancreatic cancer is higher in African Americans than in Caucasians and it is likely linked to increased exposure to certain modifiable risk factors for pancreatic cancer such as cigarette smoking, diabetes, consumption of alcohol and vitamin D deficiency, as well as genetic mutations [28]. The mortality rate of

pancreatic cancer increases with the age and it is rarely diagnosed at <55 years of age reaching the peak incidence over 70 years [28]. Furthermore, PDAC has a very poor prognosis with an overall 5-year survival rate around 7% and it is mainly attributed to late diagnosis due to the absence of symptoms during the early stages of the disease, its resistance to systemic therapies and also the lack of diagnostic biomarkers with high sensitivity and specificy [29].

1.3 Causes and risk factors

Thus far, the etiology of pancreatic cancer is not well known but many risk factors have been identified. The most common modifiable risk factor is smoking, with an up to three times higher risk of developing pancreatic cancer for smokers than for non-smokers. Obesity, alcohol consumption, dietary pattern and heavy occupational exposures to certain chemicals are also associated with an increased risk of pancreatic cancer [14,28,30]. Non-modifiable risk factors include the family history of pancreatic cancer, which contributes to an early onset of this type of cancer as well as gender, age and inherited genetic syndromes such as Hereditary Nonpolyposis Colorectal Cancer (Lynch syndrome), Peutz-Jeghers syndrome (PJS), familial atypical multiple mole melanoma (FAMMM), hereditary breast and ovarian cancer (HBOC) or Li-Fraumeni syndrome. Finally, cystic fibrosis, chronic pancreatitis, type 2 diabetes mellitus, infections and non-0 blood group are positively related to the increased risk of developing pancreatic cancer [28,31]. Noteworthy, another setting for pancreatic cancer predisposition are the somatic mutations in *KRAS*, *CDKN2A*, *TP53* and *SMAD4/DPC4* detected at different stages of PDAC precursor lesions. They are the most commonly mutated genes that promote tumorigenesis and metastasis of PDAC [32].

In particular, mutations in the gene encoding the proto-oncogenic GTPase KRAS (Kirsten rat sarcoma oncogene homolog) are detected in >90% of PDAC patients with codon G12 of exon 2 mutations being most frequent and are involved in the early stages of precursor lesions, progression and maintenance of PDAC. Other common mutations include the inactivation of tumor suppressor gene *CDKN2A* (cyclin-dependent kinase inhibitor 2A), encoding P16/INK4A protein and regulating the G1-S phase transition of the cell cycle, detected in 30-50% of pancreatic cancer cases and involved in the intermediate lesions leading to the tumor growth. In addition, homozygous mutations in the tumour suppressor gene *TP53* have been observed in late lesions with a mutation rate of 60-70% in pancreatic cancers. *TP53* is responsible for the management of cellular stress and protects genomic stability in cells, it also drives the carcinogenesis of PDAC. The tumor suppressor gene *SMAD4/DPC4* (mothers against

decapentaplegic homologue 4), encoding the Smad4 protein which regulates the transforming growth factor-beta (TGF-β) signaling, is mutated in about 50% of PDAC cases [32–34].

1.4 Pathogenesis of PDAC

PDAC is a complex and heterogeneous disease frequently arising from non-cancerous precursor lesions known as PanIN, intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN) [7,33] that can progress to invasive adenocarcinoma of the pancreas, additional lesions such as acinar-ductal metaplasia (ADM) and atypical flat lesion (AFL) are also potentially relevant in pancreatic carcinogenesis [35].

PanIN is a flat or papillary lesion that arises in the smallest pancreatic duct and is the most common non-invasive precursor lesion of PDAC that cannot be easily identified due to its small size (<0.5 cm). Based on the degree of cellular and nuclear atypia, PanIN lesions can be morphologically classified as low-grade PanINs (previously classified as PanIN-1 or PanIN-2) in which *KRAS* (codon G12) and *CDKN2A* are mutated or high-grade PanINs (previously classified as PanIN-3) in which *TP53*, *SMAD4/DPC4* and also *BRCA2* mutations are usually found [4,32,34]. Recently, telomere shortening has also been found in the early stages of PanINs development suggesting that it is an early event in PDAC pathogenesis. Telomeres are specialized structures consisting of hexameric TTAGGG repeats located at the end of chromosomal strands that protect the chromosome avoiding its deterioration [36,37].

IPMNs are glossy cystic neoplasm (≥1 cm) larger than PanINs and are predominantly papillary or rarely flat that arise from the main pancreatic duct (main-duct IPMN), branches (branch-duct IPMN) or both (combined-type IPMN) [36]. They commonly arise in the head of the pancreas (70%) and the remaining cases involve the body (20%) and tail (10%). Similarly to PanINs, based on the degree of architectural and cytological atypia, various degrees of dysplasia can be observed in neoplastic cells. Mutations in the four key PDAC driver genes (*KRAS*, *TP53*, *CDKN2A* and *SMAD4/DPC4*) were observed in IPMNs as well as somatic mutations of the oncogene *GNAS* and *RNF43* (RING-type zinc finger protein 43) encoding an E3 ubiquitin ligase that regulates the Wnt signaling pathway [38].

MCNs are mucin-producing cystic tumors arising outside the pancreatic ductal system and are characterized by columnar cells with varying degrees of dysplasia (mucin-rich columnar epithelium) and ovarian-type stroma (OTS) underlying the neoplastic epithelium. They have genetic mutations similar to those seen in other precursor lesions mentioned above, namely mutations of *KRAS*, *CDKN2A*, *TP53* and *SMAD4/DPC4* which are related to the increased

degree of dysplasia. Other genetic alterations identified in about half of MCNs include *RNF43*, conversely they do not carry *GNAS* mutations which could be a useful marker for the differentiation between IPMN and MCN [36] (**Figure 2**).

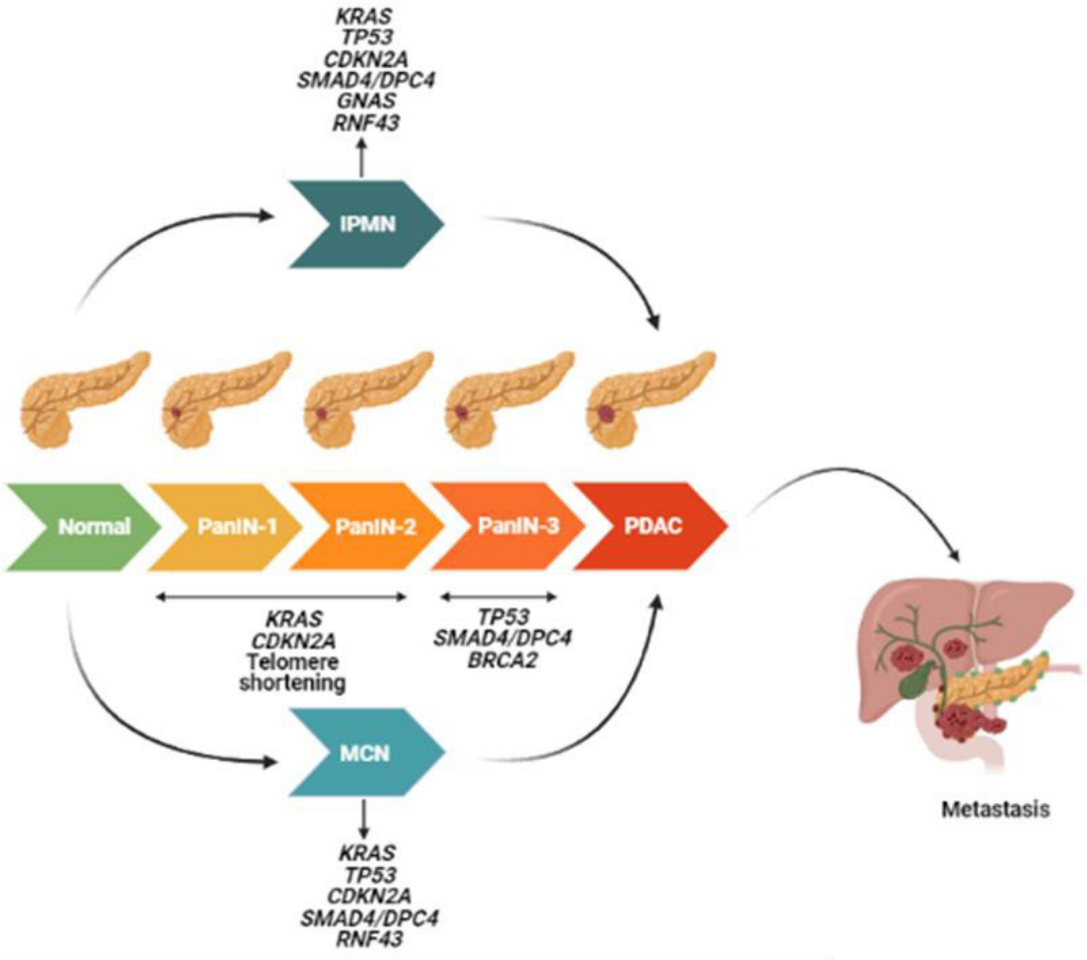

Figure 2. Overview of the precursor lesions of PDAC and associated gene abnormalities in each stage. Precursor lesions (PanIN, IPMN, MCN) give rise to PDAC through progressive stages and multiple genetic mutations that promote tumorigenesis and metastasis of the disease. Created with BioRender.com.

PanIN, pancreatic intraepithelial neoplasia; IPMN, intraductal papillary mucinous neoplasm; MCN, mucinous cystic neoplasm.

The tumor microenvironment (TME) plays a key role in PDAC pathogenesis and it also contributes to tumor growth, invasion and metastasis. It is characterized by a dense collagenous stroma called desmoplasia or desmoplastic reaction (DR) consisting of stromal cells and extracellular matrix (ECM). The latter often represents the majority of the whole tumor mass

and consists of fibronectin, collagen, integrin, laminin, glycosaminoglycan and other soluble factors such as chemokines, cytokines and growth factors [39–41]. Pancreatic cancer stroma comprises immune-suppressor cells such as T regulatory cells (Tregs), myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), as well as cancer-associated fibroblasts (CAFs), pancreatic stellate cells (PSCs), tumor-infiltrating lymphocytes (TILs) [42,43].

Notably, Sonic hedgehog (Shh) signaling, which is a highly active pathway in embryonic development, activates the PSCs leading to an increase in the stroma which further contributes to tumor progression [42].

One of the inducers of this pathway appears to be tumor hypoxia which is another feature of the PDAC microenvironment [44]. Limited vascularity can cause hypoxia which is closely related to desmoplasia [45]. As a result, it generates a physical barrier that contributes to a significant increase in interstitial pressure which leads to compression of blood vessels and hinders the delivery of conventional drugs such as gemcitabine. Therefore, the therapeutic targeting of TME components is an emerging strategy that could aid to improve the grim prognosis of PDAC patients [46].

1.5 Signs and symptoms

PDAC symptoms are usually a sign of advanced disease when the cancer has already spread to other organs. Patients may experience symptoms differently based on the size and the location of the tumor in the pancreas. The key symptoms frequently reported by PDAC patients include dull pain in the upper or middle abdomen radiating into the back related to the tumor invasion from local to distant organs as well as organ failure, ascites leading to death within three months, decreased appetite and subsequent significant weight loss closely related to anorexia, diarrhea, fatigue, decreased energy and depression. Lethargy and sudden or late onset diabetes are unique features of pancreatic cancer. Another common symptom is jaundice caused by obstruction of the biliary duct from the head of the pancreas which causes symptoms such as dark urine due to increased levels of conjugated bilirubin, pale stools and severe pruritus. Conversely, the involvement of the body and tail of the pancreas does not lead to specific and obstructive symptoms until the tumor reaches a large size. In fact, some signs and symptoms may be associated with other medical conditions. However, the early detection of pancreatic cancer still remains difficult although all of these symptoms affect quality of life of patients from the onset [47–49].

1.6 Early detection, diagnosis and staging

Currently, there is a lack of effective screening modalities for the early detection of pancreatic cancer on which the low survival rate strongly depends. Therefore, most patients with PDAC are usually diagnosed at an advanced and less curative stage [15]. The role of imaging in the early detection of pancreatic cancer is crucial to improving outcomes. A proper diagnostic technique allows a better prognosis and paves the way for personalized cancer medicine [15]. Current diagnostic imaging tools for PDAC include computed tomography (CT) and in particular multi-detector computed tomography (MDCT) is the most commonly available and best validated technique for early detection and accurate staging of pancreatic adenocarcinoma through the acquisition of high-resolution and three-dimensional images of the pancreas and surrounding vascular system. Magnetic resonance imaging (MRI) offers better soft tissue contrast resolution than CT and can detect even small tumors and distant metastases. However, it is not widely used due to its high cost and reduced availability [50].

Endoscopic ultrasound (EUS) is a highly sensitive, safe and well-tolerated procedure with the greatest benefit over CT and MRI for the detection of small pancreatic lesions (<2 cm) and also allows the cytological evaluation through aspiration with a fine needle (EUS-FNA). On the other hand, it also has some limitations such as operator dependency, invasiveness but above all the inability to detect distant metastases. Finally, positron emission tomography (PET) is the most accurate and non-invasive way that provides in-depth information of function throughout the entire body and allows the differentiation between benign and malignant lesions using the ^{18}F-fluorodeoxyglucose (FDG), glucose analog. Additionally, PET is currently performed in conjunction with CT in order to provide a more accurate diagnosis than single scan and could be useful for detecting distant metastases [15,50].

Notewhorty, an accurate staging is critical because prognosis and treatment depend on the stage of pancreatic cancer at the time of diagnosis. The most commonly used staging system for pancreatic cancer is the tumor-node-metastasis (TNM) classification system maintained by the the American Joint Committee on Cancer (AJCC) together with the Union for the International Cancer Control (UICC) [51]. The TNM classification takes into account the local extension of the primary tumor which categorizes the tumor from T0 to T4, the involvement of regional lymph nodes ranging from pN0 to pN1 as well as the absence/presence of distant metastasis such as M0 or M1, respectively [52].

Of note, the most recent AJCC 8th edition staging system for the PDAC, effective from January 2018, significantly modifies the N and T categories. In particular, the classification of T-category is based only on the tumor size (T1 with maximum tumor diameter ≤2 cm; T2 with maximum tumor diameter >2 cm but ≤4 cm; T3 with maximum tumor diameter >4 cm and T4 locally unresectable tumor due to the involvement of the celiax axis or superior mesenteric artery) and the N-category is divided into pN0 (no regional lymph node metastases), pN1 (1-3 regional lymph node metastases) and pN2 (≥4 regional lymph node metastases) in order to more accurately predict the prognosis [51,53].

1.7 Treatment and future perspectives

The treatment of pancreatic cancer includes surgery, chemotherapy, alone or in combination with radiotherapy, and palliative care administered to patients with unresectable disease in order to relieve symptoms and improve the quality of life [54].

The treatment option is based on the stage of the disease and the performance status (PS) of the patient. Indeed, the clinical staging classifies PDAC patients into four categories: (1) local or resectable, (2) borderline resectable (absence of vascular tumor contact or 180° or less contact with the superior mesenteric vein or portal vein), (3) locally advanced or unresectable and (4) metastatic [55].

Thus far, surgical resection is the only potentially curative treatment available for PDAC patients, although only 20% of patients have a resectable tumor at the time of diagnosis and 80-90% of these will have a relapse within 5 years [54]. Based on the anatomical location of the tumor, pancreaticoduodenectomy or Whipple's procedure (removal of the head of the pancreas and duodenum, distal common bile duct and proximal jejunum) is recommended in patients with adenocarcinoma located in the head of the pancreas, while distal pancreatectomy (removal of the body and tail of the pancreas) and splenectomy are recommended in patients with adenocarcinoma located in the body or tail. Finally, a total pancreatectomy is required for tumors that affect the entire length of the pancreas [52].

Chemotherapy is the mainstay of treatment in patients with advanced and metastatic disease. In 1996, gemcitabine, an analogue of the nucleoside deoxycytidine, was approved by FDA and became the first-line option for advanced PDAC for over a decade. It offers a small but significant improvement of survival compared to single agent 5-fluorouracil (5-FU) (OS 5.65 vs 4.41 months, p=0.003) as well as a clinical benefit response [56].

However, current treatments of choice for patients with metastatic PDAC and good PS (European Cooperative Oncology Group (ECOG) score 0-1) involve FOLFIRINOX (multidrug combination of 5-fluorouracil, folinic acid [leucovorin], irinotecan and oxaliplatin) and gemcitabine/albumin-bound paclitaxel (nab-paclitaxel, Abraxane®) which offer a survival benefit over gemcitabine monotherapy. In particular, FOLFIRINOX is more effective than gemcitabine monotherapy as confirmed by efficacy parameters such as OS (11.1 vs 6.8 months), PFS (6.4 vs 3.3 months) and one-year survival rate (48.4% vs 20.6%) [39]. Furthermore, the MPACT trial showed that the gemcitabine/nab-paclitaxel combination has a significantly longer OS (8.5 vs 6.7 months, $p<0.001$) for metastatic PDAC compared to gemcitabine alone. However, its safety profile is inferior to gemcitabine monotherapy, although the nab-paclitaxel-induced neuropathy appears to be reversible [57].

Recently, adjuvant gemcitabine-based chemotherapy represents another interesting approach in the management of PDAC. In particular, the gemcitabine plus erlotinib (epidermal growth factor receptor tyrosine kinase inhibitor) chemotherapy regimen is significantly superior to gemcitabine monotherapy in non-resectable PDAC patients with increased overall survival (OS), while gemcitabine plus capecitabine is associated with increased progression-free survival (PFS) but not OS [56].

Immunotherapy represents a novel and promising approach for PDAC treatment by targeting the immune checkpoint molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death 1 (PD-1) or programmed death ligand 1 (PDL-1). Unfortunately, immunotherapy has limited effects on PDAC probably due to the fact that PDAC is not "immunogenic" with poor mutated antigens that can be targeted by T cells and the dense TME of PDAC that acts as a barrier for drug delivery [58].

Cancer vaccine is another developing area of immuno-oncology that exploits different vaccination strategies: whole cell, peptide-, dendritic cell (DC)- or DNA-based vaccines. To date, the most extensively tested vaccine in PDAC is GVAX, an allogeneic irradiated whole-cell tumor vaccine in which cells are engineered to express granulocyte-macrophage colony stimulating factor (GM-CSF). GVAX combined with ipilimumab (anti- CTLA-4) improved OS of patients compared to the combination with 5-FU/cyclophosphamide which showed the same disease-free and median survival as GVAX alone [59].

A deeper knowledge of the multiple signaling pathways is critical in the field of PDAC treatment. Therefore, several clinical trials are still ongoing to evaluate the efficacy of other EGFR inhibitors, immunotherapeutic agents, as well as VEGF and PARP-inhibitors [60].

2. Pre-mRNA splicing: the spliceosome and the biochemical mechanism of splicing

Pre-mRNA splicing is a regulated process that plays a pivotal role in eukaryotic gene expression. It is a modification of the precursor mRNA (pre-mRNA) in which introns (non-coding portions of pre-mRNA) are removed and exons (coding portions of pre-mRNA) are joined together to generate the mature mRNA molecule that will be translated into protein [61]. Pre-mRNA splicing takes place within the nucleus of every eukaryotic cell and is carried out by a complex ribozyme, known as the spliceosome, which catalyzes both constitutive (mRNA spliced producing the same isoforms) and alternative splicing (different RNA isoforms from a single gene) [62]. The major spliceosome consists of five small nuclear ribonucleoproteins (snRNPs; U1, U2, U4/U6 and U5 snRNP) and approximately 150 proteins, while the minor spliceosome contains U5 snRNP and U11, U12, U4atac and U6atac which are analogs of snRNPs of the major spliceosome [63].

Chemically, pre-mRNA splicing occurs via two sequential trans-esterification reactions. In the first reaction (branching) the 2'-hydroxyl group of a specific adenosine (which forms the branch point) within the intron attacks the phosphodiester group at the 5' splice site (also referred to as the splice donor), resulting in a cleaved 5' exon and a lariat structure containing the intron and 3' exon (lariat-intron-3' exon intermediate). In the second step (exon ligation) the released free 3'-hydroxyl group end of the 5' exon sequence attacks the phosphodiester group at the 3' splice site (also referred to as the splice acceptor), joining the 5' and 3' exons together and releasing the intron sequence into a lariat to produce the mature mRNA molecule [61,64] (**Figure 3**).

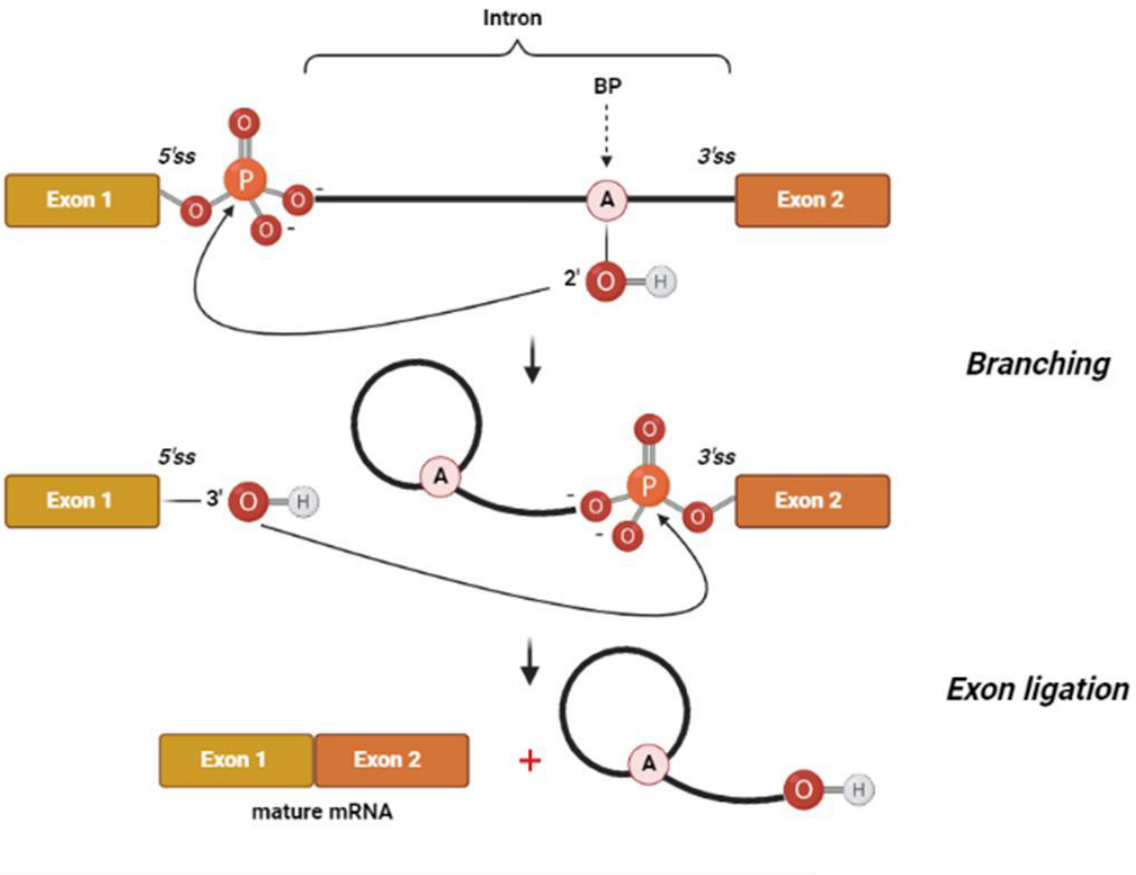

Figure 3. Graphical representation of the two-step (branching and exon ligation) trans-esterification mechanism of pre-mRNA splicing to generate the mature mRNA molecule. Created with BioRender.com.

ss, splice site; BP, branch point; A, adenosine.

Noteworthy, the specific sequence signals required to remove an intron sequence include the GURAGU consensus at the intronic 5' boundary (the 5' splice site, or 5' ss) and three elements at the 3' boundary (the 3' splice site, or 3'ss), namely a branch point sequence (or BPS) in which the adenosine forms the branch point of the lariat, a stretch of pyrimidines (polypyrimidine tract, or Py-tract) and an AG dinucleotide at the 3' end of each intron [65].

2.1 Alternative splicing: its several patterns and regulation mechanisms

Alternative splicing (AS) is an important post-transcriptional process in eukaryotic organisms that can generate multiple mRNA isoforms from a single gene and affects more than 95% of human genes. There are five main types of alternative splicing: (1) exon skipping (or cassette exons) where mature mRNA is devoid of one or more exons; (2) mutually exclusive exons in

which one of the two exons is simultaneously selected for splicing; (3) alternative 5' splice site where an alternative donor site is recognized; (4) alternative 3' splice site where an alternative acceptor splice site is used and (5) intron retention where an intron is retained in the mature mRNA [66] (**Figure 4A**).

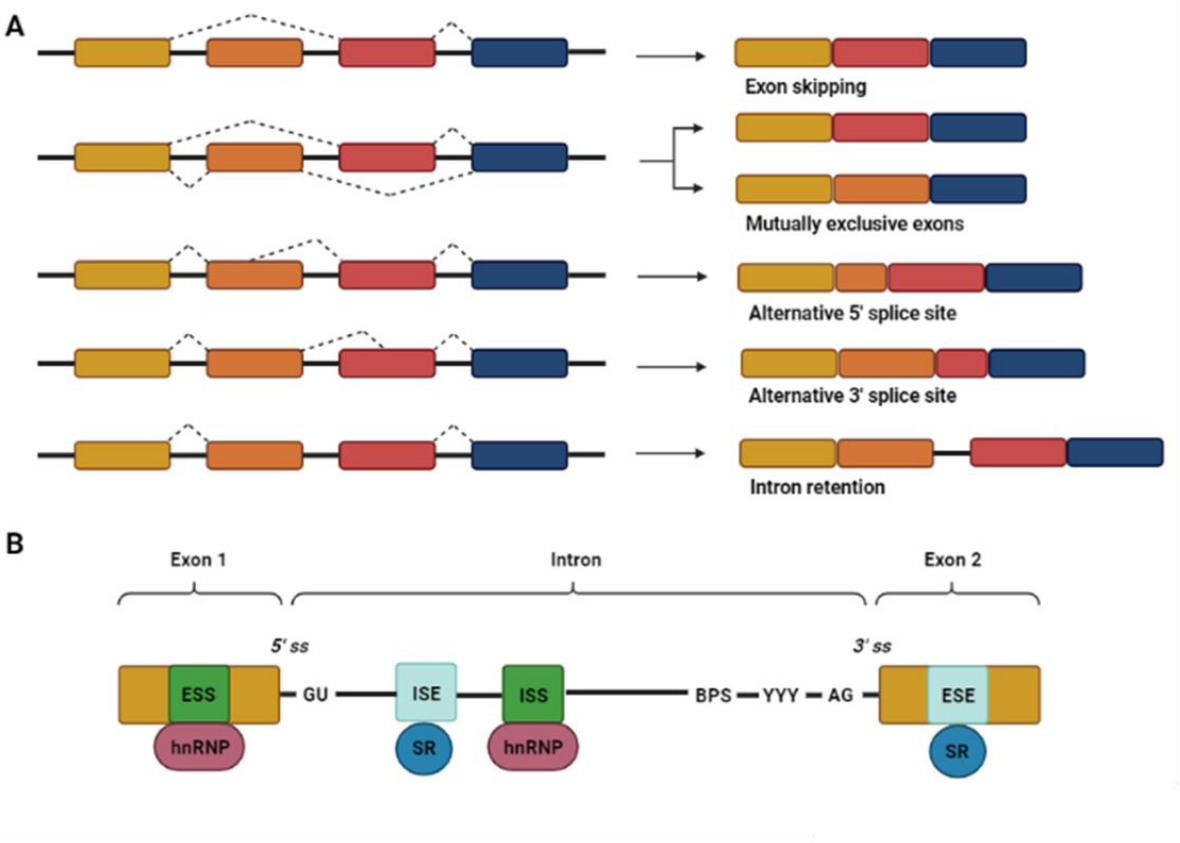

Figure 4. (A) The five major patterns of alternative splicing include: exon skipping, mutually exclusive exons, alternative 5' splice site, alternative 3' splice site and intron retention. The yellow, orange, red and blue boxes represent different exons, while the dotted black lines represent the splicing events. **(B)** Representative figure of the interplay between *cis*-acting elements (i.e. ESEs, ISEs, ESSs and ISSs) and *trans*-acting factors (i.e. SR proteins and hnRNPs) involved in the regulation of pre-mRNA splicing process. Created with BioRender.com.

ESEs, exonic splicing enhancers; ISEs, intronic splicing enhancers; ESSs, exonic splicing silencers; ISSs, intronic splicing silencers; SR proteins, serine/arginine-rich proteins; hnRNPs, heterogeneous nuclear ribonucleoproteins; ss, splice site; BPS, branch point sequence; YYY, polypyrimidine tract; GU, guanine uracil dinucleotide; AG, adenine guanine dinucleotide.

Furthermore, AS is tightly regulated by both *cis*-acting elements within the pre-mRNA and *trans*-acting factors (splicing factors) that bind to *cis* elements. The *cis*-acting elements include

short sequences that promote (enhancers) or inhibit (silencers) splicing activity such as exonic splicing enhancers (ESEs), intronic splicing enhancers (ISEs), exonic splicing silencers (ESSs) and intronic splicing silencers (ISSs), while the *trans*-acting factors include the serine/arginine-rich proteins (SR proteins) that can tipically bind to ESEs or ISEs and heterogeneous nuclear ribonucleoproteins (hnRNPs) that can bind to the ESSs or ISSs [67,68] (**Figure 4B**).

In addition, the abnormal regulation of AS is responsible for several human cancers and affects metabolism, apoptosis, cell cycle control, as well as invasion and metastasis in cancers [69].

There is a growing interest for the possible relationship between alternative splicing and PDAC, which may help to better manage the disease. Recently, it was shown that the most commonly mutated genes in PDAC (*KRAS* and *p53*) undergo a modified splicing of GTPase-activating protein (GAP) mRNAs and amplification of *KRAS* signaling as a novel mechanism through *KRAS*- and *TP53*-mutant PDACs promote cancer pathogenesis [70]. However, further efforts are needed to advance the knowledge of pre-mRNA splicing in the pathogenesis of PDAC.

2.2 SF3B complex with special focus on SF3B1

The SF3B complex is a hetero-heptameric structure of U2 snRNP which consists of seven proteins (SF3B1, SF3B2, SF3B3, SF3B4, SF3B5, SF3B6 and PHF5A) with a molecular size ranging from 10 to 155 kDa [71]. It plays a pivotal role in the branch point adenosine (BPA) recognition as well as in the early stages of spliceosome assembly during the pre-mRNA splicing process [72].

According to the Protein Data Bank (PDB) ID: 5Z56, the SF3B complex appears as a flaming torch in which SF3B1 and SF3B6 are located on the top of the flame, SF3B3 is on the bottom of the torch and the other subunits are around it. The SF3B core includes only the HEAT (Huntingtin, Elongation factor 3, protein phosphatase 2A, and the yeast kinase TOR1) domain of SF3B1, SF3B3, SF3B5 and PHF5A. In particular, the HEAT domain of SF3B1 represents the central area for RNA and protein binding in the SF3B complex. Notably, the HEAT domain contains an open conformation in the apo core structure and a closed conformation in the U2/BPS-bound SF3B state [71].

One of the most interesting SF3B components is SF3B1 (Splicing Factor 3B subunit 1) which is the largest protein (approximately 145 kDa) of the SF3B complex in the U2 snRNP of the major splicesome and it is also found in the U11/U12 snRNP of the minor spliceosome [73]. It is involved in the recognition of the branch point sequence during the pre-mRNA splicing

process. However, the pivotal role of SF3B1 is to stabilize the interaction between the U2 snRNA (small nuclear RNA) and BPS which provides the branch point adenosine as a nucleophile for splicing catalysis [73].

Structurally, the SF3B1 protein consists of an unstructured N-terminal hydrophilic domain containing multiple U2AF ligand motifs (ULMs), which can interact with the U2AF homology motif (UHM), and a C-terminal region consisting of 20 tandem HEAT repeats domain which form a superhelical structure [71,74].

Furthermore, *SF3B1* is one of the most frequently mutated genes in myelodysplastic syndromes (MDS), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), breast cancer as well as uveal melanoma, lung adenocarcinoma, prostate adenocarcinoma and pancreatic cancer [74,75].

The cancer-associated mutations in *SF3B1* are concentrated in a sequence encoding its HEAT repeat domains, particularly from H4-H12 [76], and the most recurrent mutation hotspots in *SF3B1* include R625, E622, H662, K666 and K700 [65]. In particular, the K700 mutation is most frequent in hematopoietic malignancies, while the R625 mutation in uveal melanoma; both are also present in pancreatic cancer albeit with a low frequency of 4% [77]. Recently, it was found that SF3B1 undergoes phosphorylation reactions on different residues of the N-terminal domain that are correlated with the catalytic step during the splicing process, but the function of these modifications is poorly understood [71].

2.3 Targeting splicing as potential therapeutic approach

Pre-mRNA splicing is a frequently altered process in a wide range of cancers and contributes to the pathogenicity of the disease. Therefore, it is reasonable to develop therapeutic strategies for targeting splicing in cancer cells. Currently, there is a growing interest in small molecules that modulate the activity of splicing regulators as cancer therapy drugs as well as in splice-switching antisense oligonucleotides (SSOs) [65].

In particular, since *SF3B1* is one of the most mutated genes among cancers, it is often used as a target for spliceosome modulators for the treatment of cancer. The small-molecule modulators of *SF3B1* can be classified into three classes: (1) bicycles, (2) monopyranes and (3) 12-membered macrolides that share a central pharmacophore represented by a conjugated diene, flanked by two district functional moietes. The bicycle modulators, containing two six-membered rings joined by a conjugated diene, include cytotoxic natural products derived from various bacterial sources [78].

In particular, FR901464 was extracted from *Pseudomonas sp.* in 1996 and was first described as an agent with potent cytotoxic activity against several human solid tumor cell lines, as well as being able to arrest the G1 and G2/M phases of the cell cycle. Only later, it was discovered that FR901464 inhibited pre-mRNA splicing by targeting the spliceosome component SF3B1. Additionally, spliceostatin A (SSA), which is a methylated derivative of FR901464, was shown to affect cellular splicing in low nM range of some cell lines through targeting SF3B1; other inhibitors are meayamycin B and sudemycins [78–80].

Over the years, different splicing modulators were extracted from other culture broths such as thailanstatins (thailanstatin A, B, C) from *Thailandensis burkholderia* MSMB43 which showed potent cytotoxicity. In general, the thailanstatins are more stable than FR901464 due to the lack of the C1-hydroxyl group and the presence of an extra C17-carboxylic group despite sharing a similar pharmacophore [78].

The SF3B1 protein also turned out to be the target of herboxidiene/GEX1A (belonging to the monopyranes class) and pladienolides (belonging to the macrolides class) which were originally obtained from *Streptomyces chromofuscus* A7487 and *Streptomyces platensis* Mer-11107, respectively [72].

The finding that several splicing modulators target SF3B1 has paved the way for the development of multiple synthetic derivatives. E7107, an analogue of pladienolide D, has anticancer effects and inhibits RNA splicing by binding to SF3B1. It was the first SF3B1 modulator to enter clinical trials for the treatment of solid tumors. However, its initial trial was suspended due to the development of unexpected ocular complications of two participants [73].

H3B-8800 is an orally available small molecule splicing modulator derived from pladienolide B. It was recently evaluated in a phase I clinical trial for haematological malignancies exhibiting potent antitumor effects and lower cytotoxicity [81].

As previously mentioned, SSOs have recently been an effective validated method targeting the splicing process for the treatment of cancer.

The prototype of SSOs consists of antisense oligonucleotides (ASOs) that directly target specific RNA molecules by binding to them through base pairing and inducing target degradation or interfering with the splicing process. Specifically, SSOs are short, synthetic and modified nucleic acids designed to block the binding of splicing factors to the pre-mRNA, altering the normal splicing process. This promising therapeutic approach is used for the treatment of the Duchenne muscular dystrophy and spinal muscular atrophy (SMA) which are currently being tested in clinical trials [82,83].

In conclusion, splicing modulators as well as SSOs obviously represent interesting new approaches to cancer therapy. However, considerable efforts are needed to demonstrate their clinical potential.

2.4 Splicing deregulation and anticancer drug resistance

Overcoming drug resistance in cancer treatment certainly remains the major challenge of recent decades. Among the many mechanisms involved, it is appropriate to include the abnormal alternative splicing that alters the gene expression profiles of tumor cells by inducing resistance [68].

The imbalance between pro- and anti-apoptotic factors can cause a reduced response of cancer cells to chemotherapy. Many genes involved in apoptosis regulation are highly dependent on alternative splicing to produce proteins with antagonistic functions. The pro-apoptotic B-cell CLL/lymphoma 2 (Bcl-2) family members such as Mcl-1 and Bcl-X undergo alternative splicing generating Mcl-1L/Bcl-XL (long isoforms) with anti-apoptotic function and Mcl-1S/Bcl-XS (short isoforms) with pro-apoptotic function, respectively. It was found that the anti-apoptotic Bcl-XL isoform is expressed in many types of cancers [62,84].

Furthermore, Caspase-2 (CASP-2) is also involved in the apoptosis regulation and undergoes alternative splicing producing the pro-apoptotic CASP-2L isoform and the anti-apoptotic CASP-2S isoform. The Bcl-2 family also includes the BH3-only protein, BIM, whose spliced isoforms (BIM-EL, BIM-L and BIM-S) are pro-apoptotic and play a pivotal role in drug resistance [62,85].

More importantly, alternative splicing promotes tumor resistance to immunotherapy. CD19 antigen is well known to be the target for CART-19 cellular immunotherapy. The downregulation of the splicing factor SRSF3 induces the skipping of CD19 exon 2 generating an N-terminal truncated CD19 protein without the CAR recognition site. Thus, loss of surface expression of CD19 epitopes prevents the killing of tumor cells by CART-19 inducing resistance to CART-19 immunotherapy in B-ALL (B-Cell Acute Lymphoblastic Leukemia) patients [85,86].

However, a specific example of drug resistance potentially based on the splicing process occurs in PDAC. Treatment of pancreatic cancer cells with gemcitabine leads to increased levels of the splicing factor SRSF1, which induces splicing of the MAP kinase-interacting serine/threonine-protein kinase 2 (*MNK2*) gene towards the pro-oncogenic variant MNK2b

leading to resistance to gemcitabine [68,87]. In addition, chronic exposure of PDAC cells to gemcitabine leads to resistance to gemcitabine, while also resistance to cisplatin was found. This was associated with the alternative splicing of the pyruvate kinase gene leading to an increased PKM2 isoform. High levels of PKM2 are in turn associated with increased resistance of cancer cells to gemcitabine. Conversely, the inhibition of PKM2 through an ASO, resulting in the production of the alternative PKM1 variant, leads to the sensitization of pancreatic cancer cells to gemcitabine [87,88].

Finally, further in-depth investigations are needed as the role of the splicing process in drug resistance is currently poorly understood.

3. The chemistry of heterocycles as potential strategy in cancer research

Heterocycles are structural units of many marketed drugs and form the core element of natural products such as amino acids, nucleic acids, alkaloids and vitamins. Heterocycles play a central role in the manipulation of lipophilicity, polarity and aqueous solubility. Bioisosterism may affect potency and selectivity and may improve the pharmacological, pharmacokinetic and physicochemical properties of the compounds. Therefore, in recent decades the area of heterocycle chemistry has increased interest in medicinal chemistry, paving the way for the design and synthesis of multiple new compounds [89,90].

Research efforts in Palermo over the last decade focused on the design and synthesis of imidazo[2,1-*b*][1,3,4]thiadiazole derivatives (**Figure 5**). It is a class of heterocyclic chemical compounds, discovered during the early 1950s, which result from the fusion of an imidazole ring with a [1,3,4]thiadiazole nucleus and contain a bridgehead nitrogen atom. Specifically, they are an isoster of imidazo[1,3,4]thiazole heterocycles in which the 3-CH group in the thiazole ring is replaced by 3-N atom. The presence of four heteroatoms and two condensed heterocycles with different π-conjugations can affect physicochemical and biological properties [91,92].

In addition, the versatility of the thiadiazole ring lies in its mesoionic character which facilitates the crossing of the cellular membrane and the consequent interaction with biological targets [93].

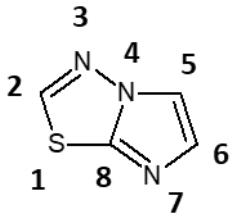

Figure 5. Chemical structure of imidazo[2,1-*b*][1,3,4]thiadiazoles.

Many imidazothiadiazole derivatives have been studied extensively and were able to inhibit several specific enzymes, leading to a wide range of biological activities *in vitro* which include antiviral, antibacterial, antitubercular, antifungal, leishmanicidal, anti-inflammatory, anticonvulsivant, antipyretic and anticancer activity. These results highlight the potential of these compounds as scaffolds for the design of therapeutic molecules [91,92].

Among the various heterocyclic compounds, indole has also received considerable attention as a valuable scaffold in drug discovery and in the design of anticancer agents. It should be mentioned that the hybridization of indole with other biologically active moieties could represent an innovative approach in order to generate new compounds with greater potency against drug-resistant tumors [90,94].

In this regard, the design of hybrid molecules characterized by the simultaneous presence of two or more biologically active scaffolds represents a new strategy that has aroused growing interest in the past few decades. This approach has allowed the development of potential anticancer drugs with better pharmacokinetic profile, multi-target mechanism of action and greater ability to counteract tumor drug resistance [95].

Recently, compounds bearing in the same structure the imidazo[2,1-*b*][1,3,4]thiadiazole and indole scaffolds have shown a relevant antiproliferative activity against PDAC cell lines, resulting in promising lead compounds for the development of new anticancer drugs [95].

On the basis of these interesting results described for the imidazothiazole nucleus [95-98], we were encouraged to continue with the same approach in order to rationally design new compounds with antiproliferative activity. Therefore, we prepared new molecules with the imidazo[2,1-*b*][1,3,4]thiadiazole scaffold substituted in position 2 with an indole nucleus. Since there is an urgent need to find new effective therapeutic strategies for the treatment of patients with PDAC, and considering the interesting antiproliferative activity showed by the imidazo[2,1-*b*][1,3,4]thiadiazole compounds towards the full NCI cancer cell panel [95,98], we

decided to extend the evaluation of their antitumor activity also against preclinical models of PDAC. This tumor has an extremely poor prognosis and is expected to become the third leading cause of cancer death by 2025 as concluded from a study in 28 European countries [27].

4. Thesis outline

This dissertation focuses on pancreatic cancer which has a constantly increasing incidence and represents one of the worst cancers worldwide.

The design of targeted and specialized therapies in combination with conventional treatments has great promise to improve cancer therapy. To this end, the current Thesis has a special focus on the rational design and synthesis of new anticancer compounds potentially active on *SF3B1*, one of the most frequently mutated genes in pancretic cancer. *SF3B1* is involved in the regulation of the pre-mRNA splicing process and is a promising therapeutic target against the cancer.

The introductory **Chapter 1** provides an overview of pancreatic cancer with a special focus on the state of the art of pancreatic ductal adenocarcinoma (PDAC), followed by a section entirely devoted to the pre-mRNA splicing process that paves the way for potential cancer cure research, providing new alternatives to the treatment of this disease.

Chapter 2 is a review on the key gemcitabine transporter, the human Equilibrative Nucleoside Transporter 1 (hENT1) which is a potential predictive biomarker in the treatment of PDAC.

Chapter 3 describes the *in vitro* antiproliferative and antimigratory effect of ten new imidazo[2,1-*b*][1,3,4]thiadiazole derivatives tested on two primary cell cultures of DMPM (Diffuse Malignant Peritoneal Mesothelioma), MesoII and STO cells.

Chapter 4 explores the emerging role of splicing deregulation in cancer by analyzing the interaction between various splicing factors and specific target miRNAs in PDAC.

Chapter 5 presents a study in which the expression levels of *SF3B1* in PDAC cells and tissues are analyzed, as well as the activity of SF3B1 modulators currently in the clinical investigation.

Lastly, **Chapter 6** examines the cytotoxic and antimigratory activity of four novel potential SF3B1 modulators (one imidazo[2,1-*b*][1,3,4]thiadiazole derivative and three indole derivatives) in the PDAC cells SUIT-2, Hs766t and Panc05.04. The effects on the splicing pattern of proto-oncogene recepteur d'origine nantais (RON) and on the hENT1 are also evaluated.

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Chapter 2

"Open Sesame?": Biomarker Status of the Human Equilibrative Nucleoside Transporter-1 and Molecular Mechanisms Influencing its Expression and Activity in the Uptake and Cytotoxicity of Gemcitabine in Pancreatic Cancer

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Simple Summary: Despite the enormous advance in biomarker discovery, many potential biomarkers of drug activity are unable to satisfy the clinical need due to inadequate sensitivity and specificity. The nucleoside transporter hENT-1 has been studied as a potential biomarker to predict the effect of the widely used anticancer drug gemcitabine in pancreatic cancer. However, several studies showed controversial results regarding the predictive value of hENT-1, prompting new analyses with larger cohorts of patients and standardized methodologies. Improved insights on molecular mechanisms underlying hENT-1 expression and activity should also help in the identification of subsets of patients who are more likely to benefit from specific treatments and improve their clinical outcome. The establishment of such biomarker is especially valuable in pancreatic cancer, which is frequently characterized by complex disease biology and high mortality.

Abstract: Pancreatic ductal adenocarcinoma (PDAC) is an extremely aggressive tumor characterized by early invasiveness, rapid progression and resistance to treatment. For more than twenty years, gemcitabine has been the main therapy for PDAC both in the palliative and adjuvant setting. After the introduction of FOLFIRINOX as an upfront treatment for metastatic disease, gemcitabine is still commonly used in combination with nab-paclitaxel as an alternative first-line regimen, as well as a monotherapy in elderly patients unfit for combination chemotherapy. As a hydrophilic nucleoside analogue, gemcitabine requires nucleoside transporters to permeate the plasma membrane, and a major role in the uptake of this drug is played by human equilibrative nucleoside transporter 1 (hENT-1). Several studies have proposed hENT-1 as a biomarker for gemcitabine efficacy in PDAC. A recent comprehensive multimodal analysis of hENT-1 status evaluated its predictive role by both immunohistochemistry (with five different antibodies), and quantitative-PCR, supporting the use of the 10D7G2 antibody. High hENT-1 levels observed with this antibody were associated with prolonged disease-free status and overall-survival in patients receiving gemcitabine adjuvant chemotherapy. This commentary aims to critically discuss this analysis and lists molecular factors influencing hENT-1 expression. Improved knowledge on these factors should help the identification of subgroups of patients who may benefit from specific therapies and overcome the limitations of traditional biomarker studies.

Keywords: pancreatic cancer; drug resistance; human equilibrative nucleoside transporter 1; clinical outcome

1. Introduction

In the story of "Ali Baba and the Forty Thieves" from the book *One Thousand and One Nights*, "Open sesame" is the magical phrase that opens the mouth of a cave in which the thieves have hidden a treasure. This statement has been commonly used to define something that allows a person to do or enter something easily, or something that unfailingly brings about a desired end. Several cellular transporters are essential for the entry (or efflux) of anticancer drugs [1,2] but, despite a number of preclinical and clinical studies on their role as biomarkers and targets, they have not yet been exploited or exploited correctly to improve clinical outcome.

The human equilibrative nucleoside transporter (hENT-1) represents a quintessential example of such a transporter. This protein is indeed the main transporter involved in the entrance of nucleoside analogs and has attracted extensive attention for its potential role as predictive biomarker for the anticancer activity of gemcitabine [3], as well as for the development of drugs bypassing this transporter in order to overcome gemcitabine resistance in pancreatic ductal adenocarcinoma (PDAC) [4–6]. In particular, we have read with great interest the recent comprehensive multimodal analysis of hENT-1 status, which has been performed by Raffenne and collaborators in the largest cohort of PDAC patients to date [3]. In the present commentary, we summarize the key findings of this analysis and discuss further insights on molecular and pharmacological factors influencing the role of nucleoside transporters in the uptake and cytotoxicity of gemcitabine in PDAC.

2. Pancreatic Ductal Adenocarcinoma (PDAC)

Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer and is amongst the deadliest solid malignancies [7]. Despite extensive genetic mapping elucidating key mechanisms in PDAC initiation and progression [8], both conventional and experimental drugs showed limited effects [7].

PDAC is indeed a highly invasive and aggressive disease with an overall 5-year survival rate lower than 10% [7]. Several factors are responsible for this grim prognosis, including late diagnosis and lack of effective therapies [9,10]. Surgery represents the only curative intervention available for patients with local disease. However, even after successful tumor resection and adjuvant chemotherapy, the 5-year survival rate is around 25–30%, with most of the patients experiencing tumor recurrence and metastatic disease within 6–24 months from

surgery [11,12]. Chemotherapy is the only treatment available for patients with advanced or metastatic disease, which includes the vast majority of diagnosed PDAC cases [7].

Unfortunately, PDAC is an inherently resistant disease, with low percentages of response rate to all current treatment regimens. Moreover, even when chemotherapy is initially effective, chemoresistance typically occurs after a few cycles, leading to disease progression and mortality [13,14]. This resistance is caused by both cellular intrinsic and extrinsic factors such as cancer stem cells (CSCs), activation of the epithelial-mesenchymal transition (EMT), and presence of a highly desmoplastic and immunosuppressive tumor microenvironment (TME) [15–18]. Understanding these molecular mechanisms of chemoresistance is an essential step towards increasing the efficacy of treatments and clinical outcome.

Despite the development of more effective multi-drug regimens such as FOLFIRINOX (a chemotherapy regimen made up of the following four drugs: FOL—folinic acid, F—fluorouracil (5-FU), IRIN—irinotecan, OX—oxaliplatin), gemcitabine is still widely used for PDAC treatment, both combined with nab-paclitaxel (Abraxane®, Celgene, Summit, NJ, USA), and as monotherapy, especially in patients who are unfit for more toxic poly-chemotherapy regimens [14]. Several studies have focused on molecular intracellular determinants of gemcitabine activity and metabolism. Among these molecular determinants, mRNA and protein expression of the equilibrative transporter-1 (hENT-1) emerged as potential predictors of drug activity in a number of preclinical and clinical studies in PDAC.

3. Nucleoside Transporters Involved in Gemcitabine Uptake

The two major classes of nucleoside transporters that have been described in mammalian cells include the concentrative nucleoside transporters (CNTs) and the equilibrative nucleoside transporters (ENTs). These transporters are transmembrane glycoproteins that localize to the cellular and mitochondrial membranes, but can also be found in lysosomes [19] and mediate the cellular uptake of nucleosides required for nucleotide synthesis in cells that lack de novo nucleotide synthesis pathways.

CNTs mediate the inward Na^+-dependent transport whereas ENTs are bi-directional facilitators of the transmembrane flux of nucleosides [20]. ENTs can be found in almost all cell types unlike CNTs, which are present in intestinal and renal epithelia [21], as well as in hepatocyte cells and in chorionic villi of human term placenta [22].

As bi-directional carriers, ENTs regulate both the influx and efflux of substrates. The human ENT homologues (hENTs) are classified into four groups: hENT-1 (SLC29A1), hENT-2 (SLC29A2), hENT-3 (SLC29A3) and hENT-4 (SLC29A4) [23]. The hENT-1 is sensitive to nitrobenzylmercaptopurine ribonucleoside (NBMPR) to which it binds with a high affinity [24]; while hENT-2 is insensitive to inhibition by NBMPR. However, the hENT1–3 shows selectivity to the NBMPR substrate which also blocks hENT-4 albeit at higher concentration than required for hENT-1 [25]. Of note, hENT-4 is better known as the plasma membrane monoamine transporter because it carries organic cations such as biogenic amines and neurotoxins. This transporter mediates the transport of adenosine in a pH-dependent manner and its activity increases in acidic conditions (optimal transport at pH 6.0) [26].

These transporters are involved in the uptake of several drugs (**Figure 1**) with different chemical structures (**Figure S1**).

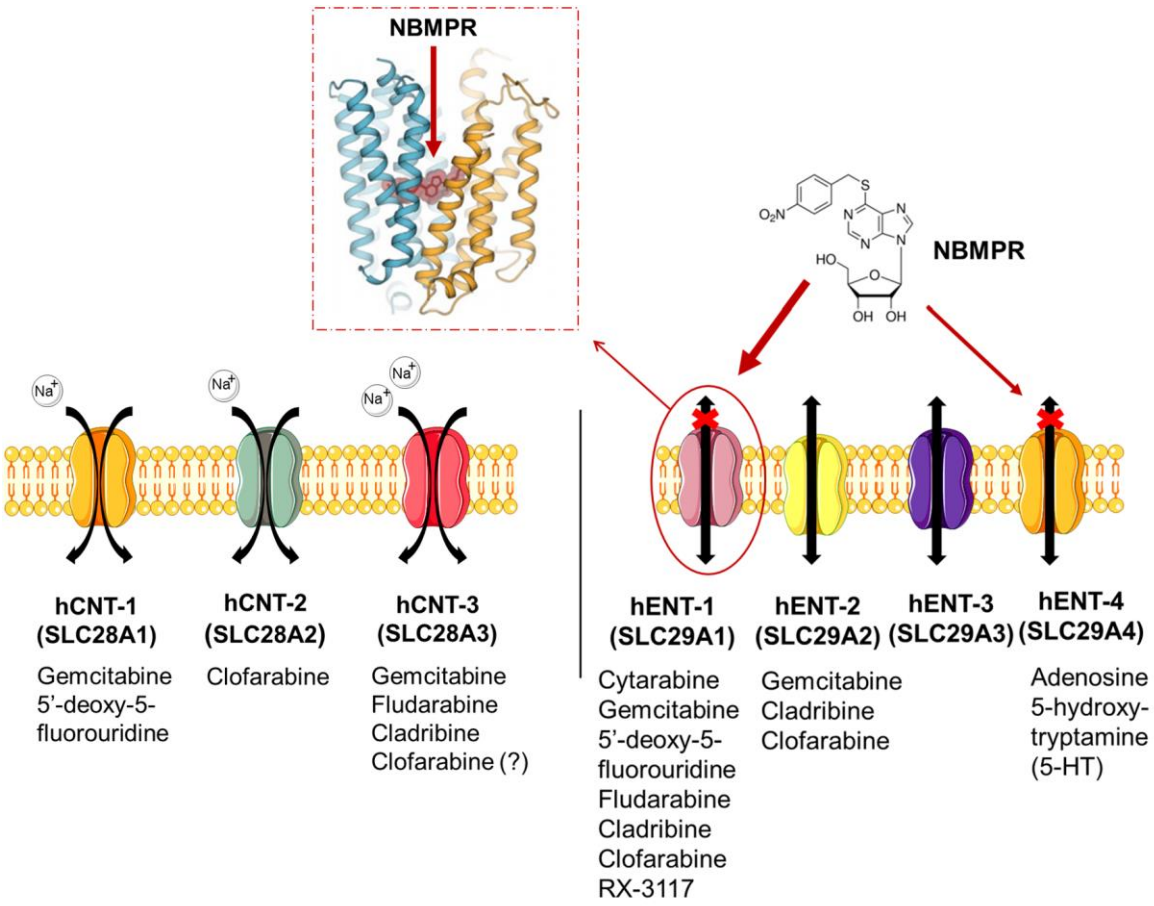

Figure 1. Nucleoside transporters in mammalian cells. CNTs facilitate the Na^+-dependent transport while ENTs are bi-directional and Na^+-independent transporters, facilitating the uptake of different anticancer drugs [27–30]. Of note, hENT-1 (es) is NBMPR-sensitive and the figure shows its crystalized

structure [30]. Acronyms: 5-HT, 5-hydroxy-tryptamine; NBMPR, nitrobenzylmercaptopurine ribonucleoside.

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) is a pyrimidine analog and relies on membrane transporters for its intracellular uptake [31,32]. The uptake of gemcitabine can be mediated by hENT-1/2 and hCNT-1/3. However, hENT-1 and hCNT-1 appear to be the most efficient transporters involved in the entry of gemcitabine into cells. Notably, hENT-1, the most widely expressed nucleoside transporter in human tissues, is overexpressed in different tumor types, including PDAC (**Figure S2A**). This is reported in the GEPIA web server, analyzing the RNA sequencing expression data of 9736 tumors and 8587 normal samples from the TCGA and the GTEx projects [33]. However, the comparison of TCGA-PAAD data of pancreatic cancer specimens with the matched non-tumor samples as well as the analysis of similarly matched transcriptomics and proteomics public datasets did not show a significance difference in hENT-1 expression levels (**Figure S2B**).

Structurally, hENT-1 is a 456-residue protein (50 kDa) with 11 transmembrane domains and three N-linked glycosylation sites, which are essential for localization, function and oligomerization. The first glycosylation site (Asn 48) is located between the first and second transmembrane domains in the hydrophilic loop, whereas the other two sites (Asn 277 and 288) are between the sixth and seventh transmembrane domains [32]. The Km for purine and pyrimidine nucleosides transport range from 0.05 mM to 0.60 mM according to a study performed in *Xenopus laevis* [34].

4. Role of hENT-1 in Gemcitabine Activity as Potential Predictive Biomarker

Several studies showed that hENT-1 expression is essential for gemcitabine cytotoxic effects [35,36]. Higher expression levels of hENT-1 have indeed been associated with higher uptake and activity of gemcitabine in cancer cells, using different preclinical models [37–40].

A number of retrospective studies on hENT-1 mRNA and protein expression with PCR and immunohistochemical (IHC) methodology demonstrated that high levels of hENT-1 correlated with a statistically significant longer survival (**Figure 2**), both in the adjuvant and in the metastatic setting, though the number of patients in the latter cohort was extremely small [41–52]. For instance, a retrospective analysis of a cohort of PDAC patients from the RTOG9704 phase III clinical trial, which compared gemcitabine with 5-FU after surgical resection, showed

an association between high tumor hENT-1 expression and increased overall survival (OS) in patients who received gemcitabine (n = 91), but not in those who received 5-FU [43]. These data support the role of hENT-1 as a specific predictive biomarker for the efficacy of gemcitabine.

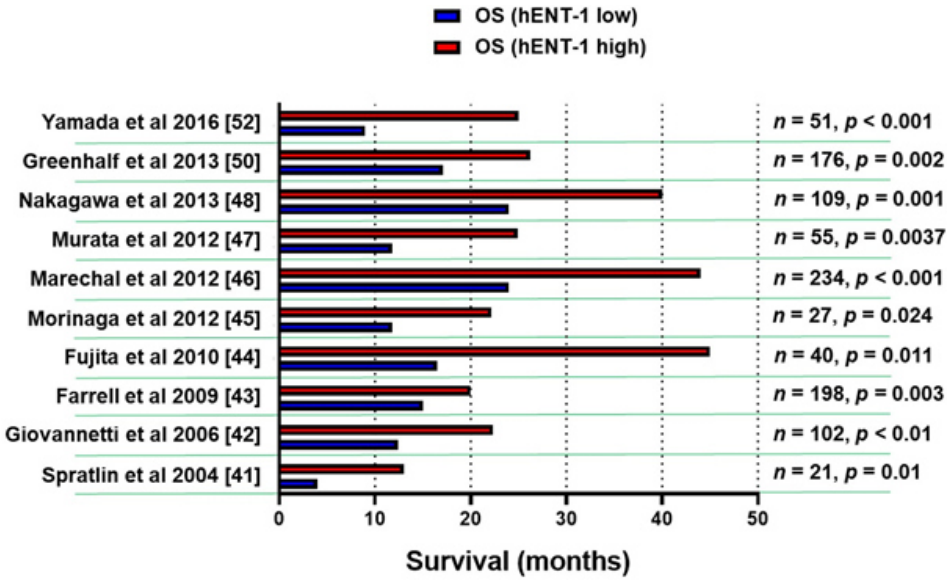

Figure 2. Association of hENT-1 expression levels and survival. Bar graph summarizing the survival data of PDAC patients in studies supporting the role of hENT-1 as a predictive biomarker for the efficacy of gemcitabine. The clinicopathological and treatment details of studies before 2014 [41–48] were reviewed systematically by Nordh et al. [49], while a more recent systematic review by Bird et al. [51] evaluated also the data from the European Study Group for Pancreatic Cancer 3 (ESPAC-3) trial [50]. n = number of patients, p = statistical p values.

Conversely, high expression levels of hENT-1 emerged as a prognostic biomarker of poor outcome in cholangiocarcinoma. Indeed, while a first study showed a significant association between disease-free survival (DFS) and high expression of membrane hENT-1 in gemcitabine-treated patients [53,54], hENT-1 overexpression was associated to high proliferation rate and significantly shorter survival in resected intrahepatic cholangiocarcinoma patients who did not receive adjuvant treatments [55]. This might be explained by the different levels of hENT-1 expression and proliferative rates in different tumor types and warrants further, larger studies.

Up to now, the largest prospective study on hENT-1 in PDAC has been performed within the European Study Group for Pancreatic Cancer 3 (ESPAC-3) trial. This study highlighted a significant association between high hENT-1 protein expression and longer OS and DFS in

PDAC patients receiving gemcitabine-based chemotherapy post-surgery [50]. Moreover, a quantitative metanalysis including 7 studies with a total of 770 patients (405 hENT-1-negative and 365 hENT-1-positive) showed that hENT-1 expression was significantly associated with both prolonged DFS (HR 0.58, 95% CI, 0.42–0.79) and OS (HR 0.52, 95% CI, 0.38–0.72) in patients receiving adjuvant gemcitabine-based therapy [52].

In contrast, Kawada et al. [56], who evaluated hENT-1 expression in PDAC patients undergoing neoadjuvant chemoradiation, showed only a trend towards statistically significant better disease-specific survival in patients with low expression of hENT-1. These results might be explained by the potential preferential eradication of tumor cells with high expression of hENT-1 by the neoadjuvant treatment before the tumor samples collection. This issue can be overcome by the use of fine-needle aspiration biopsy (FNAB) for retrieving cancer cells before treatment and resection of the tumor. Indeed, a study on pretreatment hENT-1 expression in endoscopic ultrasonography-guided FNAB specimens obtained from resectable, borderline-resectable, and locally advanced PDAC, showed that hENT-1 expression was an independent prognostic factor in both whole patients and those with resection [52]. Regardless of T3 and T4, hENT-1-positive patients with resection had significantly better prognosis than hENT-1-negative patients, whose prognosis was similar to those without resection, suggesting that the evaluation of hENT-1 expression using FNAB samples before chemoradiation provides useful information on patients who might benefit from curative-intent resection.

However, other recent studies on the evaluation of hENT-1 status using IHC in PDAC patients reported conflicting results, possibly due to the use of different antibodies. In particular, the analysis of samples from 156 patients enrolled in the CONKO-001 phase III trial did not show a significant association of high hENT-1 expression with improved median DFS or OS [57]. Similar negative results were observed within a retrospective translational subgroup analysis for hENT-1 in 130 samples from patients enrolled in the AIO-PK0104 multicenter phase III trial [58]. In both cases the researchers used the rabbit monoclonal antibody SP120 and suggested to perform a parallel study using the rabbit 10D7GD antibody. Such study was performed by Marechal and collaborators as well as by Svrcek and collaborators who reported that hENT-1 status was predictive of gemcitabine benefit in patients receiving gemcitabine-adjuvant chemotherapy when evaluated with the 10D7G2 antibody, yet no predictive value was observed when hENT-1 status was assessed using the SP120 antibody [46,59].

In contrast, Kalloger and collaborators, who performed the staining with 10D7G2 and SP120 antibodies (both optimized to run on the Ventana platform), in samples from 227 patients,

suggested that both these antibodies can be used to predict gemcitabine sensitivity in resected PDAC [60]. This study suggested also that the use of both antibodies and of the percentage of cells staining positive for hENT-1, instead of the H-score methodology, add critical information that enables the stratification of patients with good, intermediate, or poor response to adjuvant gemcitabine. However, as recognized by the authors: "these findings need to be externally validated in cohorts derived from randomized controlled trials" [60].

Overall, these controversial findings question the predictive value of the available anti-hENT-1 antibodies and call for the establishment of a standardized IHC methodology before hENT-1 status could be used as a predictive biomarker in the clinical setting.

5. Evaluation of the Study "hENT-1 Status in PDAC Patients—Are We Ready Yet?"

In a recent study Raffenne and collaborators provided the most comprehensive multimodal analysis of hENT-1 status in the largest cohort of PDAC patients to date (i.e., 471 patients with resected PDAC) [3]. In this study the expression of hENT-1 evaluated using the 10D7G2 antibody was predictive of both prolonged DFS and OS in PDAC patients receiving gemcitabine adjuvant chemotherapy. In contrast, no predictive value of gemcitabine benefit was observed when hENT-1 status was assessed using the SP120 clone when comparing surgery-gemcitabine vs. surgery-only groups. Three additional antibodies (PAB2255, MC-9777, and 11337-1-AP) manufactured by three different companies (MBL™, Woburn, MA, USA; Abnova™, Taipei, Taiwan; and Acris™-OriGene™, Rockville, MD, USA) were further evaluated to establish their potential for the analysis of hENT-1 status. None of these antibodies showed a predictive value of gemcitabine benefit over controls, providing compelling evidence that commercially available anti-hENT-1 antibodies are not suitable for the evaluation of hENT-1 status. Interestingly, all tested antibodies, except the 10D7G2 clone, detected multiple bands on Western blot that did not correspond to the expected glycosylated forms of hENT-1, hence suggesting that these antibodies may recognize and bind to non-completely functional forms of the hENT-1 protein.

Raffenne and collaborators also evaluated the predictive value of mRNA expression levels of hENT-1 which was assessed using microarray data and qRT-PCR analyses performed on formalin-fixed paraffin-embedded (FFPE) tissues from resected specimens. No difference in both DFS and OS was observed when the median hENT-1 mRNA value was used to discriminate between hENT-1 high- and low-expressing tumors. Nonetheless, an increasingly

predictive trend was detected when more stringent thresholds were employed (top 25% for OS and top 10% for DFS). Further increase of the threshold (top 10% vs. bottom 10% or bottom 90%) allowed the selection of a population of exceptional gemcitabine responders.

These results might be explained by the fact that the authors used whole tumors. Because of the dense stromal reaction, the analysis of PCR data in PDAC specimens should indeed be performed only after careful evaluation of the percentage of tumor cells and, when feasible, after laser-microdissection [61].

For instance, our PCR analysis of 22 non-microdissected (no LMD, including tumor and stroma tissues) samples showed a minor gene expression variability, with coefficient of variation values of the hENT-1 expression values ranging from 7% to 16% compared to the respective microdissected (LMD, including only tumor tissues) specimens (**Figure 3A,B**). This could potentially affect the stratification of the patients in the "low" vs. "high" expression categories and the correlation with clinical outcome. Additionally, proteomics analyses of LMD matched epithelial and stromal compartments showed an up-regulation ($p = 0.017$) of hENT-1 in the epithelial compartment (**Figure 3C**). Of note, although the presented cohort is relatively small ($n = 13$), epithelial hENT-1 expression was associated with significantly longer survival while no difference in the OS curve were observed for hENT-1 stromal expression (**Figure 3C**). Successful dissection of tumor and stromal compartment is reported in **Figure S3**. Recent studies showed the impact of LMD on the quality of both mRNA and protein content in PDAC specimens [61,62], and might explain why a not laser-assisted microdissection did not result in the association of hENT-1 expression levels with disease-specific survival, as reported by Jiraskova and collaborators in a retrospective study on a cohort of 69 resected PDAC patients treated with gemcitabine [63].

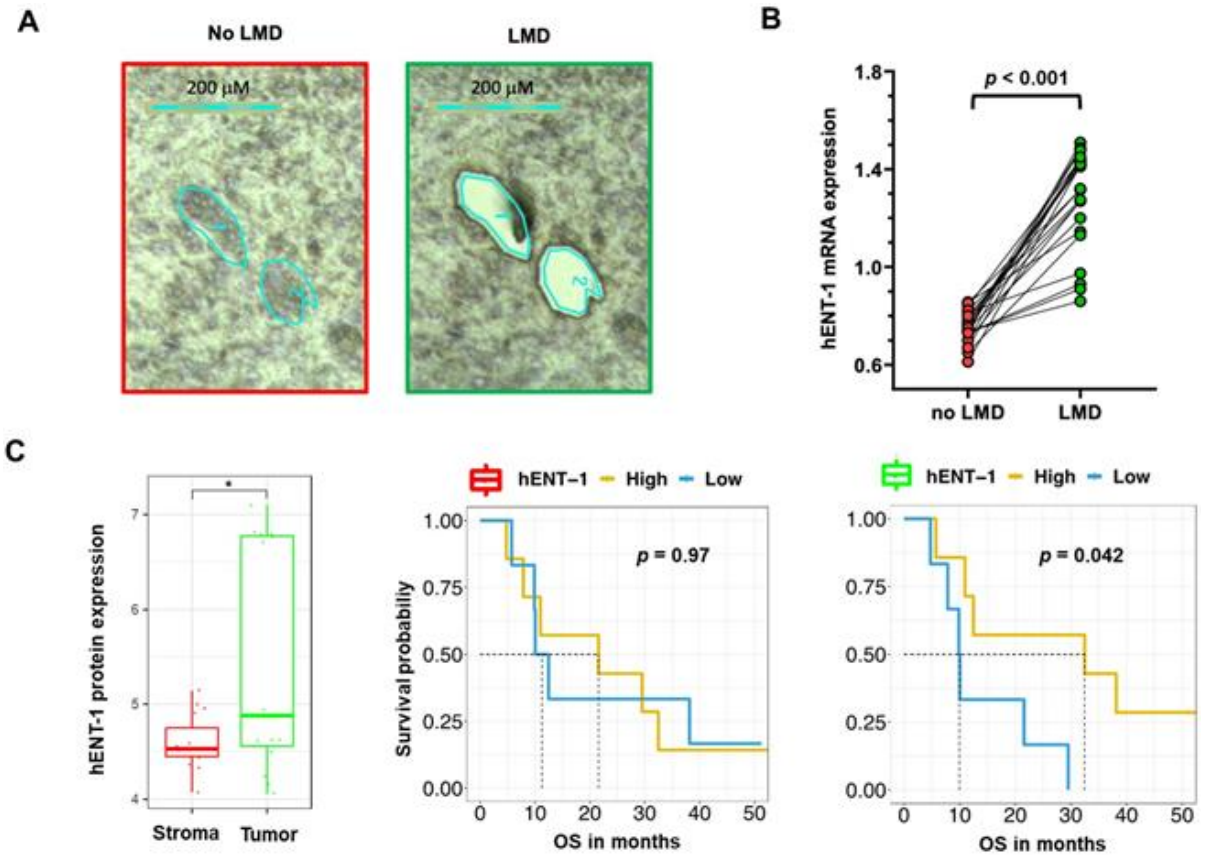

Figure 3. Impact of laser-microdissection on mRNA expression levels of hENT-1 in PDAC samples **(A)** Representative pictures of PDAC epithelium and stroma before and after laser-assisted microdissection, performed on frozen tissue with the Leica LDM7000 microscope, as described previously [62]. **(B)** Comparison between hENT-1 gene expression values in microdissected (LMD) and non-microdissected (no LMD) samples from 22 PDAC tissues. Expression of hENT-1 was evaluated by Real-Time Quantitative PCR and normalized to GAPDH expression, as described previously [42]. *t*-test statistics was performed using Graph Pad version 7. Clinicopathological features of this subgroup of patients are reported in Table S1 [64–66]. **(C)** Comparison between hENT-1 protein expression values (evaluated by nanoLC-MS/MS) in LMD stromal and tumor compartments (* = $p < 0.05$) in $n = 15$ matched samples with respective survival curves. Protein values are represented in log10(LFQ). Patients were grouped in high and low hENT-1 protein expression according to the median cutoff. [62,67]. T-test statistics and Kaplan-Meier plots were performed in R version 3.5.2.

Of note, the data of this study also suggested a limited proportional dependence between hENT-1 gene expression evaluated by qRT-PCR in FFPE samples and protein levels as assessed by immunohistochemistry with the 10D7G2 antibody [63]. This is in agreement with the study by Raffenne and collaborators, where a higher degree of concordance was observed between hENT-1 mRNA expression levels and the SP120 rabbit clone [3]. These findings suggest that

this antibody might recognize an unprocessed form of hENT-1 which is directly linked to the mRNA level, whereas the 10D7G2 clone recognizes the active glycosylated stabilized form, hence explaining its better predictive value. Remarkably, a significant correlation between mRNA level and IHC was also found, for another non-commercial rabbit anti-hENT-1 antibody developed by Pastor-Anglada and collaborators, who observed similar results in tumor cells with a different pathology [40,68].

Lastly, in the study by Raffenne and collaborators, the hENT-1 status was assessed by IHC using the 10D7G2 antibody also on coupled samples from both primary and metastatic tumors. Concordance between primary tumor and metastases was excellent for synchronous metastases. In contrast, a lower concordance was reported between metachronous metastases and primary tumors. As postulated by the authors, this discrepancy could be the result of the gemcitabine treatment that led to the selection of hENT-1-low clones in the metachronous metastases [3]. In this regard, further limitations might be represented by the relatively small sample size and other determinants influencing hENT-1 expression, such as disease stage and parameters discussed in Section 6. This is an extremely important aspect that could explain why the role of hENT-1 expression as a biomarker could not be validated when a comparison of gemcitabine with its lipophilic analog CO-101 was carried out within the prospective biomarker-stratified Low hENT1 Adenocarcinoma of the Pancreas (LEAP) trial, which enrolled PDAC patients in the metastatic setting [5]. However, another potential explanation of the lack of association between hENT-1 expression and response to gemcitabine is the use of the rabbit monoclonal antibody SP120, as reported above.

6. Factors Involved in hENT1 Regulation and Gemcitabine Activity

6.1. Genetics: Mutations and Polymorphisms

Structure and function studies have reported that hENT-1 transmembrane domains (TMDs 3–6) might be involved in interaction of nucleosides with the transporter [69]. Based on that, SenGupta et al. [70] explored the role of point mutations on glycine 179 and glycine 184 located in transmembrane domain five (TMD 5), using a GFP-tagged hENT-1 in a yeast nucleoside transporter assay system. As a result, substitution of glycine 179 with leucine, valine, or cysteine caused the lack of transporter activity without affecting its targeting to the plasma membrane. On the other hand, mutation of glycine 179 to alanine or serine influenced neither the activity of hENT-1 nor its targeting to plasma membrane. Hence, it could conceivably be

suggested that glycine 179 may have an indirect but vital role in the permeation pathway of hENT-1.

Single nucleotide polymorphisms (SNPs) have been mentioned to affect the gene expression of hENT-1 and thus influencing gemcitabine clinical efficacy [71,72]. Three SNPs were confirmed in the proximal promoter of hENT-1 by Myers et al. [72]: −1345C > G, −1050G > A, and −706G > C. Higher expression levels were observed for two haplotypes (CGC, CAG) when cloning the four naturally occurring haplotypes (CGG, CAG, CGC, GAG) into a Luciferase expression system. Individuals with such haplotypes presented increased hENT-1 expression in comparison to those with normal haplotypes.

The distribution of variants in genes involved in gemcitabine pharmacology and their association with non-small lung cancer was further characterized by Soo et al. [73]. Their results revealed that the non-synonymous variant CNT1 + 1561 G > A is correlated with increased uptake of gemcitabine and hematologic toxicity. However, as the study was limited by the small sample size, larger studies are needed to validate these findings.

6.2. Epigenetics and microRNAs

6.2.1. Epigenetics

The expression of drug transporters, drug metabolizing enzymes, and nuclear receptors, is under epigenetic control affecting the regulation of various genes and response to chemotherapeutic drugs [74]. The cellular levels of three histone modifications (H3K4me2, H3K9me2, H3K18ac), were examined by tissue microarrays from two cohorts with PDAC patients by Manuyakom and collaborators. Low H3K4me2 and H3K9me2 levels were associated with worse overall and disease-free survival (Adjusted HR: 1.48 and 1.44, respectively) [75]. Later, methylation of lysine H3K9 was extensively studied by Candelaria, et al. [76]. More specifically, they exposed CaLo cells to increasing concentrations of gemcitabine which eventually became resistant. This state was accompanied by down regulation of hENT-1. To determine whether gemcitabine resistance was associated with gene silencing induced by increased histone deacetylase activity, they performed ChIP assays, which finally showed a decrease in H3 and H4 acetylation at the hENT-1 promoter. They proposed that this mechanism could silence the expression of hENT-1 and therefore lead to gemcitabine resistance in cervical cancer cell lines.

6.2.2. microRNAs

In the recent years it has become clear that protein expression levels can be regulated by microRNAs. microRNAs are small non-coding RNAs, of 19 to 25 nucleotides, that interact with the mRNA of coding genes, directing their post-translational repression. They are known to influence various cellular processes such as cell proliferation and cell death, mainly through negative regulation of gene expression [77]. In pancreatic cancer, several miRNAs have been reported to be aberrantly expressed, including miR-34 [78], miR-21, miR-155, miR-221, and miR-222 [79]. The regulation of nucleoside transporters by microRNAs is still poorly understood. Theoretically microRNAs could target the mRNA of nucleoside transporters, down-regulating their expression levels.

Using a collection of databases of microRNA-gene interactions ("multimir" R package), 175 miRNAs emerged as miRNA potentially targeting hENT-1 (**Table S2**). Of note, four of these microRNAs are overexpressed in PDAC as reported in **Table 1**.

Table 1. MicroRNAs potentially influencing hENT1 expression.

microRNA	microRNA acc	Experiment Type	Database Source	Comments	Reference
hsa-miR-196a-3p	MIMAT0004562	PAR-CLIP	mirtarbase	Up-regulated in exosomes of PDAC's serum	[67]
hsa-miR-221-5p	MIMAT0004568	Degradome sequencing	tarbase	Up-regulated in PDAC cancer stem cells	[70]
hsa-miR-23b-3p	MIMAT0000418	Degradome sequencing	tarbase	Up-regulated in exosomes of PDAC's serum and correlated to CA19–9	[68]
hsa-miR-155-5p	MIMAT0000646	Degradome sequencing	tarbase	Up-regulated in GEM resistant PDAC cells	[69]

Of note, the presence of tumor-derived microRNAs in both tissues and body fluids offers an opportunity for their potential application as liquid biopsy-based biomarkers, and future studies should evaluate whether emerging circulating microRNAs could be a useful tool for minimally-invasive estimation of hENT-1 levels and prediction of gemcitabine activity in PDAC patients.

MiR-196a-3p is up-regulated in exosomes of pancreatic cancer cell lines and in serum's exosomes of localized PDAC patients (stage I and IIA, $n = 15$) when compared to healthy subjects ($n = 15$) [80]. However, data on outcome or to response to gemcitabine are missing.

Conversely, miR-23b-3p was found to correlate with pancreatic cancer progression in a cohort study in patients with chronic pancreatitis (CP) and pancreatic cancer. Furthermore, in this study, the authors did not provide data on gemcitabine activity, but assessed the expression level of miR-23b-3p in exosomes isolated from patients' serum demonstrating the association of miR-23b-3p to CA19–9 levels [81].

High levels of miR-155–5p were associated to gemcitabine resistance in a study conducted by Mikamori and colleagues [82]. They reported three important findings: (i) long-term exposure to gemcitabine resulted in an increasing level of miR-155–5p; (ii) miR-155–5p levels were positively associated to exosome secretion that promoted gemcitabine resistance; (iii) increasing level of miR-155-5p in PDAC cell while blocking exosome secretion did not induce gemcitabine resistance. This later finding suggests an indirect activity which might be mediated by the miRNA mediated modulation of hENT-1 levels and deserves further research.

Similarly, Zhao and colleagues validated the antagomir for miR-221-5p (a group of miRNA antisense oligonucleotides) to restore chemosensitivity in gemcitabine-resistance cell lines. This miRNA was over-expressed in PDAC cancer-stem-cell subpopulation and regulated some stemness markers such as CDK6, C5ORF41, EFNA1, IRAK3, KLF12, MAPK10, NRP1, SMAD7, SOCS6 and ZBTB41 [83].

However, more research needs to be performed to determine the prognostic and/or predictive characteristics of both tissue and circulating microRNAs regarding their role in nucleoside transport.

6.3. Tumour Microenvironment

6.3.1. Hypoxia

PDAC is characterized by a unique desmoplastic stroma and by the presence of an intense fibro-inflammatory reaction, known as desmoplastic reaction (DR). DR causes the continuous deposition of extracellular matrix components, including collagen type I and III, hyaluronic acid, and fibronectin, by activated pancreatic stellate cells (PSCs) [84]. As a result of increased intratumoral pressure and the subsequent compression of tumor vasculature, tumor cells experience hypoxia and metabolic stress [85]. Koong et al. were the first to observe hypoxia in PDAC [86], reporting areas of pancreatic carcinoma tissues with median pO_2 levels of 0-5.3 mmHg. In contrast, normal tissues had a median pO_2 level of 24 to 92.7 mmHg. Later, Buchler and collaborators [87] showed that hypoxia-inducible factor 1 (HIF-1), an important regulator of cellular response to hypoxia, is activated in PDAC in response to low oxygen conditions. High levels of HIF-1α promote angiogenesis via increased VEGF expression [87], hence promoting PDAC proliferation and metastatic potential [88]. Based on this rationale, clinical trials using drugs targeting angiogenesis have been conducted. However, despite promising preliminary results, anti-angiogenic drugs demonstrated low efficacy in PDAC. Low drug delivery due to vasculature collapse and poor tumor perfusion might explain, at least in part, the modest effectiveness of anti-angiogenic drugs (e.g., bevacizumab) in PDAC [89].

Remarkably, hypoxia also influences nucleoside transporters, as described by Eltzschig and collaborators [90]. Using *in vitro* and *in vivo* models of extracellular adenosine signalling, it was shown that hENT-1 and hENT-2 gene expression and function are negatively regulated by HIF-1α.

In particular, hENT-1 and hENT-2 are involved in the passage of adenosine through the endothelial membrane acting as bi-directional channels in normoxic conditions. In contrast, under hypoxic conditions the adenosine movement is unidirectional but predominantly inward because the extracellular adenosine concentration is much higher than the intracellular. Therefore, the repression of NTs induced by hypoxia causes an extracellular increase of adenosine concentration and signaling effects. Additionally, HIF-α forms a heterodimer with HIF-β during hypoxia which also causes the nuclear translocation of HIF-1 and the binding to the promoter of hypoxia-responsive element of hENT-1 (**Figure 4**). Thus, a downregulation of hENT1 occurs and consequently a decrease of adenosine uptake. These mechanisms represent a transcriptional pathway to limit the inflammatory response and to ensure the integrity of the vascular barrier during hypoxic conditions [91].

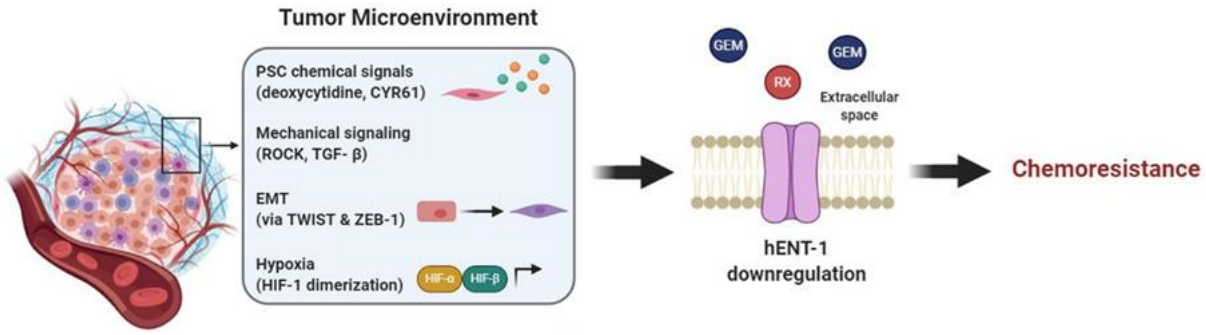

Figure 4. Tumor microenvironment affects hENT-1 expression causing chemoresistance. Components of the TME trigger PDAC hENT-1 downregulation and decreased activity. In order: PSCs secrete chemical factors, such as CYR61 and deoxycytidine, which downregulate hENT-1 and compete with drug metabolizing enzymes, respectively; mechanical signaling, such as TGF-β and ROCK, and EMT, triggered by TWIST and ZEB-1, also contribute to hENT-1 decreased activity; hypoxia induces HIF-α/HIF-B heterodimerization, which activates transcription of hypoxia-related genes further decreasing hENT-1 expression. Together these factors cause chemoresistance to drugs which are taken up via hENT-1 (e.g., gemcitabine (GEM) and RX-3117).

6.3.2. Mechanobiology

PDAC stroma, which accounts for the majority of tumor mass, is involved in resistance to gemcitabine, and a recent publication by Dalin et al. suggested a role of PSCs through an indirect influence on hENT-1. In this case the stroma compartment does not directly affect hENT-1 expression, but most likely bypasses it by producing elevated amounts of deoxycytidine. High deoxycytidine levels are taken up by PDAC cells, via hENT-1, and compete with gemcitabine's intracellular pool for phosphorylation and activation of the drug, therefore causing resistance [92]. Although further investigations are necessary [93] these promising preliminary results suggest that hENT-1 activity is indirectly influenced by the tumor stroma and that this interaction has a relevant role for PDAC gemcitabine chemoresistance.

Mechanical cues and hENT-1 are also intertwined, playing an additional role in PDAC EMT and resistance to gemcitabine. EMT is characterized by cellular physical changes, e.g., viscoelastic and stiffness properties modulation [94]. Notably, hENT-1 has been reported as a regulator of cellular mechanical properties, by means of EMT induction. Indeed, knockdown of hENT-1 was shown to induce cell elongation, stiffness reduction, increased migration potential and expression of EMT markers [95]. Consistently, recent studies reported that gemcitabine resistant cells, which had hENT-1 downregulation, were accompanied by an EMT phenotype. Additionally, these studies highlighted that the EMT was triggered by TWIST and

ZEB-1 transcription factors. By inhibiting TWIST and ZEB-1 the cells had an increased hENT-1 expression and reversed EMT phenotype, increasing gemcitabine efficacy [96,97]. These interesting results suggest that reversing cellular mechanical changes, i.e., EMT, could at least in part contrast the phenomenon of gemcitabine resistance relying on hENT-1.

Lastly, mechanical signaling, that is signaling induced by or causing mechanical stress is also involved in hENT-1 regulation. In lung cancer, hENT-1 together with ROCK1-Rho A—kinases involved in actin organization, cell contractility and motility—are regulated by miR-26b. miR-26b mimic indeed is responsible for downregulation of the aforementioned proteins, leading to reduced tumor invasion and migration [98]. The exact mechanism behind hENT-1-ROCK interaction and whether this is also valid in PDAC has yet to be confirmed. Nevertheless, since both hENT-1 and ROCK are generally overexpressed in PDAC it is not a surprise that they are involved in the aggressive behavior and chemoresistance of this tumor type. Moreover, experimental data showed that in PDAC at least one mechanical signaling pathway is responsible for hENT-1 regulation. PSCs in the tumor microenvironment are a source of cysteine-rich angiogenic inducer 61 (CYR61) and this is due to upregulated TGF-β signaling, a pathway involved in mechanical signaling, ECM remodeling and motility. Hesler et al. reported that this high concentration of CYR61 affected PDAC cells by downregulating hENT-1, therefore causing gemcitabine chemoresistance [99].

In conclusion, emerging evidence highlight the interaction of hENT-1 with tumor stroma and mechanical signaling, yet more evidence has to be obtained to have potential new targets to overcome pancreatic cancer progression and chemoresistance (**Figure 4**).

7. Discussion

The increasing use of a non-gemcitabine-based therapeutic option (FOLFIRINOX), both in the palliative and adjuvant setting, should prompt further development of predictive biomarkers for gemcitabine-based regimens. The validation of these biomarkers may indeed pave the road to the selection of patients who are likely to receive a benefit from gemcitabine-based adjuvant chemotherapy, and therefore has an immediate clinical relevance.

Remarkably, results obtained in several studies demonstrate that hENT-1 expression level is predictive of gemcitabine benefit, but only when assessed with the 10D7G2 mouse clone. Nevertheless, since this clone is not commercially available, this approach is not clinically-feasible and other strategies for the evaluation of hENT-1 status should be investigated. The

quantification of hENT-1 mRNA levels could represent an alternative technique to IHC for the clinical evaluation of hENT-1 status in PDAC patients. A predictive role for hENT-1 mRNA expression in the treatment of PDAC with gemcitabine was previously reported in PDAC laser-microdissected specimens [42]. However, Raffenne and collaborators found that the threshold required to achieve statistical significance was higher with qRT-PCR compared with IHC, probably because no laser-microdissection was performed, leading to heavy microenvironment contamination and falsely decreased mRNA level [3]. In this regard, future studies involving laser microdissection techniques and larger cohorts of patients are required to validate the analysis of mRNA as a potential strategy for the clinical assessment of hENT-1 status.

Several additional factors are involved in cancer cell sensitivity to gemcitabine. For instance, the expression level and activity of gemcitabine-activating enzymes such as deoxycytidine kinase (dCK), and inactivating enzymes such as cytidine deaminase (CDA) and nucleotidase (NT5C1A/NT5C3) may provide a rational explanation for the discrepancy between high hENT-1 level and the poor response to gemcitabine-based chemotherapies [46]. In this regard, the study of more complex gemcitabine sensitivity/resistant signatures using both preclinical models and clinical samples, as well as novel technologies is warranted. For instance, a recent study on proteomics in gemcitabine resistant PANC1 cells and xenografts did not show a significant increase ($p = 0.29$) of hENT-1 expression in the resistant cells, though the fold-change was 1.5, while revealing that proteins associated with gemcitabine resistance are correlated with microtubule regulation [100]. Notably, this data provides an explanation as to why the combination of gemcitabine with nab-paclitaxel is effective in PDAC patients. However, hENT-1 is trafficked to the plasma membrane in association with microtubules suggesting a potential correlation with this system [101]. These findings are extremely interesting because another main challenge for the use of hENT-1 as a clinical biomarker is its validation in patients undergoing treatments with combination of gemcitabine and nab-paclitaxel and not only with gemcitabine monotherapy.

The role played by different cancer cell subpopulations, such as CSCs, as well as non-cancerous cells within the TME, in gemcitabine efficacy represents another critical issue, requiring further elucidation. Of note, recent studies showed that extracellular vesicles (EVs) can confer resistance to gemcitabine by miR-155-mediated suppression of dCK, which catalyzes the rate-limiting reaction in gemcitabine activation [102]. Future studies should investigate whether miRNAs affecting hENT-1 expression could also be involved in the transfer of a resistant phenotype through EVs.

The reliability of a predictive biomarker is assessed through the analysis of sensitivity, specificity, and positive and negative predictive values. However, these parameters need extensive validation studies and quality assessment prior to approval and application in clinical setting. Therefore, the expression levels of hENT-1 should be evaluated within trials testing therapeutic strategies/drugs than can bypass hENT-1 mediated gemcitabine resistance. For instance, NUC-1031 (Acelarin®, NuCana, Edinburgh, UK), which is the first anti-cancer ProTide to enter the clinic, is a phosphoramidate modification of gemcitabine designed to overcome several mechanisms affecting gemcitabine efficacy. According to pre-clinical data, the increased hydrophobicity of NUC-1031 allows it to enter the cells bypassing the hENT-1 transporter [6], similarly to the previously tested lipid-drug conjugate CO-101 (CP-4126) [103,104]. However, the trials on CO-101 resulted in a disappointing failure in PDAC patients. In particular, in a randomized prospective study in patients with untreated metastatic PDAC, CO-101 was not superior to gemcitabine in the low tumor hENT-1 (assessed with the SP120 antibody) and an adverse effect profile similar to gemcitabine [5] was found, such as recently described for NUC-1031. Of note, hENT-1 is not the only potential mechanism mediating the favorable effect of NUC-1031, since this drug does not require the phosphorylation to difluorodeoxycytidine monophosphate (dFdCMP) by dCK and it preserves higher concentrations of the active triphosphate metabolite (dFdCTP) than gemcitabine at equimolar doses inside the tumor cells [6,105]. However, in the future trials on this drug in PDAC patients, standardized IHC technique for the detection of hENT-1 would be essential to overcome the criticisms about the previous CO-101 trial.

Several studies support the association between no or low hENT-1 expression in tumors and poor response to gemcitabine in other cancers, including bladder, biliary tract, and lung cancers. Thus, the validation of hENT-1 standardized IHC techniques would be useful also for other cancer types. For instance, Matsumura and colleagues evaluated the predictive potential of hENT-1 expression in patients with metastatic bladder cancer treated with gemcitabine-cisplatin-based combination chemotherapy. The IHC results showed that hENT-1 was localized in the cytoplasm of bladder tumor cells, and patients with high hENT-1 expression levels had a significantly longer median survival (17.3 months) compared to patients with lower levels (11.6 months) [106]. Similar results were observed by the IHC analysis of hENT-1 in a panel of patients with advanced Biliary Tract Cancer (BTC). Moreover, this study suggested that hENT-1 mediates the intracellular transport not only of gemcitabine but also of capecitabine, because a subpopulation of BTC patients treated with these two drugs showed a correlation between hENT-1 and OS [107]. Finally, Oguri and colleagues evaluated the hENT-1 expression

in non-small cell lung cancer (NSCLC) patients who received gemcitabine-containing chemotherapy, showing that the absence of hENT-1 expression may be useful to predict resistance to gemcitabine-containing chemotherapy in NSCLC [108]. Of note this study showed that the protein expression of hENT-1 in a panel of cell lines with acquired resistance to different drugs, including gemcitabine, cisplatin and paclitaxel, was similar to the expression levels in their respective parental cells. These results suggest that hENT-1 is important in inherent resistance, but does not have a role in acquired resistance. Moreover, this transporter might still be used as potential predictive biomarker of gemcitabine efficacy also after pretreatment with other drugs, such as after neoadjuvant regimens, which are gaining momentum in the multidisciplinary treatment of even potentially resectable PDAC [109].

8. Conclusions

In the present article, we explored the contradictory data related to the predictive value of hENT-1 for gemcitabine activity in PDAC. We also considered the issues related to commercial and not commercial antibodies for IHC and laser-microdissected specimens for PCR analysis. Finally, we discussed the potential impact of different biological mechanisms on hENT-1 regulation, supporting the need of integrating additional (tissue and circulating) biomarkers and further exploring the uncertainty regarding the clinical significance within larger prospective trials using standardized methodologies.

The emergence of “omics” technologies (i.e., genomics, transcriptomics, proteomics, and metabolomics) has encouraged the discovery of new biomarkers. However, the identification of solid and reproducible molecular markers is amongst the biggest challenges in personalized cancer medicine. Therefore, in the present article, we have also reported molecular mechanisms influencing the expression and activity of hENT-1, because we reckon that the integration of existing molecular knowledge should help to adjust for clinical data heterogeneity and limitation.

Last but not least, as reported in the tale of Ali Baba, in order to discover the secret of the cave, he was at right place at right time, suggesting that a relentless pursuit of the goals will lead to achieving success. Thus, persistent and appropriate studies are needed in order to validate effective biomarkers and will hopefully guide the selection of the best (sequence of) anticancer therapy in PDAC patients.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/12/11/3206/s1, Table S1: Clinicopathological characteristics of PDAC patients evaluated for hENT-1 mRNA levels. Table S2: List of MicroRNA Targeting hENT-1. Figure S1: Chemical structures of the drugs transported by nucleoside transporters. Figure S2: Studies evaluating hENT-1 expression levels in pancreatic tumors and normal specimens. Figure S3: Stromal and tumor makers for accurate dissection.

Author Contributions: F.P. and O.R. were the principal investigators and take primary responsibility for the paper; G.M., A.G. and E.G. wrote additional paragraphs and prepared the table and figures, B.P., S.C., D.S.K.L. and D.C. provided essential material and participated in the research design; G.M., F.P., I.G. and A.E.F. checked the tables and figures the paper; G.J.P., A.E.F., I.G. and E.G. edited the paper. All authors have read and agreed to the published version of the manuscript.

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Supplementary Materials Chapter 2

"Open Sesame?": Biomarker Status of Influencing the Human Equilibrative Nucleoside Transporter-1 and Molecular Mechanisms Influencing its Expression and Activity in the Uptake and Cytotoxicity of Gemcitabine in Pancreatic Cancer

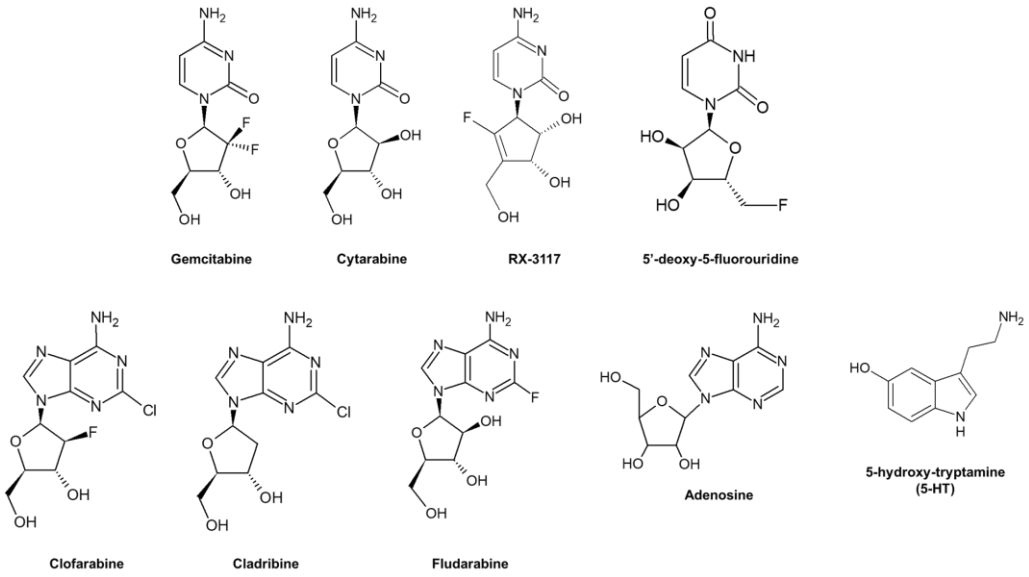
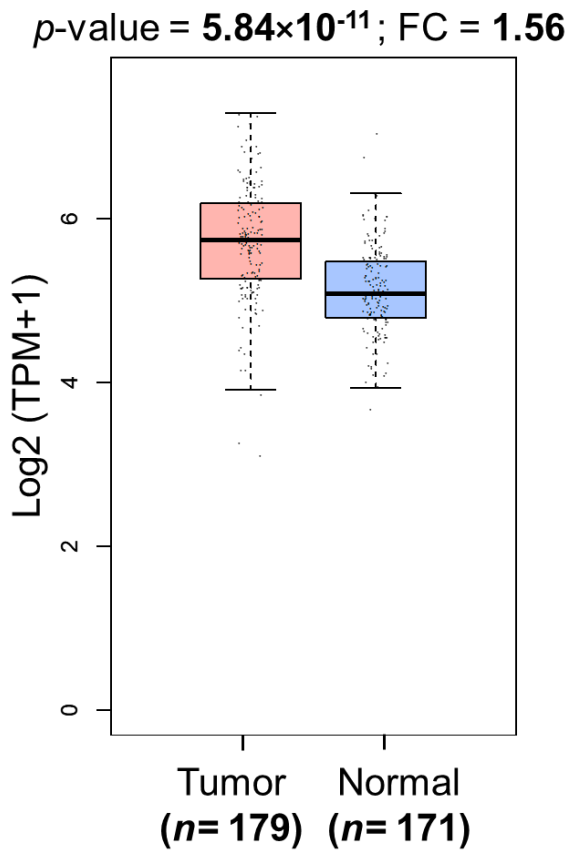


Figure S1. Chemical structures of the drugs transported by nucleoside transporters**.**

A



B Studies on tumor and non-tumor matched samples reporting hENT-1 expression

Study	Analysis type	Database source	N samples	Reference
Mao *et al.* 2017	RNA-seq	paper suppl. info: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5522086/	10	[64]
Zhang G. *et al.* 2012	microarray	GSE28735	45	[65]
Song *et al.* 2018	nanoLC MS/MS	paper suppl. info: https://www.sciencedirect.com/science/article/pii/S1936523318300597	3	[66]

Figure S2. Studies evaluating hENT-1 expression levels in pancreatic tumors and normal specimens. The nucleoside transporter hENT-1 is overexpressed in different tumor types, including PDAC analyzing the RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from the TCGA and the GTEx projects (http://gepia.cancer-pku.cn/detail.php?gene=SLC29A1) (panel **A**). However, the analysis of similarly matched transcriptomics and proteomics public datasets did not show a significance difference in hENT-1 expression levels (panel **B**).

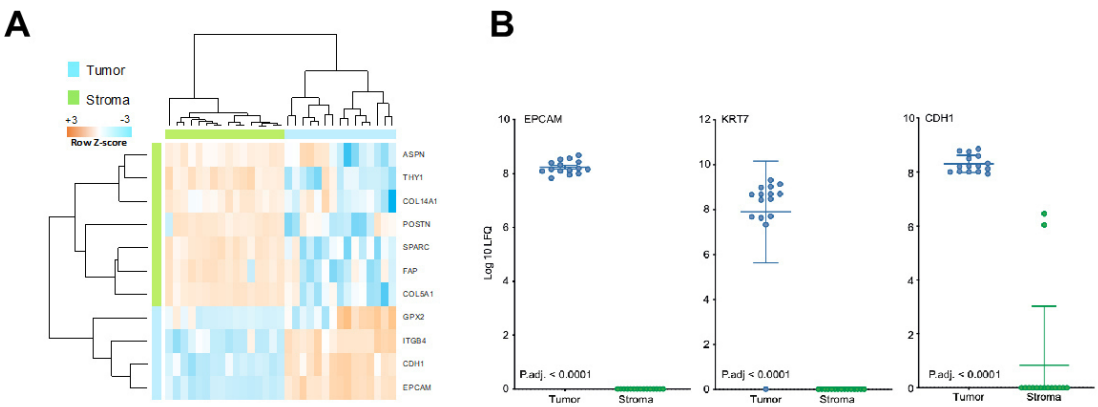


Figure S3. Stromal and tumor compartment dissection was confirmed by evaluating the protein expression of epithelial and stromal markers (panel **A**). Moreover, protein expression of specific tumor markers (EPCAM, KRT7 and CDH1) in matched stromal samples ranged from zero to very low levels, indicating a minimal stromal contamination (panel **B**).

Table S1. Clinicopathological Characteristics of PDAC Patients Evaluated for hENT-1 mRNA Levels. The expression of hENT-1 was evaluated by quantitative PCR, as described previously [1].

Age—years	
Mean (± SD)	65 (± 5)
Sex—No. (%)	
Male	12 (54.5)
Female	10 (45.5)
Stage *—No. (%)	
II	11 (50)
III	11 (50)
Grading (%)	
G1-G2§	9 (40.9)
G3	13 (59.1)

Notes: *AJCC Cancer Staging Manual, 7th Edition; §WHO grading system 2007; Abbreviations: PDAC = pancreatic ductal adenocarcinoma, No. = number of patients.

Table S2. List of MicroRNA targeting hENT-1. *Can be found in excel.*

Reference

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Chapter 3

New Imidazo[2,1-*b*][1,3,4]Thiadiazole Derivatives Inhibit FAK Phosphorylation and Potentiate the Antiproliferative Effects of Gemcitabine Through Modulation of the Human Equilibrative Nucleoside Transporter-1 in Peritoneal Mesothelioma

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Abstract

Background/Aim: A new class of imidazo[2,1-*b*][1,3,4]thiadiazole compounds have recently been evaluated as inhibitors of phosphorylation of focal adhesion kinase (FAK) in pancreatic cancer. FAK is overexpressed in mesothelioma and has recently emerged as an interesting target for the treatment of this disease.

Materials and Methods: Ten imidazo[2,1-*b*][1,3,4]thiadiazole compounds characterized by indole bicycle and a thiophene ring, were evaluated for their cytotoxic activity in two primary cell cultures of peritoneal mesothelioma, MesoII and STO cells.

Results: Compounds 1a and 1b showed promising antitumor activity with IC_{50} values in the range of 0.59 to 2.81 μM in both cell lines growing as monolayers or as spheroids. Their antiproliferative and antimigratory activity was associated with inhibition of phospho-FAK, as detected by a specific ELISA assay in STO cells. Interestingly, these compounds potentiated the antiproliferative activity of gemcitabine, and these results might be explained by the increase in the mRNA expression of the key gemcitabine transporter human equilibrative nucleoside transporter-1 (hENT-1).

Conclusion: These promising results support further studies on new imidazo[2,1-*b*][1,3,4]thiadiazole compounds as well as on the role of both FAK and hENT-1 modulation in order to develop new drug combinations for peritoneal mesothelioma.

Keywords: Mesothelioma, imidazo[2,1-*b*][1,3,4]thiadiazole compounds, FAK, gemcitabine, human equilibrative nucleoside transporter-1

Malignant mesothelioma refers to a rare but aggressive tumor derived from mesothelial cells. They form a monolayer that covers the body's serous cavities and whose main function is to provide a protective membrane for the lung (pleural), the intestine (peritoneum), the heart (pericardium) and the *tunica vaginalis*. The thorax and abdominal cavity are the primary sites for the development of cancer, with a rate of 80-90% and 10-15%, respectively [1].

Diffuse malignant peritoneal mesothelioma (DMPM) is difficult to diagnose, both clinically and histologically, and is characterized by a dismal prognosis. Most patients benefit from a multimodal treatment that includes the combination of surgery and chemotherapy. In particular, the standard of care consists in cytoreductive surgery (CRS) combined with hyperthermic intraperitoneal chemotherapy (HIPEC) [2-4]. However, many patients still suffer from disease recurrence, and new therapeutic options to implement in the current surgical and HIPEC procedures are warranted.

Though all mesotheliomas originate in serous membranes, the efficacy of conventional chemotherapy varies per location [5]. Like many other solid tumors, mesotheliomas develop as a result of different molecular aberrations. To understand these events, research is directed towards: first, to identify new molecules with antitumor activity and second, to assess the activity of compounds already known for their mechanism of action and used for the treatment of various diseases alone or in combination with other drugs. Recently, we reported the antitumor activity of a new class of imidazo[2,1-*b*][1,3,4]thiadiazole compounds on pancreatic ductal adenocarcinoma, highlighting their ability to reduce FAK phosphorylation on tyrosine residue (Y-397) [6]. Moreover, we previously observed good results with combinations of new drugs with the antimetabolite gemcitabine in preclinical models of DMPM [7]. Encouraged by these findings as well as by studies supporting 'drug repositioning' in drug discovery [8], we studied the antitumor activity of ten imidazo[2,1-*b*][1,3,4]thiadiazole compounds (**Figure 1**) on two primary cultures of DMPM cells, growing as monolayers or spheroids and evaluated the potential mechanisms underlying the pharmacological interaction with gemcitabine.

MATERIALS AND METHODS

Drugs and chemicals

The imidazo[2,1-*b*][1,3,4]thiadiazole compounds were synthesized, and dissolved in DMSO, as described previously [6]. Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12), fetal bovine serum (FBS), penicillin (50 IU/ml) and streptomycin (50 μg/ml)

were from Gibco (Gaithersburg, MD, USA). All other chemicals were from Sigma (Zwijndrecht, the Netherlands). Gemcitabine was a gift from Eli-Lilly.

Cell cultures

Human DMPM primary cultures (STO and MesoII) were derived from patients who underwent surgery [9]. The cells were maintained in F-12 for less than 20 passages, supplemented with 10% heat-inactivated-FBS, 1% penicillin/streptomycin, and routinely tested for mycoplasma.

Inhibition of cell growth

The cell growth inhibitory effect of imidazo[2,1-*b*][1,3,4]thiadiazoles was evaluated by the Sulforhodamine-B (SRB) assay, as described previously [10]. Cells were seeded into 96-well flat-bottom plates (5×10^3 cells in 100 µl/well). After 24 h, cells were treated with eight different concentrations of the compounds (from 0.3 to 40 µM) for 72 h. Thereafter, cells were fixed with 25 µl of 50% cold trichloroacetic acid and kept for at least 60 minutes at 4°C. The plates were washed gently with deionized water, dried at room temperature (RT) overnight and stained with 50 µl of 0.4% SRB solution in 1% acetic acid for 15 minutes at RT. The excess of SRB was removed on dry tissues and the plates were washed with a 1% acetic acid solution and dried at RT overnight. Finally, the SRB was dissolved in 150 µl of tris(hydroxymethyl)aminomethane solution pH 8.8 (TRIS base), and the optical density (OD) was measured at wavelenghts of 490 nm and 540 nm. The cell growth inhibition was calculated as the percentage of the OD drug-treated cells *versus* the OD of vehicle-treated cells ("negative control") (corrected for OD before drug addiction, "day-0").

Half maximal inhibitory concentration (IC_{50}) values were calculated with GraphPad Prism 7 (GraphPad, San Diego, CA, USA). In the combination studies with gemcitabine, we used the most promising compounds at their IC_{50} concentrations and gemcitabine at its IC_{25} concentration.

Wound healing assay

The *in vitro* wound-healing assay was performed as previously described [11]. MesoII and STO cells were seeded in 96-well flat-bottom plates at the density of 5×10^4 cells/well and the

confluent layer was scratched with a pin-tool. Thereafter, the medium was replaced in the control wells with only medium or with medium containing the compounds of interest. Wound closure was monitored by phase-contrast microscopy using the Leica-DMI300B microscope and pictures were captured immediately after scratch (T=0), and after 4, 8 and 20 h. Results were analyzed with the Scratch-Assay 6.2 software (Digital Cell Imaging Labs, Keerbergen, Belgium).

Spheroids assay

MesoII and STO spheroids were created as reported previously [12]. Cells were seeded at a density of 7×10^4 cells/well for MesoII and 5×10^4 cells/well for STO, in cell repellent U-bottom plates (Greiner, Kremsmünster, Austria). After three days the spheroids were treated with **1a** and **1b** at IC_{50} and $5\times IC_{50}$ concentrations. Pictures were taken every two days after replacing the medium of the control wells or adding medium with compounds to the experimental wells, and the experiment lasted seventeen days. The reduction in size of spheroids was monitored by phase-contrast microscopy and pictures were analysed with ImageJ (NIH, Bethesda, MD, USA), as described previously [13].

Enzyme-linked immunosorbent assay (ELISA) to evaluate FAK

To investigate whether our imidazothiadiazole compounds were able to reduce FAK phosphorylation at tyrosine residue 397 (FAK [pY397]) in the DMPM, we performed a quantitative analysis using a specific ELISA, as described [6]. This assay was carried out on lysates of cells treated with compounds **1a** and **1b** at $5\times IC_{50}$s concentrations for 2 h.

Quantitative-PCR

Total RNA was extracted and reverse transcribed from cells treated with **1a** and **1b** at $5\times IC_{50}$ concentrations for 24 h. The resulting cDNA was amplified by quantitative-PCR with the ABIPRISM-7500 instrument (Applied Biosystems, Foster City, CA, USA) using specific hENT-1 and primers, as previously described [12].

Statistics

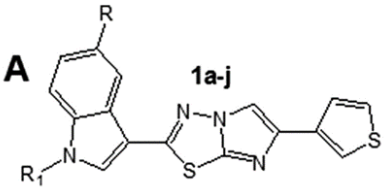
All experiments were performed in triplicate and repeated at least three times. Data were expressed as mean values±SEM and were analysed by Student's *t*-test or ANOVA followed by the Tukey's multiple comparison, setting the level of significance at $p<0.05$.

RESULTS

Antiproliferative activity

The effect of ten imidazo[2,1-*b*][1,3,4]thiadiazole compounds was evaluated on MesoII and STO cells, by the SRB assay. Only four out of the ten compounds (**1a**, **b**, **g** and **h**), showed more than 50% inhibition of growth at 10 μM and were explored in more detail. **Figure 1A** summarizes their IC_{50} values, ranging from 0.59 to 5.9 μM, with the lowest IC_{50} in STO cells (**Figure 1B**), while in MesoII cells these compounds were less effective (**Figure 1C**). The results obtained with the compounds **1a** and **1b**, prompted us to investigate their cytotoxic activity on three-dimensional (3D) models.

A



Compounds	R	R^1	IC_{50} values [μM] reported as Means ± SEM of 3 separate experiments	
			MesoII	STO
1a	H	H	2.81 ± 0.39	0.81 ± 0.35
1b	H	CH_3	2.58 ± 0.12	0.59 ± 0.11
1c	Br	CH_3	>10	>10
1d	Cl	H	>10	>10
1e	Cl	CH_3	>10	>10
1f	F	CH_3	>10	>10
1g	Br	H	5.9 ± 1.53	3.65 ± 1.03
1h	F	H	3.4 ± 0.29	1.14 ± 0.11
1i	OCH_3	H	>10	>10
1j	OCH_3	CH_3	>10	>10

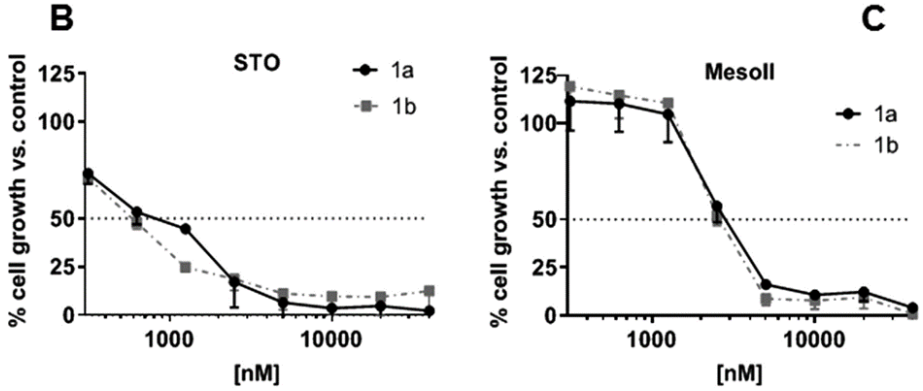


Figure 1. Antiproliferative activity of compounds 1a-j against diffuse malignant peritoneal mesothelioma cells, MesoII and STO. **(A)** Chemical backbone structure of compounds 1a-j with the list of the chemical structure of the R and R^1 substituents for each compound and the IC_{50} values in μM of each compound against the DMPM cell lines. **(B, C)** Representative growth curves of STO **(B)** and MesoII **(C)** cells after 72 h of exposure to 1a or 1b. Points, mean values obtained from one representative experiment; bars, SEM.

Volume reduction of MesoII- and STO-derived tumor spheres

Earlier studies reported that the drug activity found in the two-dimensional monolayers is different from that in 3D cell cultures [14], as the 3D model offers a more realistic representation of the tumor microenvironment, including the physical and mechanical properties, oxygen, pH and nutrients gradients, as well as drug transport [15]. Therefore, we evaluated the ability of compounds **1a** and **1b** to affect the size of spheroids of MesoII and STO cells. As shown in **Figure 2**, the spheroids decreased significantly in size over time in both cell lines. Notably, after 17 days of treatment, we found about 2-fold reduction, compared to the untreated spheroids.

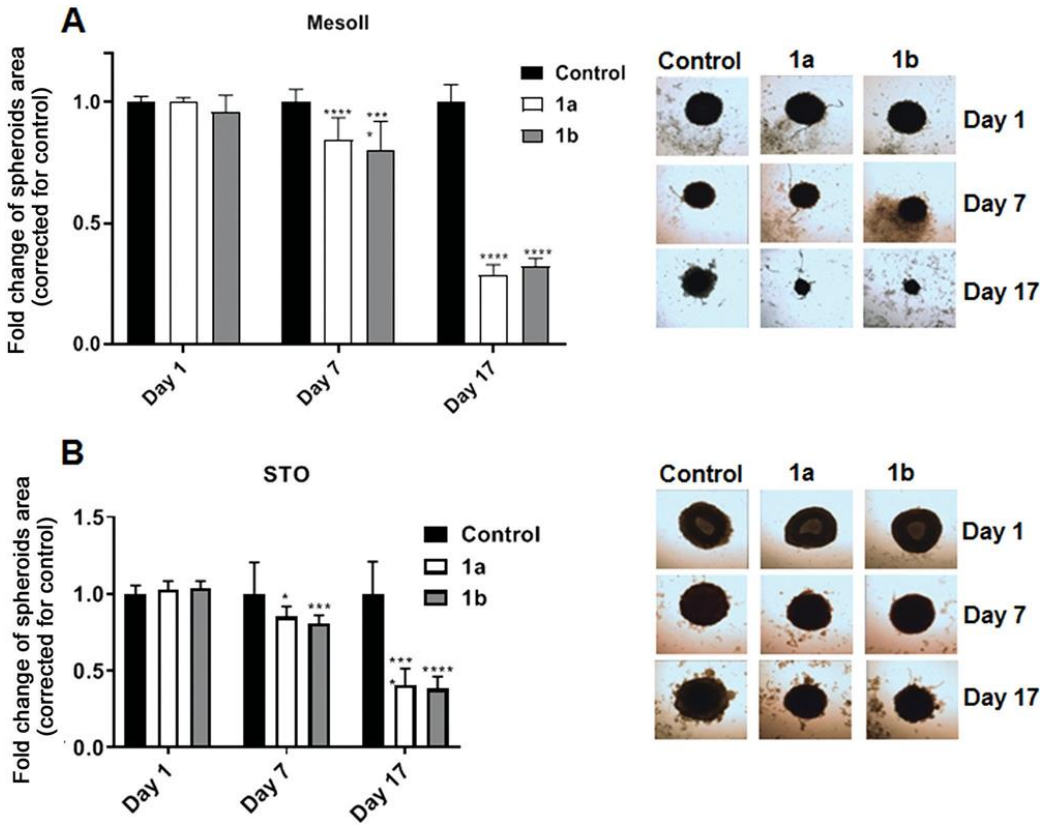


Figure 2. Size reduction of **(A)** MesoII and **(B)** STO spheroids treated with compounds 1a or 1b at 5×IC_{50} concentrations. *(Left plot)* Fold-change compared to control, on day 1, 7 and 17. *(Right pictures)* representative images of spheroids, taken with an automated phase-contrast microscope on day 1 of treatment (Original magnification 5×), and after 7 and 17 days. All *p*-values were determined by Two-way ANOVA followed by Tukey's multiple comparisons test, *p<0.05, ***p<0.001, ****p<0.0001. These values were obtained by taking the mean value of at least ten different spheroids into account.

Compounds 1a and 1b inhibited cell migration and phospho-FAK in STO cells

Secondary lesions that originate from DMPM primary site are very uncommon. However, localized and/or regional metastasis with the involvement of lymph nodes have been observed [16-18]. Furthermore, the spread of tumor cells to form new metastatic *loci* on distant organs has been reported; particularly, the pancreas and the kidneys are the main organs involved, whereas the lung, the heart and the brain are less commonly affected [19, 20]. The interesting antiproliferative activity of compounds **1a** and **1b**, prompted us to investigate their anti-migratory activity by wound-healing assay in the STO cells, which were selected because of their higher sensitivity and a replication time well-above 24 h. In these cells, we observed a reduction of migration rates by 25.8% and 20%, after 20 h from the treatment, compared to control (set at 100%) (**Figure 3A**). Statistical analyses revealed that the reduction of migration in STO cells treated with compound **1a** was significant, compared to the untreated control cells. Parallel ELISA studies revealed that both compounds **1a** and **1b** significantly reduced the phosphorylation of FAK (**Figure 3B**).

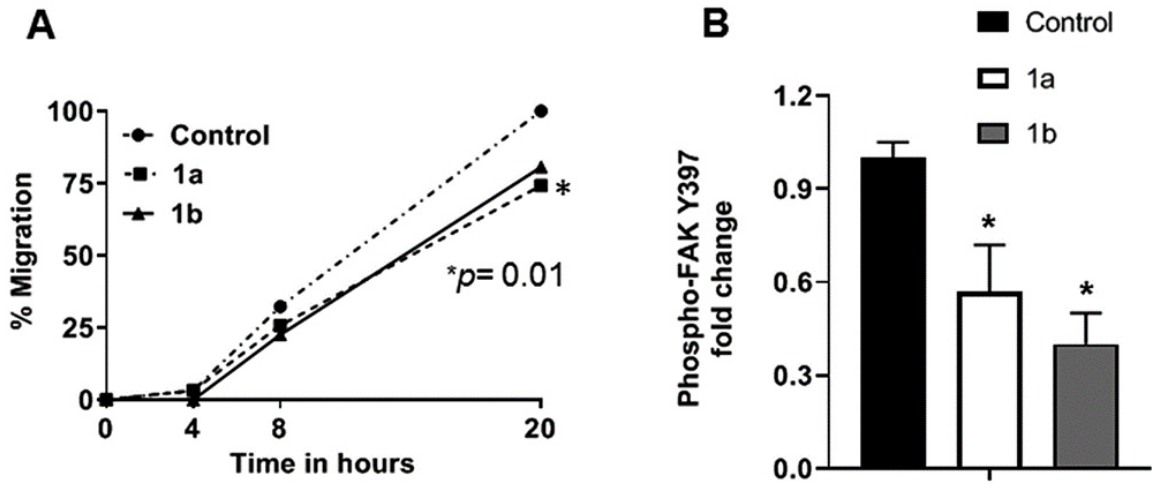


Figure 3. **(A)** Modulation of the migration rate of STO cells treated for the indicated times with the compounds 1a and 1b at concentrations of 5×IC_{50}. Mean values were obtained from the means of at least

six different scratch areas. SEM were always below 10%. **(B)** Inhibition of FAK phosphorylation at tyrosine residue 397 by compounds 1a and 1b. **p*<0.05.

Compounds 1a and 1b increased hENT-1 expression and gemcitabine cytotoxicity

Since previous data showed synergistic effects of gemcitabine with the new anticancer agents in mesothelioma cells [7, 21] we tested whether the addition of compounds **1a** and **1b** at their IC_{50} concentrations would increase the antiproliferative effects of gemcitabine. These experiments were performed in STO cells because of the strongest antiproliferative and antimigratory effects. Interestingly, the combination of both compounds **1a** and **1b** at their IC_{50} concentration with gemcitabine at its IC_{25} concentration led to a significant reduction in cell growth, which reached values around 10% compared to untreated cells (**Figure 4A**).

Finally, to investigate the molecular mechanisms underlying the interaction of gemcitabine with compounds **1a** and **1b**, we measured the modulation of the gene expression of hENT-1, which is a key determinant of gemcitabine transport. Both compounds increased hENT-1 expression significantly (**Figure 4B**), suggesting its potential role in the increased activity of gemcitabine in combination with compounds **1a** and **1b**.

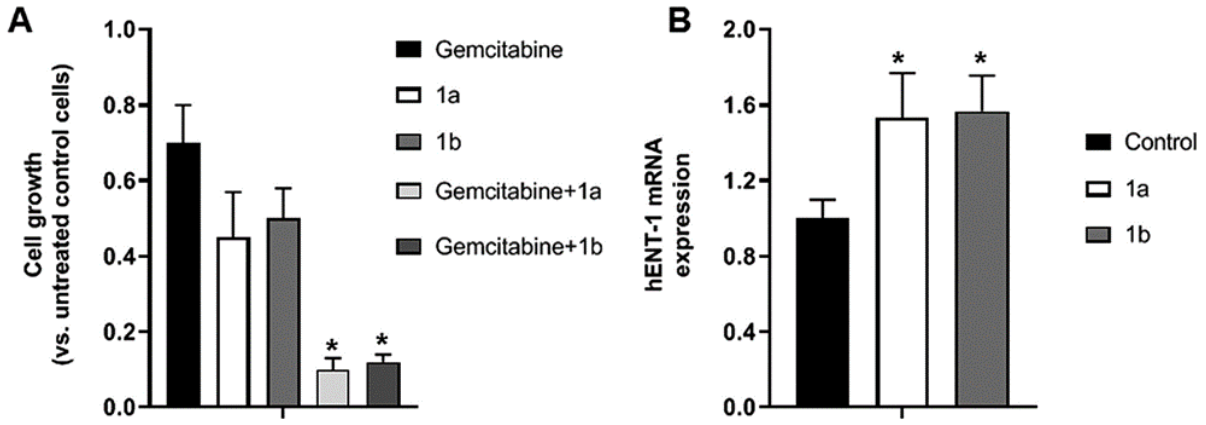


Figure 4. The combination of compounds 1a and 1b with gemcitabine led to a significant reduction in cell growth and increased hENT-1 expression. **(A)** Effect of the combination of gemcitabine, at its IC_{25} concentration, with the compounds 1a or 1b, at their IC_{50} concentrations, on the growth of STO cells. **(B)** Modulation of hENT-1 mRNA levels in STO cells. Expression was determined with quantitative-PCR by normalization with the GAPDH housekeeping gene, and the values are expressed in arbitrary units, as described previously [12]. Columns, mean values obtained from triplicate experiments. Bars, SEM; **p*<0.05.

DISCUSSION

Multimodal treatment including the combination of surgery and chemotherapy represents the standard of care for the treatment of patients with peritoneal mesothelioma; indeed, unlike radiotherapy, palliative surgery combined with chemotherapy showed a longer survival rate of patients, as demonstrated in a retrospective study conducted on Finnish patients with DMPM between 1 January 2000 and 31 December 2012 [22].

Ten imidazo[2,1-*b*][1,3,4]thiadiazole compounds, which inhibited FAK protein expression in the treatment of pancreatic cancer [6], were tested for their antiproliferative activity on two primary cell cultures of diffuse malignant peritoneal mesothelioma, namely MesoII and STO. Four compounds **1a, b, g** and **h** showed promising antitumor activity with IC_{50}s in the range from 0.59 to 5.9 μM. In particular, the compounds **1a** and **1b** showed the lowest IC_{50} in both cell lines. Similar results were observed in spheroids, inhibiting their area by approximately 2-fold compared to the controls. These are very interesting results since spheroids of mesothelioma cells are resistant to different treatments, including conventional chemotherapeutic drugs.

Moreover, the lowest IC_{50} values were also associated with the ability of compounds **1a** and **1b** to reduce cell migration of STO cells by 25.8% and 20%, respectively. These results gave more insight in the mechanism of action and led us to investigate the ability of these compounds to inhibit FAK phosphorylation, as reported previously [7]. Remarkably, both compounds were able to reduce the phosphorylation of FAK, which is a potential target in mesothelioma [23].

Moreover, these compounds potentiated the activity of gemcitabine and we might hypothesize that this effect is due to the increased mRNA expression of hENT-1, which has been associated with gemcitabine activity in different cancer cell types [24]. Of note, a previous study showed that inhibition of hENT-1-mediated transport may result from p42/44 MAPK activation in HUVEC cells after short periods of hypoxia [25]. Therefore, we hypothesize that the inhibition of FAK might cause an inhibition of its downstream target MAPK and this might in turn lead to an increase in the expression of hENT-1.

In conclusion, our novel findings should prompt further studies on imidazo[2,1-*b*][1,3,4]thiadiazole compounds as well as on the role of the modulation of FAK and hENT-1 for the rational development of new drug combinations in DMPM.

Conflicts of Interest

The Authors have no conflicts of interest to disclose in relation to this study.

Authors' Contributions

GLP, OR and CP performed chemical synthesis, experimental work and wrote the manuscript. SZ, SMC, DC, BP, and BEH, assisted with experimental work. AC, NZ, and GC provided essential material and helped to revise the manuscript. PD, GJP and EG were responsible for experimental design and helped to write the manuscript.

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Notes

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Chapter 4

Interrelationship between miRNA and splicing factors in pancreatic ductal adenocarcinoma

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers because of diagnosis at late stage and inherent/acquired chemoresistance. Recent advances in genomic profiling and biology of this disease have not yet been translated to a relevant improvement in terms of disease management and patient's survival. However, new possibilities for treatment may emerge from studies on key epigenetic factors. Deregulation of microRNA (miRNA) dependent gene expression and mRNA splicing are epigenetic processes that modulate the protein repertoire at the transcriptional level. These processes affect all aspects of PDAC pathogenesis and have great potential to unravel new therapeutic targets and/or biomarkers. Remarkably, several studies showed that they actually interact with each other in influencing PDAC progression. Some splicing factors directly interact with specific miRNAs and either facilitate or inhibit their expression, such as Rbfox2, which cleaves the well-known oncogenic miRNA miR-21. Conversely, miR-15a-5p and miR-25-3p significantly downregulate the splicing factor hnRNPA1 which acts also as a tumour suppressor gene and is involved in processing of miR-18a, which in turn, is a negative regulator of KRAS expression. Therefore, this review describes the interaction between splicing and miRNA, as well as bioinformatic tools to explore the effect of splicing modulation towards miRNA profiles, in order to exploit this interplay for the development of innovative treatments. Targeting aberrant splicing and deregulated miRNA, alone or in combination, may hopefully provide novel therapeutic approaches to fight the complex biology and the common treatment recalcitrance of PDAC.

Keywords: PDAC; splicing deregulation; miRNA; interaction; splicing modulation

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers worldwide [1,2]. Although its incidence and prevalence are lower than several other cancers, such as lung, head and neck, colorectal and breast cancer, the mortality rate almost matches the incidence rate [3]. Furthermore, early detection is difficult in PDAC because of the lack of accurate biomarkers [3,4]. Current biomarkers still have low sensitivity and specificity and, therefore, are not suitable as screening methods [5]. The problem becomes even more complicated as this type of cancer is hard to treat or to manage. PDAC has a rapid progression and only 20% of newly diagnosed patients are eligible for surgical resection, the most effective treatment option for this disease [1]. In addition, PDAC is highly resistant to any therapy upfront and tends also to rebound rapidly after first response/stabilization [3].

Recent studies provided new insights into the underlying mechanism of PDAC evolution, suggesting that, in addition to the specific mutational load, including the concurrent mutations in KRAS, TP53, p16, and DPC4, and to the tumour and stromal heterogeneity, microRNAs (miRNAs) and splicing deregulation could be major players in directing tumorigenesis and tumour evolution [6–9].

MiRNAs have been studied extensively and there is a wide array of functionally relevant miRNAs that play an important role in PDACs [7]. For instance, miR-21 has been implicated in carcinogenesis and tumour progression in many types of solid cancers, including PDAC [10,11]. It may be a strong biomarker for early detection, but lacks specificity as it is upregulated in many types of solid cancers and other diseases [12]. Another well-known miRNA in PDAC is miR-155 that is important for inflammation and metastatic processes, while miR-121 and miR-21 contribute to chemoresistance [13].

Contrary to miRNA, splicing deregulation is a relatively new concept in cancer progression, especially in solid cancers [8]. The discovery of mutations in genes encoding splicing factors increased the interests in this topic, prompting several recent studies. Splicing deregulation due to mutation of splicing factors is especially important in non-solid cancers, such as leukaemia, but their overexpression is widely observed also in solid cancers [14,15]. Despite a low mutational rate of splicing factor 3B subunit 1 (SF3B1) in mesothelioma, its overexpression is diffusely prevalent and significantly associated with increased malignant characteristics and patients survival [16]. In lung cancer, Serine and Arginine Rich Splicing Factor 2 (SRSF2) has been implicated in patient's survival and tumour progression while Serine/arginine-rich splicing factor 7 (SRSF7) is highly expressed in chemoresistant colorectal cancer [17].

Although miRNA and splicing deregulation draw extensive interest among cancer scientists, the possibility of their interaction emerged only recently. Rodriguez-Aguayo et al. [18] showed that miR-15a-5p and miR-25-3p significantly downregulate tumour suppressor gene splicing factor hnRNPA1 which is a key player in the processing of miR-18a, the negative regulator of KRAS expression. On the other hand, splicing factors themselves could affect miRNA expression as shown by Chen et al., who reported a inhibition of miR-21 by Rbfox-2 [18]. These evidences indicate that there could be a relationship between miRNA and gene splicing, which might influence different oncogenic processes and provide new crucial concepts to be exploited towards more efficient cancer treatments. Therefore, this review discusses this intricate relationship, with a focus on the basic concept of miRNA and splicing deregulation, and on how miRNA and splicing factors affect each other. Additionally, this review concludes with consideration on how to exploit this relationship for future strategies in PDAC management and treatment.

Biology of miRNA and its relevance in PDAC

By definition, a miRNA is a non-coding RNA which typically consists of 19-24 nucleotides with a pivotal role in post-transcriptional regulation [7]. MiRNAs were first discovered in *Caenorhabditis elegans* and thousands more have been identified in all kinds of organisms [19]. In humans, around 2500 different miRNAs have been identified along with their sequence, transcript annotation, and their location within the genome [20]. MiRNAs biogenesis begins with their transcription by RNA polymerase II which generates long precursors known as primary miRNA transcripts (pri-miRNAs) (**Figure S1**). This transcript has a wide variability in their length, but it typically ranges between 100 and 1000 base pairs. The pri-miRNAs are then processed by Drosha-DGCR8 ribonuclease complex in the nucleus, producing 70-100 nucleotides long intermediate pre-miRNAs with hairpin shape. Then, this intermediate will be transported into the cytoplasm by Exportin-5 and RanGTP6 where it will further be processed by the endoribonuclease Dicer (also known as endoribonuclease RNase III). Dicer cleaves the terminal loop and produces mature double stranded 19-24 nts RNA. One strand of this mature miRNA will be degraded, while the other is incorporated into Argonaute heteromultimer protein to form highly specialized RNA Induced Silencing Complex (RISC) [21]. The seed sequence in miRNA leads to the complex towards target mRNA by means of RNA-RNA base pairing. The target mRNAs can either be degraded or translationally repressed, which depends on whether the seed sequence matches the target sequence within the mRNA. Complete base

pairing between the seed sequences and target mRNAs usually leads to degradation of mRNA while incomplete pairing results in translational suppression [22]. However, this phenomenon also underlies the reason why miRNA is so versatile. It has been shown that a single miRNA can indeed control mRNAs from several genes, while a single mRNA can be targeted by several different miRNAs [23]. In addition, it is estimated that 60% of all genes are controlled by miRNAs which further underscores their importance in the control of gene expression [24]. MiRNAs are also involved in important cellular processes, such as cell proliferation, metabolism, differentiation, apoptosis, and cell signalling [25].

Extensive studies on miRNA gave insight in its important role in many types of cancer, including PDAC. Cancer cells are known to have aberrant miRNA expression: where tumour suppressors' miRNAs are often downregulated, miRNAs promoting carcinogenesis or tumour progression is usually over-expressed [25]. This deregulation often leads to aberrant cellular processes, such as uncontrolled mitosis, apoptosis, drug resistance, invasion, metastasis and angiogenesis [7,13,14,25]. The first evidence of miRNA dysregulation in PDAC was reported by Poy et al. [26] through a profiling study using mouse pancreas. Follow-up studies using different types of samples confirmed the initial finding that PDAC has a specific miRNA expression profile [17,27,28]. In particular, a study comparing PDAC tissue with adjacent normal pancreatic tissue reported a total of 158 miRNAs differentially expressed [29]. Fifty-one miRNAs were upregulated including miR-196, miR-200a, miR-21, and miR-27a, while 107 miRNAs were downregulated, with miR-96, miR-200, and miR-217 being the most significant [29].

In **Table 1** we report an overview of the clinical evidence on miRNA deregulation in PDAC as well as the most interesting preclinical findings on candidate miRNAs emerging from these studies. Schultz and colleagues [28] reported that 43 miRNAs were upregulated while 41 were downregulated when comparing paraffin-embedded PDAC tissue samples with normal ones. The expression of key miRNAs was also different between resectable and non-resectable PDAC patients as reported by Calatayud et al. [30]. Around 22 miRNAs were differentially expressed with miR-64, miR-136, miR-196, miR-492, and miR-622 being the most significant. A separate study by Papaconstantinou et al. showed a different but also some consistent results [31]; miR-21, miR-155, miR-205, miR-221, and miR-222 were consistently overexpressed while miR-31, miR-122, miR-146, and miR-375 were downregulated in PDAC samples. Preclinical and functional analysis showed that miR-21 and miR-155 are the only two miRNAs that were consistently overexpressed and linked to cancer progression [17]. However, several profiling studies showed consistent overexpression of miR-21, miR-155, and miR-221, while

miR-34 and miR-145 were downregulated [32]. Remarkably, miR-21 and miR-155 obtained from pancreatic tissue could differentiate malignant from benign lesions with high accuracy [33] and have both been proven to be able to differentiate between pancreatic intra-epithelial neoplasia (PanIN) with normal pancreatic [34,35].

Table 1. MiRNAs aberrantly expressed in PDAC samples.

miRNA	Expression level (N)		Tissue type	N Samples	Method	Reference
	Normal Tissue	PDAC				
miR-21	Low	High	FFPE	Pancreatic cancer (n=165); Normal Pancreas (n=35)	RT-PCR	[30]
	Low	High	Fresh Tissue	Pancreatic Cancer (n=88); Normal Pancreas (n=98)	qRT-PCR	[31]
	Low	High	Biopsy	Metastatic (n=31); Non-metastatic (n=50)	q-PCR	[43]
	Low	High	FFPE	Adjuvant therapy (n=52); Non-adjuvant therapy (n=27)	qRT-PCR	[45]
	Low	High	FFPE	Normal pancreas (n=12); Pancreatitis (n=45); PDAC (n=80)	In-situ Hybridization	[171]
	Low	High	Plasma	PDAC (n=32); Normal healthy (n=30)	qRT-PCR	[9]
miR-155	Low	High	Fresh Tissue	Pancreatic cancer (n=88); Normal pancreas (n=98)	qRT-PCR	[31]
	Low	High	FFPE	Pancreatic lesions (n=55)	qRT-PCR	[33]

	Low	High	Plasma	Pancreatic cancer (n=40); Normal pancreas (n=25)	q-PCR	[172]
	Low (N=80)	High (N=80)	FFPE	Pancreatic cancer (n=80); Normal pancreas (n=80)	In-situ Hybridization	[173]
miR-205	Low (N=98)	High (N=88)	Fresh Tissue	Pancreatic cancer (n=88); Normal pancreas (n=98)	qRT-PCR	[30]
	Low (N=17)	High (N=34)	FFPE	Pancreatic cancer (n=34); Normal pancreas (n=17)	qRT-PCR	[173]
	Low (N=17)	High (N=47)	Serum	Pancreatic cancer (n=47); Normal pancreas (n=17)	qRT-PCR	[173]
miR-205	Low (N=5)	High (N=5)	Fresh Tissue	Pancreatic cancer (n=5); Normal pancreas (n=5)	MiRNA Microarray	[174]
miR-196b, miR-217, miR-411, miR-198	High (N=28)	Low (N=170)	FFPE	Pancreatic cancer (n=170); Normal pancreas (n=28)	RT-PCR	[28]
miR-210, miR-222	Low (N=98)	High (N=88)	Fresh Tissue	Pancreatic cancer (n=88); Normal pancreas (n=98)	qRT-PCR	[31]
miR-375	High (N=35)	Low (N=165)	FFPE	Pancreatic cancer (n=165); Normal pancreas (n=35)	RT-PCR	[30]
miR-377	High (N=30)	Low (N=30)	Snap-frozen sample	Pancreatic cancer (n=30); Normal adjacent tissue (n=30)	qRT-PCR	[175,176]
miR-127	High (N=42)	Low (N=42)	Snap-frozen sample	Pancreatic cancer (n=42); Normal adjacent tissue (n=42)	qRT-PCR	[158]

miR-181d	Low (N=37)	High (N=37)	Snap-frozen sample	Pancreatic cancer (n=37); Normal adjacent tissue (n=37)	qRT-PCR	[177]
miR-107	Low (N=80)	High (N=100)	Plasma	Pancreatic cancer (n=100); Normal pancreas (n=80)	qRT-PCR	[178]
miR-1290	Low (N=267)	High (N=167)	Plasma	Pancreatic cancer (n=167); Normal pancreas (n=267)	ddPCR	[179]

Clinically, miRNAs have been assessed to differentiate benign and malignant lesions, determining the stage of PDAC, as a biomarker for metastasis, and to predict the therapeutic outcome, as illustrated in **Figure 1**. The potential role of specific miRNA in early diagnostics is particularly important in PDAC since screening modalities are very limited and the disease tends to be diagnosed in advanced stage which has a high risk of metastasis and low therapeutic response [3]. Furthermore, miR-155 is increasingly expressed as early as PanIN-1 while miR-21 is beginning to be abundant in PanIN-2 and -3, suggesting that miR-21 is more suitable for advanced disease marker. Another microRNA that has increased expression in advanced PanIN (PanIN-3) is miR-196b while the expressions of miR-133, miR-185, miR-200c, and miR-34c are higher in low-grade neoplasia [36].

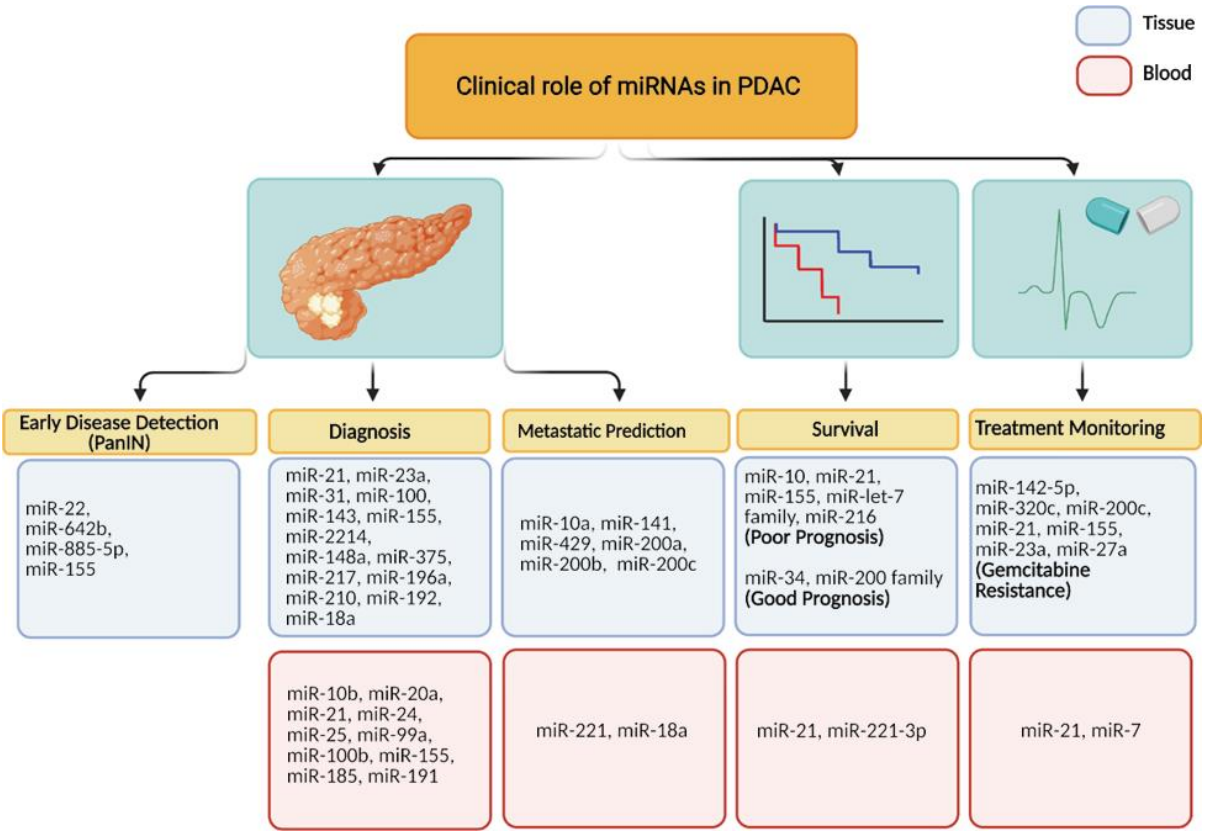

Figure 1. Clinical role of miRNAs in early PDAC detection, diagnosis, metastatic prediction, survival and treatment monitoring. The scheme shows different miRNAs, tissue and blood-derived, which could serve as biomarkers for discriminating the different stages of the disease, as well as for early diagnosis and metastasis prediction. Furthermore, some miRNAs could be associated with prognosis and monitoring of PDAC patients.

The expression levels of miR-21 and miR-155 were also up-regulated also in invasive intraductal papillary mucinous neoplasms (IPMNs) of the pancreas, compared to non-invasive IPMNs, as well as in non-invasive IPMNs compared with normal tissues. Conversely, miR-101 levels were significantly higher in non-invasive IPMNs and normal tissues compared with invasive IPMNs. Furthermore, miR-21 emerged as an independent prognostic biomarker in invasive IPMNs [37].

The information on circulating miRNAs in preneoplastic lesions is limited while circulating miR-10b, miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-100b, miR-155, miR-185, and miR-191 showed a high accuracy in differentiating PDAC with pancreatitis and normal pancreas. Regarding metastasis, both miR-21 and miR-155 have been proven to actively play important role in inducing cell migration and metastasis [38]. However, other microRNA, such as miR-10b, miR-200b, and miR-200c, miR-218, miR-194, and miR-429 are also emerging

biomarkers for metastasis in PDAC [39]. In blood, only miR-221 and miR-18a had been evaluated to be significantly associated with metastasis [40,41]. Unsurprisingly, all of the aforementioned miRNAs are also associated with patient's prognosis. The tissue expression of miR-10, miR-21, miR-155, miR-let-7 family, and miR-216 are known to predict an unfavourable prognosis, while expression of miR-34 and miR-200 family correlated with a better prognosis. For circulating miRNAs, only miR-21 and miR-221-3p have been consistently proven as indicators of poor prognosis. Despite the urgency in detecting metastasis in pancreatic cancer, there are only limited number of studies regarding the predictive value of microRNA in metastasis which limit their clinical validation and application.

MiR-21 in combination with miR-23a and miR-27a was also associated with more malignant PDAC phenotype and shorter overall survival after tumour resection [42]. In addition, expression of miR-21 determines PDAC response against gemcitabine with a lower expression correlating with better treatment outcome [43–45]. However, tumoural miR-21 overexpression emerged in a pooled meta-analysis assessing miRNAs as prognostic biomarkers in PDAC, independent of other clinicopathologic factors, including adjuvant chemotherapy use [44].

Differential expression of miRNA is not only observed in tissue samples but also in blood. For instance, miR-18a, miR-21, miR-22, miR-24, miR-25, miR-27a, miR-155, miR-185, miR-191, miR-196a, miR-642b and miR-885-5p were significantly upregulated in PDAC patients' blood plasma [17,41]. Most importantly, blood-based miRNA profiling not only helps to differentiate PDAC patients from healthy individuals but also from other conditions that usually are considered as differential diagnosis such as acute or chronic pancreatitis and benign pancreatic tumours [32]. Another recent study showed that miR-486-5p and miR-938 could differentiate patients with PDAC from those who were healthy or had pancreatitis [46]. Additionally, circulating miRNAs can also be used as therapeutic biomarker. For example, downregulation of miR-181a-5p after FOLFIRINOX therapy correlates with better survival in PDAC but not in those who were treated with nab-paclitaxel and gemcitabine [47].

MiRNA dysregulation drives tumorigenesis through a close link with cellular signalling and metabolism. Several studies demonstrated that miR-21 enhanced PI3K/AKT and MAPK/ERK signalling that promote cell proliferation [48–50]. MiR-21 also suppresses the expression of phosphatase and tensin homolog (PTEN) and programmed cell death protein 4 (PDCD4) which facilitate cellular invasion induced by TGF-β signalling [51,52]. MiR-21 is also known to activate pancreatic stellate cells and cancer associated fibroblasts (CAF) to actively produce extracellular matrix proteins which contribute to its dense stroma [53,54]. On the other hand,

miR-155 suppresses suppressor of cytokine signalling 1 (SOCS1) and MLH1 expression within cancer cells and enhance cancer invasion [55,56]. MiR-155 knock down is known to reduce membrane-type 1 matrix metalloproteinase (MT1-MMP), EGFR, and K-Ras expression in PDAC cell lines which led to lower proliferation rates and colony formation [57]. The functionality of other miRNAs has also been studied but seems less clear compared to miR-21 and miR-155.

Splicing factors and alternative splicing in PDAC

During malignant transformation, cancer cells experience aberrant splicing processes which result from mutations at the splice sites, mutations of splicing factors, and/or over/under expression of certain splicing factors [14]. Splicing deregulation could suppress protein expression by directing the inappropriately spliced mRNAs towards non-sense mediated decay or producing more active splice variants of oncogenic proteins [8,9,14]. The clinical application and implication of this process have also been studied both as therapeutic targets and biomarkers [8,15,58] and only recently received attention in PDAC.

Normal splicing is a post-transcriptional process where the introns are removed from primary transcripts, leaving only exons in the final transcript [14] (**Figure 2a**). In alternative splicing, exons can also be removed and different transcripts can be produced from a single gene [8]. RNA splicing occurs in the nucleus and is facilitated by splicing factors (SFs) which will assemble themselves in a sequential manner during the splicing process. The typical process is initiated by binding of a small nuclear ribonucleoprotein (snRNPs) to the primary transcript. Initially, U1 snRNP binds to the 5' splice site (5'SS) while U2 snRNP binds to a branch point at the other end of the intron. U1 and U2 then attract more snRNPs (U5, U4/U6) which then form a complete spliceosome and bend the intronic section, forming a lariat-like structure in which the 5' side of the intron is ligated to the branch point. Then, U4 is removed while the 5'SS site is hydrolysed [15]. In this process, the two extremities of the exons are held together by the spliceosome complex. Consecutively, the 3'SS is cut and the two exons are ligated, forming the final transcript that will be transported to the cytoplasm for translation [8,15,58].

Splicing is regulated by a wide array of splicing factors which bind to specific sites in primary transcripts [15,59]. The binding sites of those factors can be located in an exon or intron and can induce or repress the splicing process. The most important splicing factors and their binding sites are presented in **Figure 2b**. Splicing factors act early in the splicing process, facilitating

snRNPs binding to primary transcripts [59]. Typically, SRSF2 binds the exonic splicing enhancer in exons flanking the intron and facilitate U1 and U2 binding. It connects to U1 by 70 K linker protein while its interaction with U2 is much more complex. It interacts with the U2 Small Nuclear RNA Auxiliary Factors U2AF1 and U2AF2, as well as with Zinc Finger CCCH-Type, RNA Binding Motif and Serine/Arginine Rich 2 (ZRSR2) and RNA-binding motif 10 (RBM10) in facilitating U2 binding. Additionally, SF3B1 facilitates U2 binding by interacting with a branch point site [15]. After U1 and U4 dissociate from spliceosome, all of the splicing factors are also dissociated except SF3B1 which firmly binds to U2 and SRSF2.

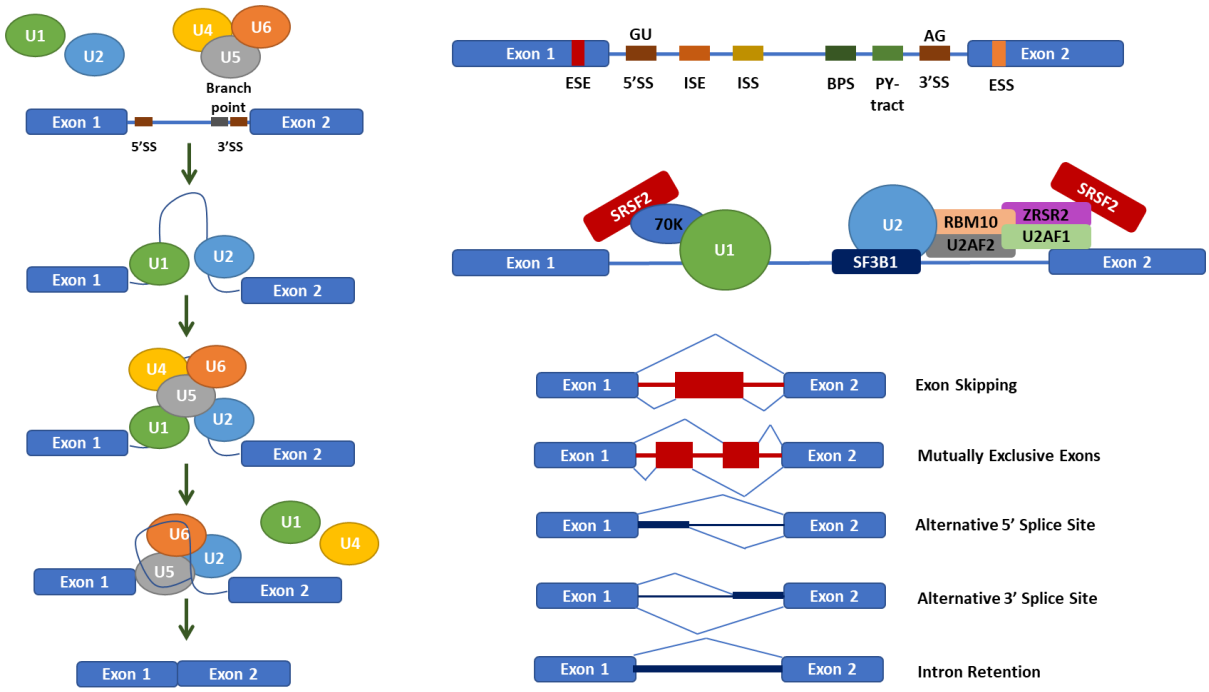

Figure 2. The mechanism of splicing mediated by splicing factors. The splicing is initiated by binding of U1 at 5'SS and U2 at 3'SS, bending the intron segment. Both SFs (U1 and U2) then recruit another SF which induced loop formation, cleaved the intron segment and ligated the exons [14,15]. Splicing is regulated by splicing factors which bind the primary transcript at several regulatory sites. Several essential regulatory sites and splicing factors are presented in the right part. SRSF2 is particularly important in initiating splicing by facilitating U1 and U2 binding to the primary transcript while SF3B1 mediates U2 binding to BPS. After U1 and U4 detached from spliceosome, only SRSF2 and SF3B1 remain in the complex while the other SFs detached [59]. ESE: Exonic splicing enhancer; ESS: Exonic splicing suppressor; ISE and ISS: Intronic splicing enhancer/suppressor; 5'SS and 3'SS: 5' or 3' splice site; BPS: Branch point site; Py-tract: Polypyrimidine-tract.

RNA splicing and alternative splicing are crucial steps in protein expression and the isoforms of the proteins that are expressed by a certain gene are determined by these processes [8]. In cancer, these processes can be altered and this alteration can drive carcinogenesis [60]. In fact, in many types of cancer, splicing factors are either mutated or overexpressed, which strongly indicates an aberrant splicing process in cancer [8,15].

Mutations in splicing factors have been identified in several types of cancer as the driving force of carcinogenesis, most notably in haematologic cancers. SF mutations are detected in 78% of refractory anaemia with ringed sideroblasts and 60% of chronic myelomonocytic leukaemia (CMML) while it only happens in less than 5% in pancreatic, lung, breast, and head and neck cancer [15]. However, despite a lower mutational frequency, several SFs are over/under expressed in solid cancer including PDACs [8,14]. For example, SF3B1 and heterogeneous nuclear ribonucleoprotein K (HNRNPK) are consistently overexpressed in PDAC and are linked to an unfavourable prognosis [61–64]. Several important SFs in PDAC and their functions are summarized in **Table 2**.

Table 2. Splicing factors in PDAC and their biological and clinical effects in preclinical studies.

Splicing Factors	Biological and Clinical Significance	Reference
SRSF1	Upregulated in PDAC	[76,80]
	Upregulated by Myc	[76,80]
	Promote resistance to gemcitabine	[76]
	Promote oncogenic splice variant of Bcl-xs, ΔRON and MCL-1s alternative splicing preferring their oncogenic variant	[77–79]
SRSF6	Increased proliferation and cellular transformation	[72]
	Prognostic factor for PDAC	[75]
SF3B1	Overexpression is associated with poor prognosis	[15,79]
	Mutated in 4% PDAC with mutation associated with better survival	[65]
	Important in branch point regulation and alters the splicing process of several oncogenes and tumor suppressor genes	[8,14]
Rbfox2	Often downregulated as its control cellular proliferation	[97]
	Moderate upregulation in cancer tissue increased invasive potential	[98]
	May specify the mesenchymal tissue-specific splicing profiles both in normal and in cancer tissues	[8]

HnRNPs	Superfamily of RNA-binding proteins	[8]
	hnRNPA2B1 and hnRNPA1 altered Bcl-x alternative splicing and facilitate KRAS expression	[8,112]
	Higher expression associated with poor survival	[8]
HnRNPK	Wide range of effect including alteration in alternative splicing, gene transcription and RNA stability	[8,59,63,106,108, 113]
PTBP1	Altered PKM expression by favouring PKM2 and induces a Warburg effect	[70,124]
	Upregulation of PTBP1 after chronic exposure to gemcitabine, conferring resistance against the drug	[70]

Of note, in PDAC, SF3B1 and U2AF1 are the only known SFs with mutations and occur at a very low frequency [15,65]. However, these mutations are interesting because they can be targeted and induce synthetic lethality [66]. Furthermore, it appeared that PDAC relies on the normal form of SFB31, U2AF1, and RBM10 since patients with these mutations tend to have better prognosis compared to the wild types [9,14,15].

A more frequent form of splicing deregulation in PDAC consists in the overexpression of SFs [8]. Several SFs are upregulated in PDAC such as SF3B1, SRSF1, SRSF6, hnRNPK, Heterogeneous Nuclear Ribonucleoprotein A1 (hnRNPA1), and Polypyrimidine Tract Binding Protein 1 (PTBP1), while Rbfox2 tends to be downregulated. These splicing factors are thought to mediate many of PDACs unique characteristics, such as dense stromal, low immunogenicity, immune avoidance, as well as early metastasis, and invasion [9,64,65]. The pivotal role of splicing deregulation in PDAC was described by Wang et al. [8,58,59,61,64–66] who compared alternative splicing in PDAC to normal pancreatic tissue through Affymetrix exon array.

Alternative splicing tends to occur in genes encoding extracellular matrix (ECM), ECM-receptor interaction, and focal adhesion protein. In addition, pyruvate kinase and acyl-CoA synthetase long-chain family member 5 (ACSL5) were also present, which suggests that alternative splicing may have an impact on tumour metabolism.

Splicing deregulation drives pancreatic carcinogenesis by shifting the expression of pivotal oncogene and tumour suppressor proteins [5,8,14]. A clear example is the shifting of RON isoform expression of the tyrosine kinase receptor recepteur d'origine nantais (RON) [67,68]. Normally, RON has a low expression in normal pancreatic epithelial cells, but its expression increases gradually from low to high grade pancreatic intra-epithelial neoplasia [68]. In PDAC,

it is expressed in 69–96% of cases [68]. However, it is not only its higher expression that makes RON so important in PDAC; RON has indeed also several splicing alterations which lack exon 10, 11, or have 5 + 6 exon skipping [67]. These isoforms are constitutively active and, therefore, have more oncogenic potential compares to native isoform [67].

Another example is Pyruvate Kinase M2 (PKM2) whose expression has been observed in almost all types of tumours [69]. Normally, pancreatic epithelial cells express PKM1 instead of embryonic PKM2. This shift is mediated by PTBP1 which is also overexpressed in PDAC [70]. PTBP1 associates directly to intron 8 of PKM mRNA and induces alternative splicing. Therefore, the higher expression of PKM2 facilitates an oncogenic glycolytic metabolism and increases cancer cell resistance towards genotoxic drugs. This effect was confirmed by a knock-down study where PDAC cell lines with suppressed expression of PTBP1 or PKM2 are much more sensitive to Gemcitabine.

Deregulated splicing factors in PDAC and their interaction with miRNA expression

Several important splicing factor aberrations have been identified in PDACs and, most recently, their association with miRNA expression has been described in different cancer types. Although many known splicing factors are deregulated in cancer, PDAC is relatively unexplored and tends to be limited to SF3B1. Overall, there are four splicing factors which have been studied in more detail and which will be discussed in detail.

SRSF6

SRSF6 is one of the most important splicing factors in PDAC and is often upregulated but not mutated [8,71]. SRSF6 is classified as an oncogenic splicing factor since it enhances cellular proliferation. Jensen et al. [72] reported that SRSF6 overexpression induces excessive keratinocyte hyperplasia in sensitized skin. Cohen-Eliav et al. [73] reported that SRSF6 overexpression increased proliferation rate of immortalized lung epithelial cells, transforming these into malignant cells. In addition, the human protein atlas considers SRSF6 as a potential prognostic marker in renal cancer, liver cancer, and PDAC [74]. Interestingly, and in contrast with the other two above-mentioned cancers, lower SRSF6 expression is correlated with poor prognosis in PDAC.

Keeping with these findings, Li et al. [75] showed that miR-193a-5p downregulates SRSF6, increasing the metastatic potential of PDAC cell lines. Apparently, SRSF6 downregulation was beneficial for cancer cells because it enhanced the invasive properties through the alteration of oxoglutarate dehydrogenase-like and ECM1 protein by alternative splicing. Thus, SRSF6 has a dual, apparently contradictive function since high expression of SRSF6 induces PDAC while a downregulation promotes tumour invasiveness. SRSF6 downregulation probably occurs late in the PDAC evolution, whereas at an early stage of carcinogenesis SRSF6 upregulation is preferred due to its beneficial effect on promoting cellular proliferation and survival. However, further studies in primary cells as well as in patient samples are needed to confirm this hypothesis.

SRSF1

SRSF1 is a well-characterized SR protein in cancer and one of SR proteins that is overexpressed in different cancers, including PDAC [8,59,71]. SRSF1 is a versatile protein, promoting carcinogenesis through several important mechanisms including increased proliferation rate and apoptosis resistance [76]. Furthermore, SRSF1 is a known splicing factor that influences the expression of Bcl-x, RON and MCL-1 isoform expression, changing their anti-apoptotic to pro-mitotic variants in cancer [77–79]. High expression of SRSF1 is induced by the Myc oncogene which is commonly upregulated in many cancers including PDAC [80].

Interestingly, besides its role in processing mRNA splicing, SRSF1 facilitates miRNA processing. SRSF1 is indeed involved in the final cleaving process mediated by Drosha, serving as an auxiliary factor [81]. The miR-7 family depends on SRSF1 for its maturation. However, miR-7 itself would suppress the expression of SRSF1, forming a negative feedback loop. Other miRNAs that depend on SRSF1 include miR-221, miR-222 and miR-17-92 [82]. MiR-221/222 contribute to the progression of PDAC by increasing the expression of MMP-2 and MMP-9, increasing stromal remodelling and the invasive properties of the cancer cells [83]. For miR-17-92 which consists of four members, namely miR-17, miR-18, miR-19, and miR-92, an oncogenic effect was shown [84–87]. In PDAC, miR-19 actually promoted invadopodia and increased the invasiveness [88]. Therefore, it is important to further investigate the interaction between SRSF1 with miR-7, miR-221/222, and miR-17-92 to better understand the feedback loops that exist among them and also to assess why their overexpression favour carcinogenesis instead of tumour suppression.

SF3B1

SF3B1 is a well-known protein with a key role in PDAC [8,14,15]. It has the highest mutation rate among splicing factors and is also often overexpressed in PDAC [15]. Furthermore, this is one of the only three splicing factors that can be inhibited by small-molecule inhibitors so far, making its therapeutic potential higher than other SFs [89].

However, despite the wealth of information regarding the biological role of SF3B1 and its modulation, its relationship with miRNA is poorly understood. Their association has only been studied by Aslan et al. [90] in myelodisplastic syndrome in which they reported that the SF3B1 mutation was associated with global downregulation of tumour suppressor miRNAs from the let-7 family, especially miR-103a and miR-423. However, the mechanism of downregulation was not evaluated and there is still the possibility that this downregulation is not directly related to a SF3B1 mutation but might relate to other signalling aberrations.

Pianigiani et al. [91] demonstrated that there is a relationship between SF3B1 and splice site overlapping miRNAs (SO-miRNA). The precursors of these miRNAs are generated on the intron-exon junctions, from which the name 'splice-site' belongs. SF3B1 knockdown did not affect SO-miRNAs in HeLa and HaCAT cells, but increased the level of 52 SO-miRNA including miR-636, miR-6510-5p, miR-3614-3p, miR-3655, miR-3656, miR-4260, miR-5187-3p, miR-7109-5p, and miR- 8069. Some of these miRNAs are classified as tumour suppressors [92–95]. However, these *in vitro* results cannot be generalized to PDAC and gene silencing using siRNA is different than protein inhibition by a small molecule because there might be a different active site involved in miRNA processing than the inhibited site. Nevertheless, this finding suggests that SF3B1 modulation could have an additional beneficial effect by enhancing tumour suppressor miRNA expression. In addition, when these miRNAs are secreted to the extracellular compartment, they could also serve as biomarker for therapeutic monitoring.

Rbfox2

The RNA-binding Fox (Rbfox) proteins (Rbfox1, Rbfox2 and Rbfox3) constitute an important class of regulators of alternative splicing, and Rbfox2 (RBM9) can influence small and non-coding RNA in PDAC [8,59,96]. This RNA-binding protein is highly conserved in mammals [97], and it is different from Rbfox1 and Rbfox3, whose expression is limited to neuron and muscle cells. Rbfox2 is indeed widely expressed, especially in stem cells, haematopoietic stem cells, and embryos, where it regulates cellular proliferation [8]. In PDAC, Rbfox2 is

downregulated, similar to other types of cancer [96]. These findings seem controversial because Rbfox2 is essential for cancer cell invasion, and the level of Rbfox2 increased moderately after the induction of EMT [98]. However, the same study showed that after the initial induction the levels of Rbfox2 were decreased. Notably, Rbfox2 is also subject to regulation by other proteins, and this might also momentary increase its expression [99]. Moreover, this mechanism might enable cancer cells to exploit the EMT promoting ability while evading excessive tumour suppressing effect by Rbfox2.

Rbfox2 is known to upregulate tumour suppressor miRNA, such as miR-20b and miR-107 while cleaving the oncomiR-21 [18–100]. The regulation of miRNA expression by Rbfox2 is mediated by direct binding to cognate sequences in miRNA or indirectly affects miRNA expression by altering Dicer expression. Of note, mutations in the DICER gene as well as in other components of the miRNA biogenesis pathway are not commonly detected in PDAC, and miRNA upregulation is more common than downregulation [100–102]. Additionally, several studies showed that miRNAs are broadly required for the development and maintenance of pancreatic cell lineages and play a role in carcinogenesis [103–105]. These findings suggest that miRNAs play a pivotal role in pancreatic tumorigenesis, and that loss of function mutations in the miRNA processing machinery are selected against during tumour evolution, but the impact of Dicer in later stages of pancreatic tumorigenesis or progression remains limited.

HnRNPs

Heterogeneous nuclear ribonucleoproteins (HnRNPs) are essential members of the RNA-binding proteins (RBPs) that act as regulators of alternative splicing, particularly, in linking the primary transcript with splicing machinery [106]. Several of its family members have been studied in relation to their role in carcinogenesis [106–108]. In PDAC, HnRNPA2B1 and HnRNPA1 are known for their role in tumour progression by shifting the Bcl-x isoform expression and facilitating KRAS expression, respectively [18,109]. In addition, HnRNPs are prognostic factors in PDAC with a higher expression associated with significantly shorter survival [108].

While there is no direct evidence of HnRNP and miRNA interaction in PDAC, their interaction has been identified in ovarian cancer. Aguayo et al. [18] reported that HnRNPA1 suppresses miR-18a expression in docetaxel-resistant ovarian cancer cell lines. MiR-18a normally suppresses KRAS expression, but its downregulation enhanced KRAS expression and

facilitated resistance to docetaxel [110,111]. MiR-18a has also been investigated in PDAC and elicited the same effect towards KRAS [112]. HnRNPA1 expression was also suppressed by miR-15a-5p and miR-25-3p [18]. These two miRNAs are also known as tumour suppressor miRNAs in PDAC [7]. Therefore, the same molecular mechanism might exist in PDAC, and inhibiting or blocking HnRNPs could be explored as a new way to fight chemoresistance in this disease.

Another member of HnRNPs that was recently investigated regarding its role in PDAC is HnRNPK [6,8,14,106]. Much of the biology of HnRNPKs is still under investigation because these proteins are not only involved in RNA splicing but also in DNA transcription and RNA stability [113]. They are also responsible for the downregulation of some tumour suppressor genes in PDAC [63,64]. Remarkably, HnRNPKs interact with miR-223, an oncomiR that enhances cell proliferation and migration [63]. These effects have been attributed to downregulation to miR-223 targets FBXW7 and PDS5B, two tumour suppressor proteins which inhibit cellular migration and induce apoptosis [63,114]. A similar finding on the importance of miR-223 was also found in pancreatic cancer cells when using the naturally occurring isoflavonic phytoestrogen genistein that inhibited miR-223 expression which in turn enhanced FBXW7 expression [115]. These effects resulted in inhibition of cell growth and induction of apoptosis.

PTBP1

PTBP1 has been investigated for its role in PDAC metabolism [70,108]. The expression of PTBP1 was increased in two Gemcitabine resistant PDAC cell lines (PANC-1 and Pt45P1) where it modulated alternative splicing alteration of PKM, resulting in overexpression of the cancer-related PKM2 isoform, whose high expression also correlated with worse prognosis in PDAC patients [70]. PTBP1 is also considered a prognostic factor and a potential therapeutic target due to its role in enhancing PDAC metabolism [108].

The only proven miRNA that directly interacts with PTBP1 in PDAC is miR-124, which directly downregulates PTBP1 mRNA and shifts PKM isoform expression from PKM2 to PKM1. The importance of miR-124 and PTBP1 interaction was shown by ectopic expression of miR-124 or administration of PTBP1 siRNA which increased sensitivity to gemcitabine and relieved autophagy in gemcitabine resistant PDAC cell lines [116]. However, in PDAC, miR-124 is mostly downregulated which facilitates increased PTBP1 expression, favouring Warburg

effect [117,118]. In neural differentiation, Yeom et al. [119] observed that PTBP1 could repress miR-124 maturation by directly binding to pri-miR-124 and blocked transcript cleavage by DROSHA. Therefore, we can assume that a low expression of miR-124 could also result from increased expression of PTBP1 and this potential feedback loop adds to the complexity of splicing factors-miRNA interaction in cancer.

Another miRNA known to interact with PTBP1 is miR-133b [119]. Although there are no data regarding their interaction in PDAC, miR-133b is downregulated in PDAC and has been considered as a tumour suppressor miRNA based on findings in other cancer types [120–123]. In colorectal cancer, miR-133b silenced PTBP1 expression and inhibited the Warburg effect by promoting the expression of the PKM1 isoform [124]. Due to its low expression in PDAC, miR-133b could also exert a similar effect in PDAC. Despite the limited direct evidence, there is a strong indication of interaction between splicing deregulation and miRNA in PDAC. A summary of relevant splicing factors and the miRNAs that interact with each other as well as their main biological effects is presented in **Table 3**. Remarkably, further studies exploring this field of research are now extremely timely since splicing inhibitors are becoming available as novel anticancer drugs and could offer new therapeutic strategy for PDAC.

Table 3. Relevant splicing factors and miRNAs affecting key aggressive biological features of PDAC in preclinical studies.

Main Effect	Splicing Factor	Associated miRNA	Interaction	Biological Impact	Reference
PROLIFERATION					
	SF3B1	miR-636, miR-6510-5p, miR-3614-3p, miR-3655, miR-3656, miR-4260, miR-5187-3p, miR-7109-5p, miR-8069, miR-155-3p, miR-148a-3p, miR-98-5p, and miR-21-3p	Upregulation miR-636, miR-6510-5p, miR-3614-3p, miR-3655, miR-3656, miR-4260, miR-5187-3p, miR-7109-5p, and miR-8069 Downregulation miR-155-3p, miR-148a-3p, miR-98-5p, and miR-21-3p	Decreased cellular proliferation Enhanced keratinocyte differentiation	[43,91]
	HnRNPs	miR-18a, miR-15a-5p, miR-25-3p	Downregulation of miR-18a by HnRNPA1 Suppression of HnRNPA1 by miR-15a-5p and miR-25-3p	Increased KRAS activation resulted from miR-18a downregulation	[18]
		miR-223	Increased expression of miR-223 by HnRNPK	Increased cancer cell proliferation by FBXW7 and PDS5B suppression	[63]
EPITHELIAL-TO-MESENCHYMAL TRANSITION AND METASTASIS					
	SRSF1	miR-7 family, miR-17, miR-18	Facilitate miR-7 family biosynthesis Increased miR-221/222 expression	Increased invasion and metastasis through MMP-2 and -9 upregulation	[81,83]

SRSF1	miR-19, miR-92	Facilitate miR-17-92 family biosynthesis	Increased invasion and metastasis through MMP-2 and -9 upregulation	[82,83]
SRSF6	miR-193a-5p	SRSF6 downregulation by miR-193a-5p	Facilitate metastasis by alteration in oxoglutarate dehydrogenase-like (OGDHL) and extracellular matrix protein 1 (ECM1) alternative splicing	[75]
Rbfox2	miR-20b, miR-21	Upregulation of miR-20b Suppressing miR-21 expression	Considered as anti-cancer splicing factors; suppressing cellular proliferation at normal tissue	[180]
	miR-107	Upregulation of miR-107	Considered as anti-cancer splicing factors; suppressing cellular proliferation at normal tissue Enhancing EMT in PDAC when moderately increased in PDAC	[181]
TUMOUR METABOLISM				
PTBP1	miR-124	In neuron: PTBP1 suppress miR-124 cleavage (not confirmed in cancer)	Enhanced resistance against gemcitabine	[116,119]

miR-124, miR-133b	PTBP1 downregulation by miR-124 and miR-133b	Altered cancer metabolism favouring Warburg effect by promoting PKM2 expression	[124]

5 miRNA profiling methods

Accurate detection and quantification of miRNAs represent a major challenge due to the small size of miRNAs (approximately 22 nucleotides), the high sequence homology among members of the same family and the low abundance in biofluids. Currently, miRNAs profiling is a growing field of study, although conventional methods for detecting miRNAs still remain the gold-standards used to confirm the results of new detection techniques [125].

Northern blot is a widely used historical method to measure the expression of miRNAs ranging from the primitive miRNA to the mature form. It is based on molecular hybridization and gel electrophoresis and is able to simultaneously determine the size of miRNAs. However, Northern blot has several disadvantages: it is a time-consuming technique, requires large amounts of samples and reagents, with low sensitivity (pM-nM range) and low throughput [126].

Current miRNA detection strategies include reverse transcription-quantitative polymerase chain reaction (RT-qPCR), which is so far the undeniably gold-standard method for routine testing, especially for diagnostic purposes. It is commonly used to detect miRNAs at any stage of maturation, but does not allow the identification of new miRNAs. RT-qPCR is less time-consuming technique than Northern blotting and displays higher sensitivity, specificity and reproducibility than Northern blot. In addition, it converts small miRNA sequences into longer sequences by adding a poly(A) tail (poly(A)-tailed RT-qPCR) or a stem-loop structure (stem-loop RT-qPCR) overcoming the primer design limitation [127]. An innovation is represented by the ddPCR (droplet digital PCR) which offers greater performance, improved sensitivity, and accuracy as it allows for absolute quantification of miRNAs without the need for a reference gene [128].

PCR techniques cannot detect nucleotide sequences in cells and tissue sections, while in situ hybridization (ISH) can visualize miRNAs within cells and can determine the

spatiotemporal expression of miRNAs, elucidating their biological role as well as their pathologic involvement in numerous diseases [129]. This technique is labour intensive and is limited by its low-throughput nature but the recent development of directly labelled fluorescence probes and multiplexed miRNA ISH methods allowed to detect multiple miRNAs per reaction.

Microarray is a hybridization-based method suitable for relative quantification. Locked nucleic acids (LNAs) can be incorporated into capture probes to normalize the melting temperature (Tm) whose variance is related to miRNA GC content [128,130]. The strength of this method is the multiplexed detection of multiple miRNAs in a single reaction, although it cannot discriminate between miRNA variants and has poor sensitivity compared to RNA-seq because it lacks the amplification step. On the other hand, microarray assays are fast, expensive and high-throughput [131].

Finally, Next-Generation Sequencing (NGS) is a highly accurate miRNA profiling technique that can simultaneously measure expression level and sequence changes, as well as detect unknown miRNAs. It should be noted that NGS has the highest multiplexing capability as specific primers are not required for each targeted miRNA detection [125,132]. Drawbacks to NGS include time-consuming for converting a sample into a library for sequencing, expensive analyses due to sophisticated software and qualified personnel for data analysis and it is not a fully automated technique as well [126].

Since multiplexing capability plays a crucial role in miRNAs detection, in addition to the above-mentioned multiplexing approaches, it is worth citing the suspension arrays (i.e., on-particle), which represent promising emerging methods for highly multiplex analysis of complex samples due to the versatility of the encoded microspheres used in conjunction with flow cytometry [133]. Furthermore, Rondelez et al. recently reported an isothermal amplification mechanism for multiplex and digital detection of miRNAs using the rational building of a molecular circuit that suppresses non-specific amplification due to cross-talk reactions [134]. In conclusion, extensive efforts have been made so far to develop efficient and sensitive methods for miRNA detection, but there still remains a need for a standardized method that should be highly sensitive, specific and multiplexable.

Predicting the effect of splicing modulation and its effect towards miRNA profile of PDAC

Splicing modulation is a new emerging therapeutic approach that had been tested in several types of cancer either pre-clinically or clinically [15,66,89,135–137]. Splicing modulation is promising because of its high potency to induce apoptosis and suppress cellular migration. Cancer cells harbouring mutations in genes encoding splicing factors are the most promising targets [15,89,136,137]. However, splicing modulation could also be applied in cancer cells with splicing factors overexpression [9,89,135].

The important role of splicing deregulation in PDAC carcinogenesis and the potency of several splicing modulators might increase the potential application of splicing inhibitors in PDAC. However, there have not yet been studies evaluating the efficacy of splicing inhibitors/modulators in PDAC. Preclinical studies have demonstrated that the SF3B1 inhibitors pladienolide B and E7107 were effective in gastric cancer, cervical cancer, and peritoneal mesothelioma [135,138–140]. In particular, pladienolide B has high efficacy in gastric cancer with complete tumour elimination in SCID mice in just 2 weeks and has an IC_{50} in the nanomolar range. Similar findings were observed in peritoneal mesothelioma where pladienolide B and E7107 inhibited cell proliferation and migration [135]. Remarkably, *in vivo* treatment with E7107 resulted in complete regression of peritoneal tumours in the second week. SF3B1 inhibition also showed similar efficacy in cervical cancer and cutaneous squamous cell carcinoma but, in these tumours they apparently showed more efficacy towards cells with mutated p53 [140].

Unfortunately, clinical trials with splicing modulators have been limited by toxicity. Indeed, in a phase I pharmacokinetic and pharmacodynamic study of E7107 in advanced solid tumours, when using doses above 4.3 mg/m^2, several patients suffered from gastrointestinal side effects, such as diarrhoea, vomiting, dehydration, and in two cases there was vision loss [141].

H3B-8800 is another SF3B1 inhibitor and entered phase I clinical trial in 2016, with a focus on patients with MDS, AML and CMML (NCT02841540). Initial results revealed dose- dependent target engagement, a predictable pharmacokinetic profile and a favourable safety profile, even with prolonged dosing. Although objective therapeutic responses have not been achieved to date, 14% of patients had reduced requirements for red blood cell or platelet transfusions [142].

A number of other drugs targeting splicing factors have shown encouraging preclinical effects in mouse models of cancer, such as inhibitors of SRPK and CLK protein kinases that phosphorylate SR proteins and thereby inhibit angiogenesis by inducing changes in the alternative splicing of VEGF [143,144]. Other splicing inhibitors targeting a variety of

spliceosomal components also reduce cancer cell proliferation *in vitro* [145–148], but their effects in animal models of cancer are not yet known.

However, toxicity may be prevented by the use of a lower dose of splicing modulators, and the risk of reduced efficacy can be avoided by rationale combinations with different antitumor strategies, including modulation of selected miRNAs.

There are no data yet on the potential effect of splicing inhibitors on miRNA in PDAC. The most plausible candidates as therapeutic targets are PTBP1 and HnRNPK because their role has been already established in PDAC [63,70]. Moreover, SF3B1 has the advantage as a therapeutic target due to the availability of small-molecule inhibitors [89,137,139,140,149]. Of note, SF3B1 inhibition resulted in upregulation of tumour suppressor SO-miRNAs in a cervical cancer cell line [91]. Therefore, similar studies should be performed to demonstrate this effect in PDAC cell lines.

The effect of splicing inhibitors targeting those three splicing factors might however be predicted using available data. Calabreta and colleagues [70] provided initial evidence that targeting the splicing factor PTBP1 in gemcitabine resistant PDAC cell line by siRNA shifted PKM isoform expression towards PKM1 which was accompanied by increased sensitivity towards gemcitabine and an enhanced level of cleaved caspase-3. Li et al. [116] studied the long non-coding ROR in PDAC and found that PTBP1 was the target of tumour suppressor miR-124 which could effectively block its expression. However, in PDAC, long non-coding ROR acts as sponge that binds miR-124, preventing it to regulate PTBP1 expression and increasing PKM2 expression. This study suggested that miR-124 could be used as a marker for Warburg effect in PDAC as well as a therapeutic agent candidate to target PTBP1 in gemcitabine resistant PDAC. However, the other targets of miR-124 should be elucidated to minimize unfavourable off-target effects.

Another potential SF target candidate is HnRNPK which is known for its role in enhancing cancer cellular proliferation, invasion and metastasis in PDAC [63]. HnRNPK is associated with miR-223 which suppressed FBXW7 as previously described. However, the sister chromatid cohesion protein PDS5 homolog B (PDS5B) is another important target of miR-223. The downregulation of miR-223 led to increased expression of PDS5B which resulted in inhibition of cellular proliferation and migration [114].

Inhibition of SF3B1 could possibly be effective and may produce the most pronounced miRNA profile changes in PDAC. In cervical cancer, SF3B1 inhibition resulted in an increase of several

tumour suppressor miRNA, most notably miR-636, miR-6510-5p, miR-3614-3p, miR-3655, miR-3656, miR-4260, miR-5187-3p, miR-7109-5p, and miR-8069 [96]. In addition, four miRNAs were downregulated, namely miR-155-3p, miR-148a-3p, miR-98-5p, and miR-21-3p. Apparently, SF3B1 inhibition can suppress the expression of miR-155 and miR-21 which play important roles in PDAC. However, this should be further investigated in PDAC preclinical models.

The effect of upregulation of tumour suppressor miRNAs or downregulation of oncogenic miRNAs is expected to have a wide impact [25]. A summary of potential effects of splicing modulation on the miRNA profile in PDAC as well as their biological effects is depicted in **Figure 3**. For example, miR-21 and miR-155 have many targets that are involved in carcinogenesis and metastasis [11,12,50,53,55,56]. Suppression of these oncogenic miRNAs can thus potentially lead to tumour suppression and inhibition of metastasis. However, these miRNAs can also serve as potential biomarkers of tumour progression or response to treatment, and could improve the clinical management of PDAC patients by monitoring the modulation of these miRNAs in samples that can be collected during treatment/follow-up, such as in liquid biopsy studies.

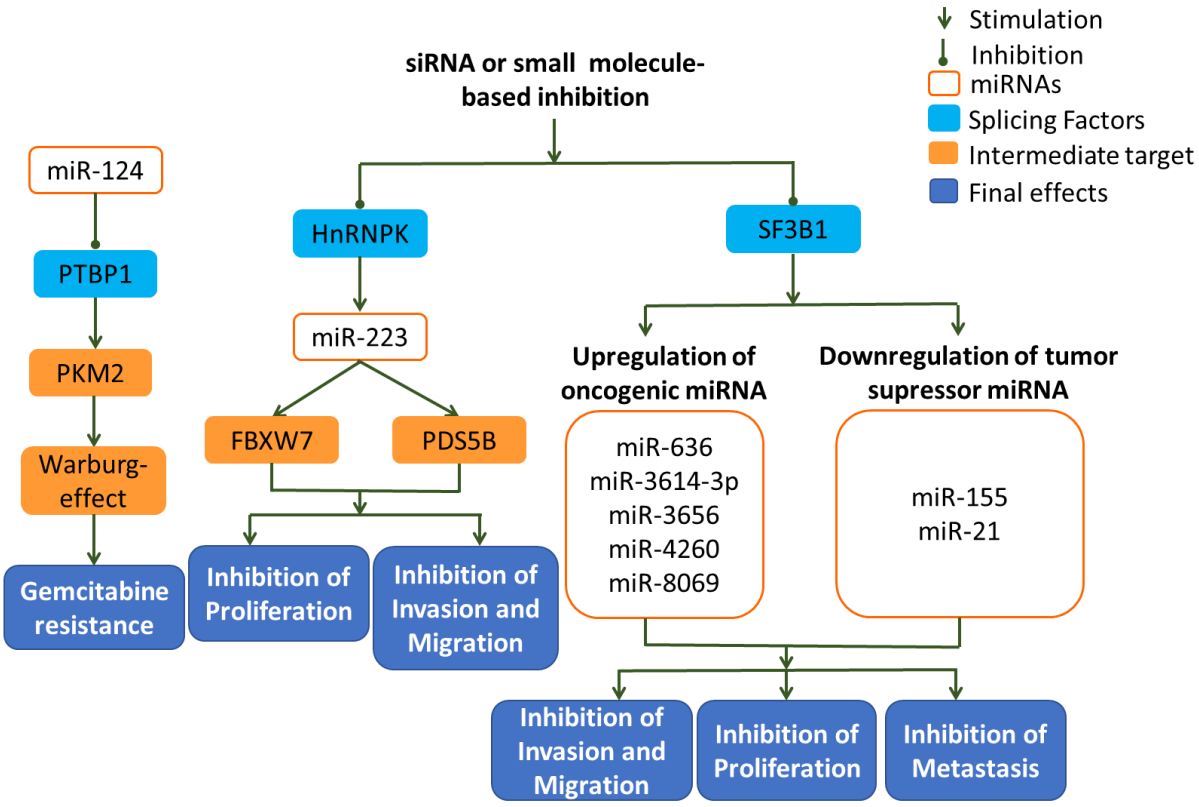

Figure 3. The interaction of relevant splicing factors in PDAC and their associated miRNA. PTBP1 and HnRNPK are considered as the relevant targets in pancreatic cancer and have demonstrated their interaction with miRNAs in PDAC (miR-124 and miR-223, respectively). Despite lack of evidence in PDAC, cervical cancer experiment demonstrated that SF3B1 inhibition resulted in extensive change in miRNA expression and potentially brings more profound effects than PTBP1 and HnRNPK [63,70,89,91].

Bioinformatic tools to predict the effect of splicing modulation towards miRNA profiles

Bioinformatics uses advanced computing, mathematics and biological knowledge to store, manage, analyse and get insights into biological data. In recent years, there has been a boom of publicly available computational tools, online data analysis modules, biological data repositories, and bioinformatics workflow management systems [150]. In order to assess how splicing modulation can affect miRNA profiles, alternative splicing detection tools such as rMATS [151], SUPPA2 [152] or MISO [153] can first detect differential splicing between conditions, after which splicing motif analysis tools like MEME [154] or RNAContext [155] can use these splicing motifs to identify regulators of alternatively spliced junctions. Lastly, potential miRNA targets of splicing modulation can be detected using miRNA-target databases such as mirTarBase [156] where experimentally validated miRNA-target interactions are curated, or mirDB [157] where the predictive algorithm MirTarget is used to analyse thousands of miRNA-target interactions from high-throughput experiments. An example of analysis pipeline to identify splicing factors modulated by miRNAs is reported in **Figure 4**.

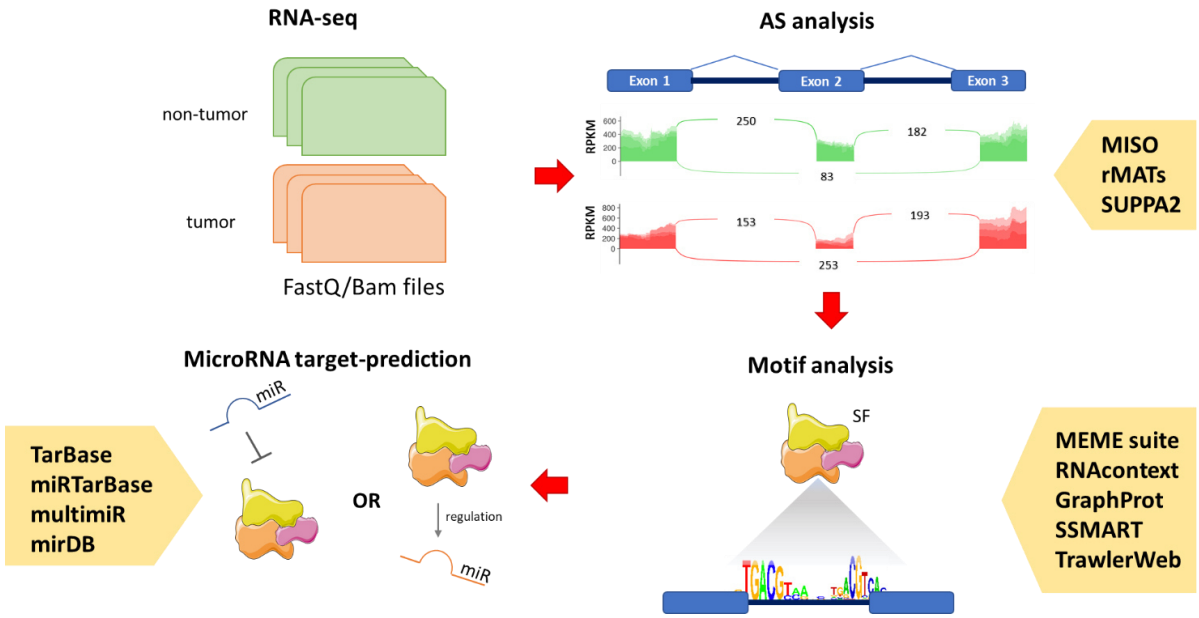

Figure 4. Bioinformatic pipeline for splicing factor and miRNA modulation discovery with RNA-seq data. Raw data or mapped BAM files are used as input for alternative splicing (AS) tools such as MISO, rMATs and SUPPA2 to identify differential alternative splicing events. Next, a motif analysis is performed to identify the splicing factor (SF) specific for that RNA isoform. Lastly, miRNA-target databases are used to retrieve possible miRNA targeting SF. Additional miRNA profile can be useful to detect miRNA modulation through SFs.

The first step is the identification of alternative spliced (AS) events. While there is a plethora of available tools, no current tool can be regarded as the golden standard and the matter of choice strictly depends on the research question and familiarity. Research into which tools are superior is currently incomplete. One example of a tool that detects differential alternative spliced RNA transcripts is MISO, published in 2010 [157]. This statistical model estimates expression of alternatively spliced exons and isoforms using mapped reads as input format. MISO then uses Bayesian inference to compute the probability that an RNA-seq read originated from a particular isoform. Despite being the most cited and used tool for alternative spliced differential analysis, it has no longer been maintained since its publication, and it has high computational time. rMATS [151] is a more recent and often-cited tool used in differential splicing analysis, it analyses replicates and includes a function to handle paired and unpaired replicates. Another common tool recently published is SUPPA2. This type of algorithm requires two biological replicates because it accounts for biological variability, which is important for the reliability of the estimations that are drawn from the data. However, it can work with multiple conditions and includes the possibility to perform hierarchical clustering on

differentially spliced events to identify common regulatory mechanisms. Other tools capable of detecting AS and compare AS patterns between sample groups are DEXSEq, SplicingCompass, Altanalyze, BitSeq, EBSeq, and Cuffdiff2 whose performances are extensively reviewed in Lahat and Grellsheid [158].

Once differentially expressed AS events are identified, prediction of splicing factors is the second step of the analysis. Motif analysis tools may be used to identify the direct regulators of alternative spliced junctions. For example, MEME suite [154] provides a unified portal of online discovery tools for DNA binding sites and protein interaction domains. MEME is an online web-based module that includes three sequence scanning algorithms that allow to scan different DNA and protein databases. Next, transcription factor motif can be further analysed for putative functions using GOMo tool [159,160]. Previous tools such as RNAcontext [155] and GraphProt [143] work with classification and regression model settings and are not capable of *de novo* sequence-structure motifs. Other tools for motif analysis recently published are SSMART [161] and TrawlerWeb [162] which is a web version of the previous published standalone tool. Trawlerweb is currently the fastest online de novo motif discovery tool and it displays resulting scores allowing the user to prioritize the choice for validation experiments. Validation analysis, such as *in vitro*/*in vivo* binding assays, cross-linking immunoprecipitation sequencing (CLIP-seq), minigene splicing reporter assays (*invitro*) or antisense oligonucleotides which block splicing factor-binding sites are needed to validate results from the (splicing) motif analysis.

The last step of the analysis is the identification of putative miRNAs that are targeting the splicing factors based on previously performed motif analysis. Nowadays there is a plethora of miRNAs-target databases available on the web, such as miRTarBase [156], mirDB [157], miRBase [163] and TarBase [164]. In addition, a new R package multimiR [165] includes a compilation of around 50 million records in human and mouse from 14 different databases and it expands on miRNAs involved in drug response and disease annotation.

However, an integrative analysis with miRNA profile can be useful to detect miRNA modulation (e.g., inhibition) through the predicted SFs. Nowadays, there are several ways to analyse miRNA-seq profiles, and here we describe the general downstream analysis pipeline with the most commonly used tools.

After trimming and quality check, the resulting reads are aligned to a reference database containing miRNA sequences. miRbase [163] is the primary database of published miRNA sequences which is often used for miRNA mapping. A broader database of small-RNA and

miRNA sequences is mirGeneDB 2.0 [166] resulting in more precise annotation while avoiding misleading miRNA annotation from other types of small-RNAs. The read sequences are mapped to reference databases through mapping tools. There is an increasing number of mapping tools for small-RNA sequences and the most used are: miRanalyzer [167], miRDeep2 [168] and sRNAbench [169]. All these tools rely on Bowtie algorithm [170] (allowing mismatches and improving speed of alignment).

sRNAbench and its downstream analysis tool sRNAtoolbox includes an automatic processing of the five most used library preparation protocols (including new reference genomes from Ensembl, NCBI and MirGeneDB), a consensus differential expression analysis, target prediction, analysis of unmapped reads, batch mode to profile several samples at once with the same set of parameters and improved visualization and mapping statistics. This also enables users with a 'non-bioinformatics' background to analyse small-RNA high-throughput data from raw fastq files (standard output files from sequencing machines) to post-processed data for differential analysis and miRNA-target prediction.

The ever-expanding field of bioinformatic studies and the enormous availability of wet-lab data has given rise to several predictive models that are extremely useful for target prediction and to prioritize experimental validation targets.

Conclusions and Future Perspectives

The interaction of miRNAs and splicing deregulation is an understudied field, but evidence of their close interconnection is increasing. Currently, the application of miRNAs is focused on their role as biomarker while splicing inhibitors are under investigation as a novel therapeutic strategy.

Increasing evidence shows that splicing deregulation resulting from mutation or overexpression can produce a pronounced aberration in miRNA expression in different cancer types, including PDAC. For instance, the upregulation of tumour suppressing miRNAs may mediate an anti-cancer effect of splicing modulation as was shown by the inhibition of PTBP1 and SF3B1 in cervical cancer. Potentially similar inhibitory effects and the impact of other SFs on cancer progression and miRNA profile still need to be investigated in PDAC. Moreover, specific miRNAs could be used as a target to downregulate specific SFs and also for combined therapeutic approaches.

Remarkably, novel bioinformatics tools are providing extensive data that can be used to deepen our knowledge in the biological effects of the interplay between splicing and miRNAs, as well as several predictive models for target prediction in order to prioritize future experimental and clinical validation. In-depth analysis of PDAC aberrant splicing patterns associated with miRNA profiling may indeed further provide mechanistic insight to successfully target key PDAC drivers. Targeting aberrant splicing and the reciprocal interaction with deregulated miRNA could therefore provide more effective therapeutic approach to combat the complex biology of PDAC and its chemoresistant features.

Authors' contribution

D.I.S, G.M., I.B.M., O.R., G.J.P, E.G. wrote the manuscript; D.I.S., O.R. and G.M. designed figures and tables; G.J.P, E.G., P.D. and S.C. extensively revised the manuscript.

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Supplementary Materials Chapter 4

Interrelationship between miRNA and splicing factors in pancreatic ductal adenocarcinoma

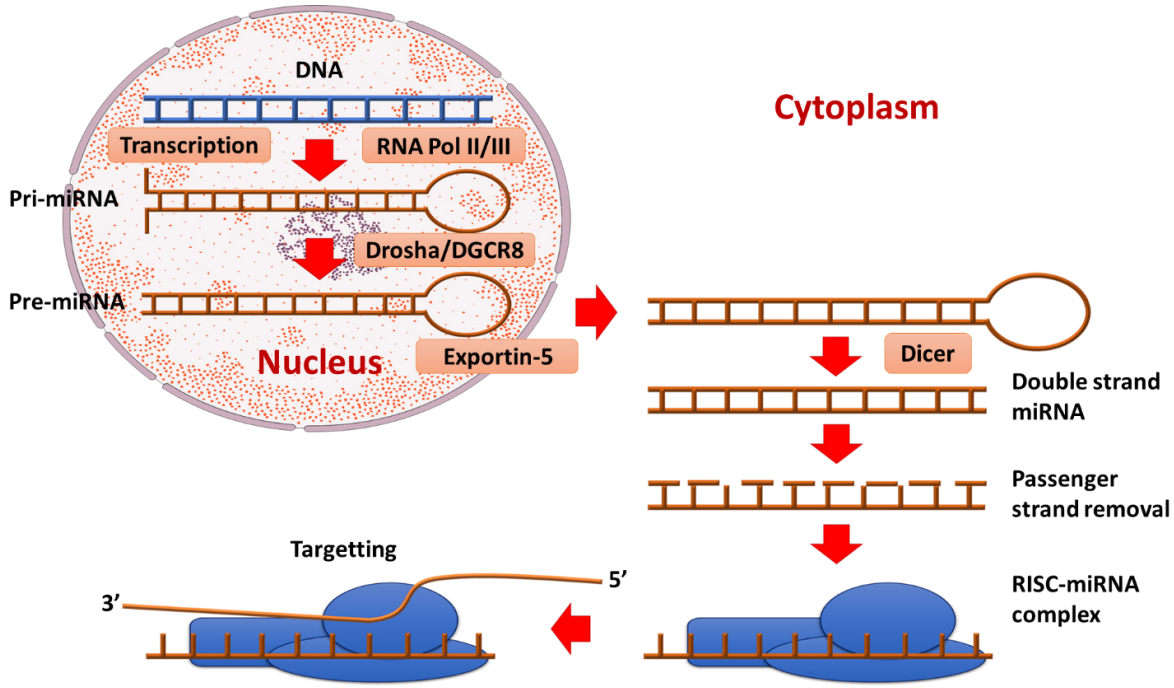

Figure S1. The biosynthesis process of miRNA. miRNA is transcribed from DNA by RNA pol II which produced a hairpin-shaped transcript. DROSHA removes the overhanging sequence and exportin-5 transports the pre-miRNA to cytoplasm where Dicer removes the loop section. After removal of passenger strand, mature miRNA forms the RISC complex with Argo proteins which can target and degrade any mRNA with compatible sequence [21,22].

Chapter 5

Exploring splicing modulation as a novel strategy against pancreatic cancer

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Abstract

Introduction: Current prognostic and therapeutic strategies to treat pancreatic ductal adenocarcinoma (PDAC) patients fail to deliver successful cures and the survival rates remain dramatically low. Splicing deregulation is a new hallmark of cancer and modulators of the key splicing factor SF3B1 proved highly efficient against a range of solid and hematological malignancies. However, they have never been tested in PDAC. Therefore, in this study, we explored the still largely uncharacterized alternative splicing profile and the expression levels of the splicing factor SF3B1 in PDAC cells and tissues as a source of novel potential therapeutic targets and predictors of treatment outcome.

Methods: We performed a transcriptome-wide characterization of splicing profiles in 5 PDAC primary cell cultures compared to 2 immortalized normal ductal epithelial cell lines to identify differentially spliced genes. The differentially spliced genes were then subjected to enrichment analyses using different sources. The efficacy of two SF3B1 modulators (Pladienolide-B and E7107) was evaluated in PDAC cells. Subsequently E7107 activity was confirmed in orthotopic *in vivo* models. Antiproliferative activity was assessed by sulforhodamine B assay, while the effect of E7107 on cell migration and RON splicing was evaluated by wound healing assay and PCR, respectively. Lastly, SF3B1 expression was evaluated in tissue microarrays (TMA), including paraffin-embedded PDAC samples from 87 patients.

Results: RNA-sequencing based differential splicing analysis comparing PDAC cultures to 2 non-malignant cell lines revealed a total of 420 significant differential splicing events affecting 340 genes largely involved in the regulation of mRNA splicing, gene expression, and nucleic acid metabolism, as revealed by Gene Ontology analysis. All the PDAC cells displayed a high sensitivity toward SF3B1 modulation by Pladienolide-B and E7107, with IC_{50} values in the low nanomolar range. Moreover, both compounds significantly reduced cellular migration, associated with splicing alteration of RON. E7107 showed promising results also *in vivo*, while SF3B1 expression in the TMAs was significantly correlated with overall survival and progression-free survival with a hazard ratio of 1.79 (95%CI: 1.14-2.80; p=0.011) and 1.77 (1.14-2.7; p=0.012), respectively.

Conclusion: SF3B1 is a potential prognostic factor as well as a therapeutic target in pancreatic cancer, and its modulation affects proliferation, migration, and alternative splicing of key oncogenes. These results support further studies on this novel therapeutic approach for the treatment of PDAC.

Keywords

PDAC, splicing, SF3B1, Pladienolide-B, E7107

INTRODUCTION

From 2014 to 2021, the five-year survival rate for pancreatic ductal adenocarcinoma (PDAC) patients increased from 6% to 10% [1]. However, the global incidence of PDAC has also been rising steeply in the last years. This tumour is predicted to become the second leading cause of cancer-related deaths by 2030. This dismal trend is due to late diagnosis and invariable high chemoresistance, making PDAC a priority for studies on novel biomarkers for early detection and on more effective treatments [2].

Large-scale cancer genomics studies have defined subtypes in many cancer types, such as lung and breast cancer, often resulting in clinically relevant approaches to improving patient care. Similar studies have been performed in PDAC, providing insights into pathogenesis's key mechanisms. Except for BRCA1/BRCA2 mutations for PARP1 inhibitors [3], unfortunately, this increased knowledge in the underlying genetics of PDAC has not yet been translated to the identification of “actionable” therapeutic targets, and new precision medicine initiatives are warranted [4].

However, the emerging role for extensive splicing aberrations in this tumor type, as recently reported by Kahles and collaborators [5], offers a powerful rationale to redirect focus from the genetic approaches to the wider epigenetic regulation of PDAC chemoresistance.

Alternative splicing (AS) is the essential process in eukaryotic gene expression by which non-coding intron sequences are removed from the pre-mRNA transcripts, and specific exons are included or excluded from mature mRNAs. AS is operated by several splicing factors (SFs) assembled as a multi-protein complex called the spliceosome. Multiple transcripts generated from a single gene can be translated into proteins, often executing distinct functions. Defects in SF genes, such as changes in expression levels or mutations, can produce aberrant mRNA splicing patterns on a genome-wide scale. This is a common feature of several cancer types, both hematological malignancies and solid tumors [6,7]. Several SF alterations have been described in pancreatic cancer (PC). One single nucleotide polymorphism in SF3A1 was significantly associated with the risk of developing cancer combined with environmental factors like smoking and alcohol consumption [8]. Non-silent SF3B1 mutations were described in

PDAC patients [9], while SF3B4 is downregulated in PDAC cells, and its overexpression enhances cell growth and motility [10].

Splicing alterations also have prognostic value and contribute significantly to tumorigenesis [7,11]. A large genomic study involving 32 cancer types from the Cancer Genome Atlas (TCGA), including PDAC, identified thousands of cancer-specific alternative splicing events [5]. In search of new potential prognostic predictors for PDAC, Yu and collaborators recently investigated aberrant AS patterns using RNA-seq data from TCGA and SpliceSeq databases [12] and revealed that aberrant AS events of several SF genes were significantly associated with overall survival [13]. Similarly, a study on 43 PDAC tissues, showed that expression of aberrant splice variants predominantly affects the extracellular matrix-associated genes and focal adhesion genes, suggesting their role as diagnostic and therapeutic targets [14].

In light of these studies, splicing deregulation is emerging as an important feature of PDAC pathogenesis and could represent a novel susceptibility to be exploited for cancer therapy. Pharmacological modulation of spliceosome activity via small molecules targeting the SF3b complex (e.g. FR901464, GEX1A, and pladienolides) has shown potential in several pre-clinical studies [15,16]. E7107, an improved pladienolide derivative tested in a Phase 1 trial, stabilized the disease in 8 out of 40 cancer patients and reached partial response in one metastatic PC patient. Unfortunately, the incidence of two cases of vision impairment led to the discontinuation of this trial [17]. However, a similar splicing modulator, H3B-8800, is now in Phase 1 clinical trial (NCT02841540) [18,19].

In this study, we explore the rationale of splicing as a new target in PDAC. We characterized the global splicing landscape of 5 primary PDAC cell cultures and tested the antitumor effects of the SF3b modulator E7107 and pladienolide *in vitro* and *in vivo*, through orthotopic mice models. Furthermore, we analyzed the expression levels of SF3B1 in patient tissues and established its association with clinical outcomes.

METHODS

Patient material and immunohistochemistry

Tumor specimens of 87 PDAC patients from the University of Pisa were preserved in paraffin blocks and selected for pathological examination. Tissue micro-arrays (TMAs) were constructed as described in PMID: 32860207 and SF3B1 expression was analyzed by immunohistochemistry using a monoclonal antibody (D221-3, MBL CO. LTD., Japan).

Pathological examination and scoring (high or low) were performed by two researchers, blinded to the clinical patient outcome. Patients were categorized according to their SF3B1 levels using a 6-grade scoring system, as described in [20].

Splicing modulators

E7107 was provided by H3 Biomedicine (Cambridge, MA, USA). Pladienolide-B (Plad-B) was purchased from Cayman Chemical (Ann Arbor, USA).

Cell lines and culture conditions

Five primary pancreatic ductal adenocarcinoma cell lines (PDAC-1, -2, -3, -4 and -5) were isolated as previously described [21,22] and two non-malignant pancreatic epithelial cell lines (HPDE and HPNE), were obtained from American Type Culture Collections (ATCC) [23]. Cells were grown in RPMI (Lonza, Switzerland) supplemented with 10% fetal calf serum (S0750-500, VWR, Amsterdam, The Netherlands) and incubated at 37°C, 5% CO_2. Primary cells were used until passage n.20.

Drug sensitivity and wound healing assays

Cell viability upon exposure to SF3b modulators was measured by sulforhodamine B (SRB) assay. PDAC-1 and -3 cells were seeded at a density of $3*10^4$ cells/well while PDAC-2 and -5 at $5*10^4$ cells/well and tested as previously described [24]. All experiments were conducted in triplicate. For the wound healing assay, PDAC-1 and PDAC-3 cells were seeded at a density of $15*10^4$ cells/well and tested as previously described [20] using 10 nM or 30 nM drug concentration. "% of migrated cells compared to T=0" was calculated per each timepoint as: |Average scratch area Tn – T0|/(Average scratch area T0)*100.

RNA sequencing, differential splicing (rMATS) analysis, and RT-PCR

Total RNA from PDAC and normal epithelial pancreatic cells was extracted with RNeasy mini Kit (QIAgen) and processed as previously described [20,24,25]. Briefly, cDNA sequencing libraries were prepared with the Illumina TruSeq Stranded mRNA Library Prep LT Kit and Agencount AMPureXP beads. Single-end, 100 bp-reads were obtained from HT-v4-SR100

Chip (8 lanes) on Illumina HiSeq 2500 System and subjected to quality check. rMATS version 3.2.5 [26] was used to detect unique AS splicing events (ES, A5SS, A3SS and RI, except for mutually exclusive exons), using default parameters and FDR < 0.05 was taken as cut-off for significance. The enrichment tool gProfiler [27] version r1741_e90_eg37 was used for pathway analysis of genes affected by AS using Gene Ontology (GO) terms as a source database.

End-point RT-PCR analysis of treated PDAC cells was carried out as previously described. Primer sequences:

Mcl-1_Fw: (5'-GCCAAGGACACAAAGCCAAT-3')

Mcl-1_Rev: (5'-GCTCCTACTCCAGCAACACC-3')

Bcl-x_ex2_Fw: (5'-GGGCATTCAGTGACCTGACA-3')

Bcl-x_ex3_Rev: (5'-GGGAGGGTAGAGTGGATGGT-3')

RON_Ex10_Fw: (5'-CCTGAATATGTGGTCCGAGACCCCCAG-3')

RON_Ex12_Rev: (5'-CTAGCTGCTTCCTCCGCCACCAGTA-3')

Orthotopic mouse model and E7107 treatment

Nu/nu female mice were purchased from Harlan Laboratories (Madison, WI, USA). In accordance with the European Community Council Directive 2010/63/EU for laboratory animal care and the Dutch Law on animal experimentation, animal experiments were performed. The working protocol was validated and approved by the local committee on animal experimentation of the VU University Medical Center (DEC HEMA14-01). $1*10^6$ PDAC-5 cells were injected orthotopically as previously described [28]. Mice were subjected to PET-MRI scan one week after tumor induction. [18F]-FDG was administered via intraocular injection (5 MBq/mouse) and scan was performed with nanoPET-MRI (Mediso, Hungary). Data and images were acquired, normalized and analyzed as previously described [20,29].

E7107 *in vivo* test and flow cytometry-based assessment of tumor burden

One week after tumor induction, one daily dose of 2.5 mg/kg E7107 was administered intraperitoneally for four consecutive days. Control group was administered with vehicle (PBS + 10% ethanol + 5% Tween-80). Mice were monitored for two weeks before sacrifice. Single-

cell suspensions were obtained by dissociating tumor tissues with gentleMACS™ C Tubes (Miltenyi Biotec Inc., USA) and Falcon 40 µm Cell Strainer (Corning). Samples were first stained with 7-AAD (Via-Probe^{TM}), washed with FACS buffer (PBS, 0.1% HSA, 0.05%) and stained with FITC-labelled Mouse Anti-Human CD44 (560977) and APC-labelled Mouse Anti-Human CD24 (658331, BD Bioscience, USA). After washing and resuspension in FACS buffer, 50 µl of Flow-Count^{TM} Fluorospheres (7547053, Beckman Coulter) were added to each sample. Fluorescence was measured using BD FACS Fortessa flow cytometer. The analysis was performed using BD FACS Diva software version 8.0.1.1.

Statistical analysis and visualization

Clinical outcome was correlated with N-stage, grade, resection margins, and SF3B1 expression by means of a univariate analysis using a Mantel-Cox (Log-rank, Chi-Square) test. Survival rates were calculated using the Log-Rank test and visualized by the Kaplan-Meier method. A multivariate model to assess the prognostic relevance of SF3B1 expression was obtained by selecting independent prognostic factors among all clinicopathological parameters (selected as covariates) and using the backward stepwise elimination (Wald) method. Statistical analysis for clinical data was performed with SPSS version 23 (IBM, USA). Graphs and statistical analysis for *in vitro* experiments were produced with GraphPad Prism version 8 and R version 3.5.0.

RESULTS

Primary PDAC cells are characterized by globally altered splicing profiles and sensitivity to splicing modulators

We first characterized the splicing profiles of 5 primary cultures that were already characterized as representative models of primary PDACs [22,30]. To this end, we performed a whole-transcriptome splicing profile analysis on PDAC cells and 2 non-malignant pancreatic epithelial cell lines (HPDE and HPNE, **Figure 1A-B**). We characterized 420 significant differential splicing events (**Figure 1A**) by which 340 genes were largely involved in the regulation of mRNA splicing, gene expression, and nucleic acid metabolism, as revealed by enrichment analysis on GO terms (**Figure 1C**, **Table 1**).

Under the hypothesis that altered splicing could constitute a vulnerability for PDAC cells, we tested the inhibitory effects of SF3B1 modulators on cell growth and migration. All PDAC

cultures were extremely sensitive to E7107 and Plad-B exposure, with IC_{50} values within low nanomolar ranges (**Figure 2A** and **Supplemental Figure 1A**).

Since one of the major hallmarks and problems in the therapy of PDAC is its early local and systemic dissemination, we evaluated whether E7107 might affect PDAC cell migration. Using a 24-hour wound-healing assay in the two relatively most resistant cells (PDAC-1 and -3) we showed that incubation with 10 and 30 nM E7107 and Plad-B resulted in the inhibition of cell migration compared to untreated controls in all tested time points (**Figure 2B**, **Supplemental Figure 1B**). Remarkably, this effect was associated with the mis-splicing of the proto-oncogene RON. Our PDAC cells express the truncated variant ΔRON which plays an important role in cancer cell motility due to its constantly activated kinase function [31,32]. A 24-hour exposure to 2.5 and 25 nM E7107 or Plad-B caused intron retention in RON transcript and decreased transcript abundance, probably due to nonsense-mediated decay (**Figure 2C**, **Supplemental Figure 1C**, **Supplemental Figure 2**).

E7107 inhibits *in vivo* tumor growth

PDAC orthotopic mouse models were generated by injecting PDAC-5 cells in the pancreas of immune-deficient mice (**Figure 3A**). One week after surgery, E7107 was administered intraperitoneally according to the treatment schedule (**Figure 3B**), and animals were monitored for three weeks until vehicle-treated mice showed tumor masses in the proximity of the pancreas. The efficacy of E7107 was assessed on explanted tumors by measuring the number of engrafted PDAC-5 cells via flow-cytometry detection of the cell surface markers CD44 and CD24 [33,34] (**Figure 3C**, **Supplemental Figure 3A**). E7107-treated animals showed a significantly lower amount of tumor cells compared to the control group (**Figure 3D**). Parallel analysis of mice weight, showing similar values in treated and control mice, suggested that this treatment did not induce toxicity (**Supplemental Figure 3B**).

High SF3B1 expression is a negative prognostic factor in PDAC

Finally, we investigated whether the expression levels of SF3B1 protein correlate with the clinical outcome of PDAC patients. To this end, we constructed tissue microarrays (TMAs) from paraffin-embedded histological specimens of 87 PDAC patients and determined SF3B1 protein expression by immunohistochemistry. Multivariate analysis, including well-established prognostic factors (age, grade, stage, and resection radicality) confirmed the independent

prognostic value of SF3B1 and patient age (**Figure 4A**). Additionally, the log-rank test revealed a significant correlation between high SF3B1 levels and shorter overall and progression-free survival (*p*-value 0.0068 and 0.0066, respectively), as illustrated by the Kaplan-Meier curves in **Figure 4B**.

DISCUSSION

This is the first study that demonstrates that the modulation of the core spliceosomal protein SF3B1 has strong antiproliferative and anti-migratory effects in a panel of primary PDAC models starting from deep sequencing and innovative analyses of their splicing profiles.

Interestingly, the functions highly enriched in our dataset included regulation of mRNA processing, in particular pre-mRNA splicing (which is a known self-regulatory mechanism of many splicing factors).

Recent reports provide evidence for the efficacy of spliceosome modulators in PDAC [17]; however, extensive pre-clinical testing aimed at elucidating the rationale of splicing modulation in PDAC is still lacking. In this study, we first characterized the splicing landscape of primary PDAC cultures compared to normal cell lines using RNA-seq. In agreement with previous studies [13], we detected differentially spliced variants mainly of ES and RI type affecting genes involved in RNA splicing and metabolism. These data point at widespread splicing deregulation of PDAC cells that can be used as a therapeutic target.

E7107 and Plad-B showed potent *in vitro* antitumor activity in primary PDAC cells. This is in line with previous results in other tumor types [20,25]. However, a recent study reports that almost 24% of the 119 examined spliceosomal genes (i.e. PRPF40A and SNRNP27) were differentially expressed in PDAC compared to the normal pancreas, which further underscores the relevance of alternative splicing in PDAC biology [14].

Two recent studies interrogating the molecular structure of U2 snRNP bound with Plad-B and E7107 revealed that the conformation of the SF3B1 catalytic pocket, in association with PHF5A, is critical for BPS recognition and these compounds compete for the pre-mRNA substrate in a dose-dependent fashion [35,36]. Splicing modulators showed promising results as single agents in a range of adult solid tumors (i.e., breast, colon, and non-small cell lung cancer) and hematological malignancies in both *in vitro* and *in vivo* models [37,38]. Moreover, combinations of sudemycins with other chemotherapeutics (i.e., with BCL-2/BCL-XL antagonists and the tyrosine kinase inhibitor ibrutinib) proved effective in CLL [39,40]. In adult

secondary AML, spliceosome modulation by E7107 specifically decreased leukemic stem cell burden in xenograft models while sparing the normal hematopoietic stem cells [41]. Importantly, the latter, as well as and other studies, demonstrated superior cytotoxic activity of spliceosome inhibitors against cancerous cells as compared to normal cells, suggesting a potential therapeutic window [42]. This might be explained by the differential expression/activity of SF3B1 and other SFs in normal and cancer cells. However, further experiments on SF3B1 knock out cells and the relation between SF3B1 gene and protein expression in PDAC are warranted.

Migration processes are controlled by transcription factors, tyrosine kinases, and alternatively spliced transcripts such as ΔRON. RON is a tyrosine-kinase receptor that belongs to the MET family, which plays an important role in the carcinogenesis of many human solid cancers [31,43–45]. RON itself has been reported to play a crucial role in pancreatic cancer tumorigenesis and metastasis as it is expressed in 93% of PDAC specimens [43]. Our data clearly show that SF3b modulation impairs this variant's splicing pattern, and this effect is reflected by a reduction in cell migratory capacities *in vitro*. This finding is consistent with previous findings by our group in DMPM, which show a similar splicing pattern after treatment with splicing inhibitors [20]. Moreover, similar findings were observed in previous studies in different PDAC models, with increased levels of specific splice variants of CXCL12, MUC4, and RHAMM genes associated with increased invasiveness and metastasis [46–48]. Although the precise mechanism of action remains elusive, migration inhibition and specific splice variant expression are likely involved.

To our knowledge, this is also the first study evaluating the antitumor activity of the SF3B1 modulator E7107 in orthotopic models of PDAC. *In vivo* administration of E7107 led to a slight but significant reduction of tumor burden, without relevant toxicity. However, using a single end-point was an important limitation of this study. Future studies should consider the use of bioluminescent orthotopic models that can be used for longitudinal monitoring of tumor growth before randomization and after drug administration [20].

Ultimately, our analysis performed on a large cohort of PDAC patients revealed that the overexpression of SF3B1 protein is associated with a significantly worse prognosis and is an independent risk factor. These data are in agreement with our previous findings in mesothelioma samples [20] and support further studies that should lead to the implementation of splicing modulators into the clinic. While we found that patients characterized by overexpression of SF3B1 protein have inferior treatment outcome to those with low expression

of this protein, due to global splicing perturbation previously shown in PDAC cells as compared to normal pancreatic cells, these notoriously chemo-resistant tumors might indeed be particularly sensitive to the new class of SF3B1 modulators. This type of personalized therapy approach holds promise for more effective interventions. In conclusion, novel therapeutic approaches are urgently needed for PDAC, an aggressive tumor characterized by poor survival rates and refractoriness to current therapeutic regimes. Our clinical data assign new prognostic roles to SF3B1, which prompt validation studies and may be extended to other spliceosome components. Finally, our *in vitro* and *in vivo* efficacy data support the rationale that drugs modulating the spliceosomal activity could constitute an attractive therapeutic option for PDAC patients.

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Conflicts of interest:

The authors report no conflict of interest

Figures

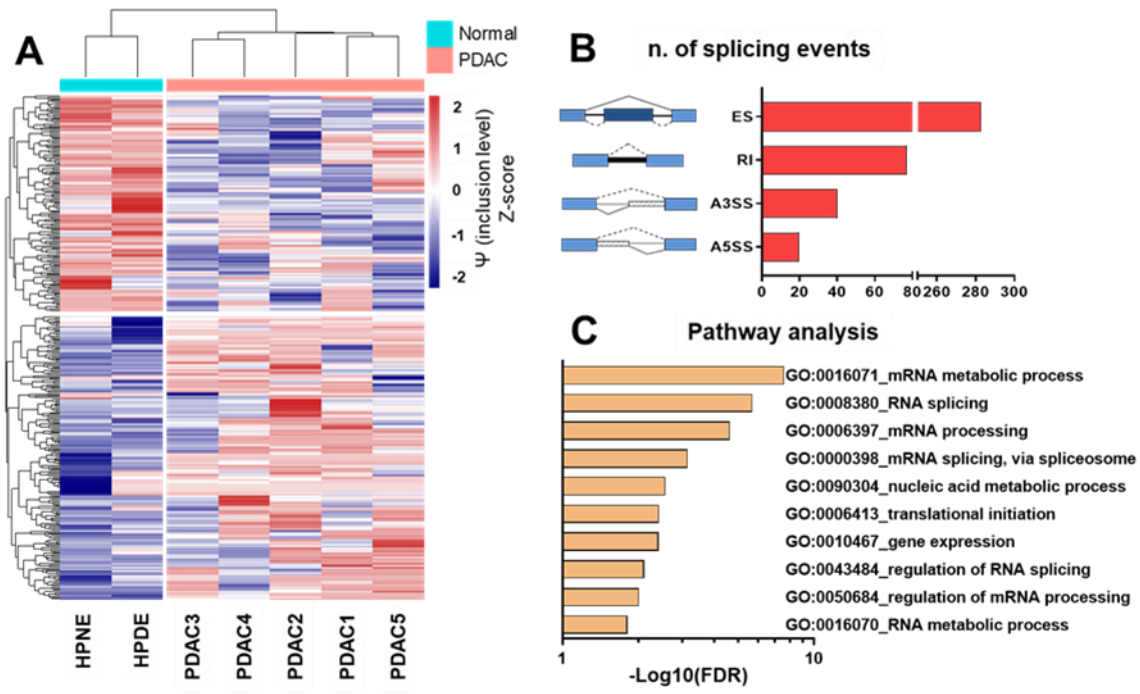
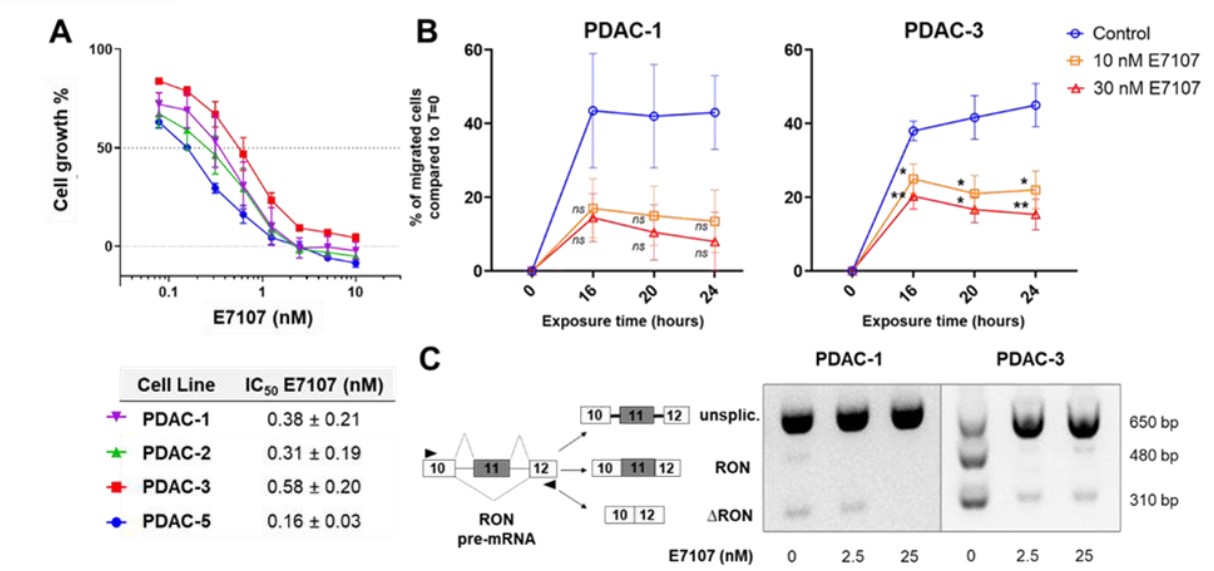


Figure 1. Global splicing profiles of PDAC primary cells differ from normal epithelial pancreatic cells. The figure depicts the results of differential splicing analysis performed on transcriptomics data of 5 PDAC primary cells versus 2 normal epithelial pancreatic cells.

(A) Hierarchical clustering performed using all significant differential splicing events (n=420). Inclusion levels (or Percentage Spliced-In, Y) per each event were Z-score-normalized and plotted as a heatmap in R version 3.5.0. The colored bar over the heatmap represents the phenotype of analyzed cells (Cyan: normal cells; Red: PDAC).

(B) Schematic representation of alternative splicing event types as detected by rMATS algorithm. ES: exon skipping (dark blue exon can be excluded from the mRNA transcript); RI: retained intron (thick black line represents the intron that is included in the mRNA); A3SS: alternative 3' splice site; A5SS: alternative 5' splice site (depending on the position of the splice site, part of introns are included in the mRNA and depicted as striped boxes). The number of significant splicing events (FDR<0.05) per each type are reported in the bar graph.

(C) Gene Ontology (GO) enrichment analysis using all genes affected by significant differential splicing events (FDR<0.05). The bar plot represents the 10 major GO terms including the largest numbers of genes in each term.



Cell Line	IC_{50} E7107 (nM)
PDAC-1	0.38 ± 0.21
PDAC-2	0.31 ± 0.19
PDAC-3	0.58 ± 0.20
PDAC-5	0.16 ± 0.03

Figure 2. Splicing modulator E7107 inhibits PDAC cell growth and migration *in vitro*.

(A) Dose-dependent inhibition of cell growth by E7107 in four PDAC primary cell lines (PDAC-1, -2, -3 and -5). Cell viability was determined using SRB assay upon 72 h treatment with increasing concentrations of E7107. Data points represent mean percentage of cell growth relative to untreated cells ± SD of three independent experiments. IC_{50} values ± SD (drug concentration that inhibits 50% of cell growth) were determined by graphical interpolation of dose-response curves and reported in the table below. PDAC-4 cells were not tested due to sub-optimal growth pattern.

(B) Wound healing assay performed for PDAC-1 and PDAC-3 cells incubated with 10 and 30 nM E7107 for 16, 20 and 24 hours after creating wound tracks in 96-well plates. Points represent % of migrated cells within the wound track ± SEM of three independent experiments (at least 6 wells per condition in each experiment). Asterisks indicate statistical significance compared to untreated cells at each timepoint (**P=0.01, *P=0.05, Student`s t-test). Growth pattern and cell adhesion of PDAC-2, -4 and -5 cells were not suitable for this assay.

(C) Splicing profiles of RON for PDAC-1 and PDAC-3 cells assessed by RT-PCR after 24 hours incubation with 2.5 and 25 nM E7107. Schemes of pre-mRNA structures with primer annealing sites (black triangles) and predicted PCR products are shown on the left.

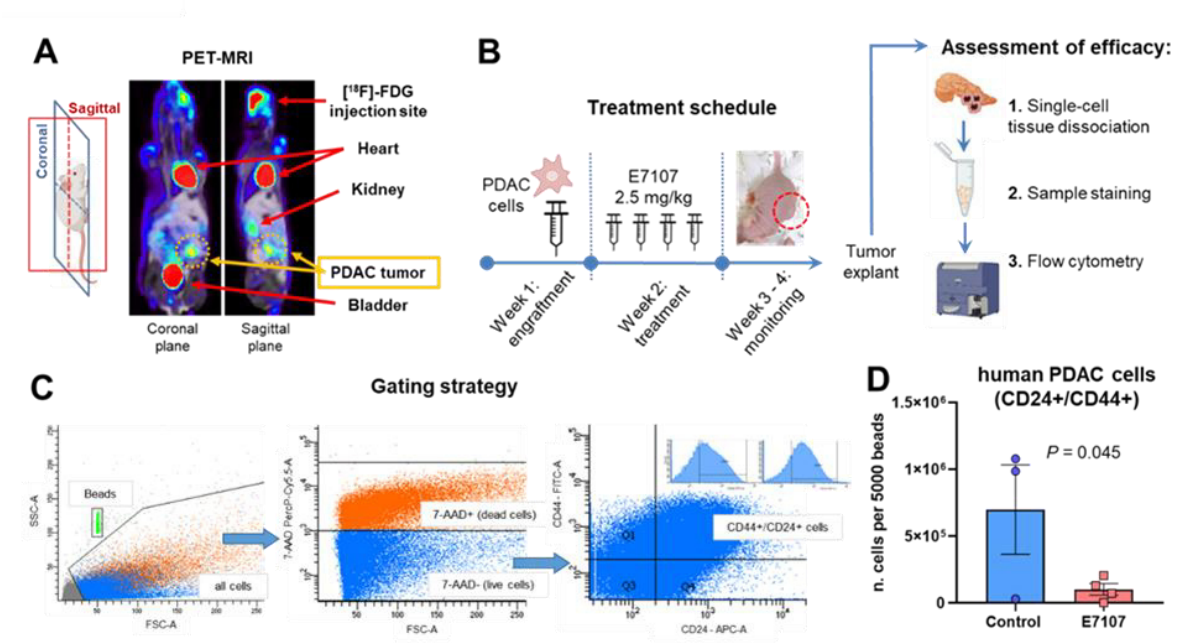


Figure 3. E7107 impairs tumor growth in an orthotopic PDAC mouse model.

(A) PET-MRI scan of PDAC orthotopic model performed one week after surgery. [18F]-FDG tracer was administered via intraocular injection.

(B) Experimental setup: PDAC-engrafted mice were randomized one week after surgery (4 animals per arm) and E7107 was administered four times (four doses of 2.5 mg/kg). Animals were monitored until sacrifice. Tumor burden was assessed via flow-cytometry. Pancreas and tumor infiltrates (see Supplemental Figure 4) were homogenized to obtain single-cell suspension, washed and stained with 7AAD for viability assessment and with antibodies binding specifically to hCD44 (FITC-labelled) and hCD24 (APC-labelled). Fluorescent beads were added to each sample before flow-cytometry measurement.

(C) Gating strategy: events were acquired by counting 5000 beads per sample. Viable human PDAC cells (7-AAD-negative) expressing both hCD44 and hCD24 surface markers were discriminated from murine cells.

(D) Semi-quantitative assessment of tumor burden in E7107-treated mice compared to untreated controls. Bar graph represents the number of human PDAC cells (CD24+/CD44+) measured every 5000 beads in each sample (Student`s t-test). One data point from the control group was excluded due to a technical error in sample labelling.

A

Clinical characteristics, outcome and SF3B1 protein expression in PDAC patients

Univariate analysis		N	OS, months (95% CI)	P	PFS, months (95% CI)	P
Age, years	≤65	42	21.5 (14.4 - 28.6)	*0.013**	15 (12.2 - 17.8)	*0.011**
	>65	45	18 (16.1 - 19.9)		12 (8.7 - 15.3)	
Sex	Male	42	19 (13.1 - 24.9)	*0.509*	15.5 (8.8 - 15.2)	*0.181*
	Female	45	19 (13.7 - 24.3)		12 (8.3 - 22.7)	
Nodal Status	N0	20	19.5 (6.3 - 32.6)	*0.526*	14 (11.4 - 16.6)	*0.965*
	N1	67	19 (16.7 - 21.3)		14 (10.4 - 17.5)	
Resection Margin	R0	65	19 (15.7 - 22.3)	*0.411*	14.7 (11.9 - 17.5)	*0.399*
	R1	22	19 (13.7 - 24.3)		13 (10 - 15)	
Grade	1 or 2	50	19 (14.5 - 23.50)	*0.247*	14 (11.1 - 16.9)	*0.192*
	3	37	19 (15.6 - 22.3)		14 (10.3 - 17.7)	
SF3B1 expression	Low	47	23 (20.7 - 25.3)	*0.007**	18 (13.3 - 22.7)	*0.007**
	High	40	18 (16 – 20)		12 (10.3 - 13.7)	

Multivariate analysis	df	Risk of death HR (95% CI)	P	Risk of relapse HR (95% CI)	P
Age	1	1.7 (1.1 - 2.7)	*0.019**	1.7 (1.1 - 2.8)	*0.016**
SF3B1 expression	1	1.8 (1.1 - 2.8)	*0.011**	1.8 (1.1 - 2.7)	*0.012**

CI, Confidence Intervals; df, degrees of freedom; OS, Overall Survival; PFS, Progression Free Survival; HR, Hazard ratio

B

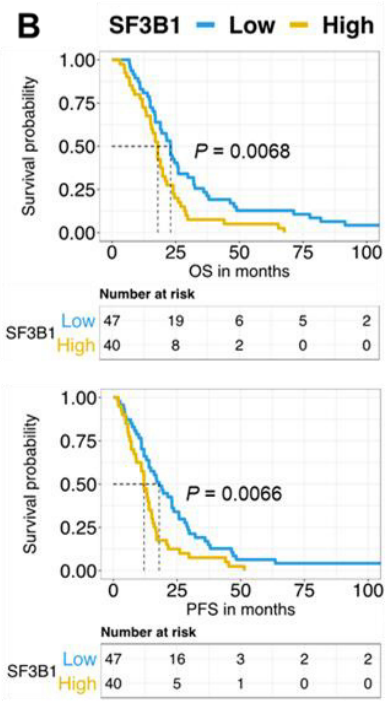


Figure 4. SF3B1 protein expression is an independent predictor of the clinical outcome in PDAC patients.

(A) Clinicopathological characteristics and SF3B1 protein expression in relation to the clinical outcome (overall survival – OS; progression-free survival – PFS) in PDAC patients. The results of univariate and multivariate Cox regression analysis are shown.

(B) Survival analysis of SF3B1 protein expression in relation to OS and PFS in PDAC patients. P-values were determined by the Log-rank test and plotted using the Kaplan-Meier method.

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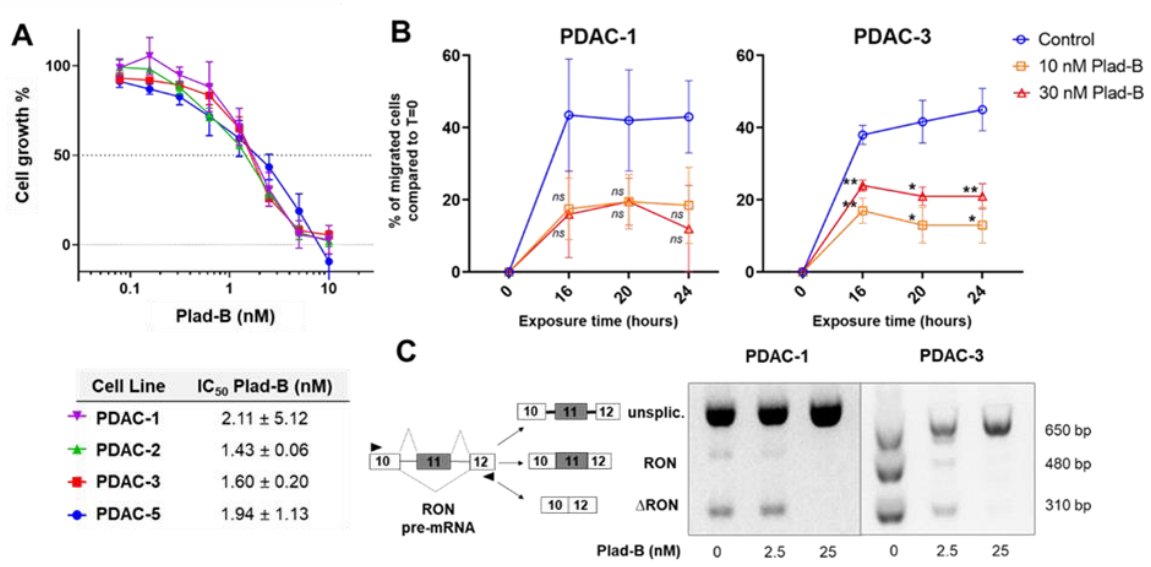
Supplementary Materials Chapter 5

Exploring splicing modulation as a novel strategy against pancreatic cancer



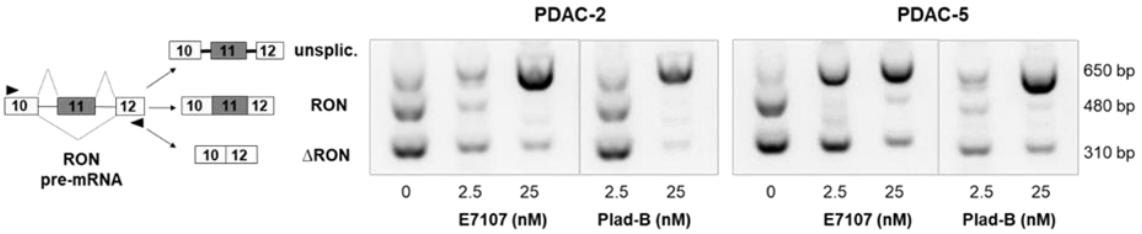
Cell Line	IC_{50} Plad-B (nM)
PDAC-1	2.11 ± 5.12
PDAC-2	1.43 ± 0.06
PDAC-3	1.60 ± 0.20
PDAC-5	1.94 ± 1.13

Supplemental Figure 1. Splicing modulator Plad-B inhibits PDAC cell growth and migration *in vitro*.

(A) Dose-dependent inhibition of cell growth by Plad-B in four PDAC primary cell lines (PDAC-1, -2, -3 and -5). Cell viability was determined using SRB assay upon 72 h treatment with increasing concentrations of Plad-B. Data points represent mean percentage of cell growth relative to untreated cells ± SD of three independent experiments. IC_{50} values ± SD (drug concentration that inhibits 50% of cell growth) were determined by graphical interpolation of dose-response curves and reported in the table below. PDAC-4 cells were not tested due to sub-optimal growth pattern.

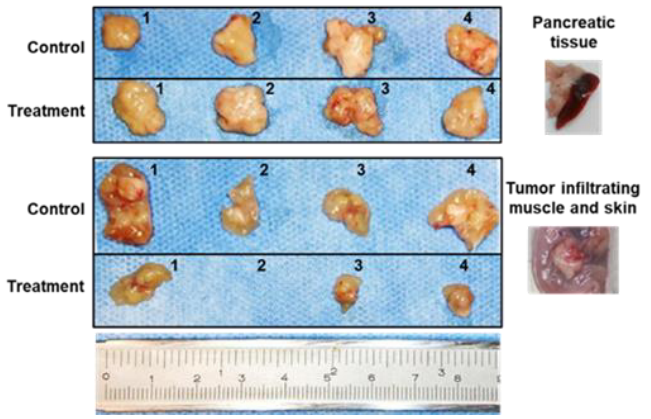
(B) Wound healing assay performed for PDAC-1 and PDAC-3 cells incubated with 10 and 30 nM Plad-B for 16, 20 and 24 hours after creating wound tracks in 96-well plates. Points represent % of migrated cells within the wound track ± SEM of three independent experiments (at least 6 wells per condition in each experiment). Asterisks indicate statistical significance compared to untreated cells at each timepoint (**P=0.01, *P=0.05, Student`s t-test). Growth pattern and cell adhesion of PDAC-2, -4 and -5 cells were not suitable for this assay.

(C) Splicing profiles of RON for PDAC-1 and PDAC-3 cells assessed by RT-PCR after 24 hours incubation with 2.5 and 25 nM Plad-B. Schemes of pre-mRNA structures with primer annealing sites (black triangles) and predicted PCR products are shown on the left.



Supplemental Figure 2. Splicing profiles of RON for PDAC-2 and PDAC-5 cells assessed by RT-PCR after 24 hours incubation with 2.5 and 25 nM E7107 and Plad-B. Schemes of pre-mRNA structures with primer annealing sites (black triangles) and predicted PCR products are shown on the left.

A



B

	Average weight (g)	
Day	**Control**	**E7107**
1 (start treat.)	20,8±1,6	21,6±2,3
2	21,4±1,6	21,2±2,2
3	20,8±1,6	20,2±2,3
4 (end treatm.)	21,2±1,6	19,8±2,1
7	21,7±1,5	20,5±2,2
14	21,4±1,5	22,3±2
21	22,4±1,9	23,2±2,1

Supplemental Figure 3. (A) Macroscopic analysis of explanted PDAC tumors prior single-cell dissociation and FACS analysis. Pancreatic tissue and tumor infiltrating muscle and skin tissues at the site of surgery are shown. **(B)** Average weight of mice ± SD from control and E7107-treated groups.

Tables

Table 1. Significant differential splicing events detected by rMATS analysis (FDR<0.05).

Event ID	Gene Symbol	FDR	Δψ	AS type	Event ID	Gene Symbol	FDR	Δψ	AS type
10354	YWHAH	0	0,485	ES	7761	APP	0,000125122	-0,031	ES
10355	YWHAH	0	0,477	ES	8128	RP11-33B1.1	0,000158217	-0,551	ES
39953	USMG5	0	0,21	ES	27608	MTMR14	0,000185501	-0,289	ES
59274	PPIA	2,21E-09	0,029	ES	18477	OSER1-AS1	0,000204045	0,672	ES
53209	EIF3B	7,90E-09	-0,091	ES	32735	ZNF90	0,000204045	-0,526	ES
39952	USMG5	9,20E-08	0,215	ES	37152	SNHG8	0,000214847	0,334	ES
67093	ALDOA	4,39E-07	0,034	ES	55608	TBC1D2	0,000217331	-0,312	ES
46614	UQCRH	2,38E-06	0,049	ES	10583	C17orf61-PLSCR3	0,000248823	0,398	ES
13015	ACTN1	1,09E-05	0,061	ES	64468	FAM192A	0,00025913	-0,551	ES
29010	ASPH	1,09E-05	0,031	ES	9809	LTBP4	0,00025913	-0,517	ES
41458	TRA2A	1,09E-05	-0,271	ES	31341	NT5C3B	0,000266412	-0,098	ES
41456	TRA2A	1,58E-05	-0,277	ES	56205	HNRNPH1	0,000302674	-0,022	ES
52891	NPC2	1,93E-05	-0,05	ES	19340	KIF23	0,00031007	0,211	ES
37644	ATP5I	1,95E-05	0,093	ES	37154	SNHG8	0,000310581	0,376	ES
66057	MYL6	2,25E-05	0,316	ES	42768	SPATS2L	0,000313612	-0,169	ES
33201	RPLP0	2,32E-05	0,06	ES	40918	ULK3	0,00040195	-0,621	ES
52894	NPC2	2,32E-05	-0,049	ES	48038	PTBP1	0,000500699	-0,288	ES
7560	MYOF	2,32E-05	0,111	ES	56186	HNRNPH1	0,000587596	0,115	ES
33195	RPLP0	3,44E-05	0,039	ES	50220	GEMIN4	0,000630635	0,532	ES
60285	CTNND1	3,44E-05	-0,049	ES	39605	AFTPH	0,000651456	0,423	ES
25441	NUDT2	5,59E-05	-0,555	ES	65111	SNHG5	0,000751404	0,061	ES
34986	RBM39	5,78E-05	-0,107	ES	51393	CHCHD7	0,000813681	0,62	ES
30879	MYO6	6,56E-05	-0,605	ES	52892	NPC2	0,001075372	-0,038	ES
33825	SRSF7	6,56E-05	-0,063	ES	49498	IFI27	0,001143991	-0,127	ES
18711	CLCC1	7,55E-05	-0,318	ES	49417	HNRNPL	0,001216096	-0,051	ES
52765	CNN2	7,55E-05	0,047	ES	17447	PTPN13	0,00143665	-0,093	ES
55215	APLP2	7,55E-05	-0,132	ES	56195	HNRNPH1	0,00151278	0,051	ES
20591	SNRNP70	7,88E-05	0,029	ES	41579	WDR45B	0,001632097	-0,143	ES
6086	AGBL5	7,88E-05	-0,722	ES	37756	C3orf67	0,001642123	0,559	ES
34841	ACIN1	8,15E-05	0,073	ES	33938	MBD1	0,001674044	-0,284	ES
17366	TPM1	9,99E-05	-0,287	ES	64696	NONO	0,001674044	0,072	ES
29016	ASPH	0,000106082	-0,025	ES	9801	LTBP4	0,001674044	-0,125	ES
53471	GBAP1	0,000106082	0,717	ES	26650	SUZ12P1	0,001733568	0,458	ES
60509	RP11-421L21.3	0,000106082	0,572	ES	52162	STAU2	0,001776312	0,46	ES
33824	SRSF7	0,000106442	-0,045	ES	48660	PARP12	0,00189901	-0,199	ES

Event ID	Gene Symbol	FDR	Δψ	AS type
57712	DNMT3B	0,00199866	-0,678	ES
6484	CAST	0,002088041	0,076	ES
11529	VTI1A	0,002094588	-0,501	ES
58664	RABGAP1L	0,002120505	-0,402	ES
46470	WDR89	0,002254592	-0,509	ES
48153	SLC25A43	0,002254592	0,458	ES
30105	DMWD	0,002520987	-0,306	ES
42550	FAM126A	0,002573289	-0,068	ES
27233	HNRNPDL	0,002576635	0,045	ES
65040	LCORL	0,002576635	0,465	ES
8883	SAP30L	0,002576635	0,238	ES
49499	IFI27	0,002608841	-0,114	ES
64470	FAM192A	0,002835556	-0,546	ES
46278	MORF4L2	0,003203174	0,068	ES
17516	SRP14	0,003493104	0,011	ES
17927	ASNS	0,003499653	0,095	ES
53729	SYTL3	0,003712143	0,382	ES
6308	PRDM5	0,003712143	0,59	ES
47626	HKR1	0,003857307	0,698	ES
39830	ELMOD3	0,003863742	-0,184	ES
22562	FANCL	0,003867942	0,268	ES
59269	PPIA	0,003867942	0,016	ES
61878	PUS1	0,003867942	-0,045	ES
62536	IMPDH2	0,004097256	0,029	ES
15123	PLSCR1	0,004136022	0,125	ES
3277	SEC14L1	0,004623205	-0,246	ES
40759	TMEM241	0,004702661	-0,241	ES
3549	EIF2A	0,004780257	-0,066	ES
50363	SLC38A2	0,004821345	-0,052	ES
3506	IL4R	0,00496805	-0,067	ES
23177	EDEM2	0,005176751	-0,187	ES
55997	HAPLN3	0,005176751	0,373	ES
58779	RTFDC1	0,005494791	-0,36	ES
63429	ZNF213-AS1	0,005841893	0,401	ES
29767	ANXA2	0,006176075	0,021	ES
5395	CD44	0,006326866	0,077	ES
29901	FAT1	0,006349348	0,412	ES
33200	RPLP0	0,006402198	0,052	ES
5352	CD44	0,006407047	0,1	ES
23171	EDEM2	0,006526736	-0,139	ES
5498	MAPK13	0,006526736	0,142	ES
42030	TNNT1	0,006798022	-0,201	ES
47990	PLD3	0,006798022	0,533	ES
55981	PCBP2	0,007145562	0,143	ES
36770	RP11-706O15.1	0,007535082	-0,348	ES
5394	CD44	0,007535082	-0,019	ES
65116	SNHG5	0,007535082	0,1	ES
65790	C8orf59	0,007535082	0,168	ES
65791	C8orf59	0,007736952	0,155	ES
5905	UBE3A	0,007982561	0,467	ES
23604	USP34	0,008366815	0,159	ES
29721	TMEM263	0,008450521	-0,265	ES
8532	MND1	0,008450521	0,144	ES
54164	HMBOX1	0,00862438	-0,485	ES
45039	MIF4GD	0,008719905	0,216	ES
37111	ZNF782	0,009094369	-0,412	ES
46270	MORF4L2	0,009581954	0,044	ES
47192	PPT1	0,009689581	-0,043	ES
62534	IMPDH2	0,009689581	0,066	ES
65792	C8orf59	0,009689581	0,158	ES
66586	ZFR	0,009689581	0,08	ES
27246	RDH13	0,009766898	-0,226	ES
3546	EIF2A	0,009806831	-0,048	ES
21986	FAM49B	0,010007121	-0,289	ES
39184	FDPS	0,010726211	0,133	ES
7554	MYOF	0,01113932	-0,045	ES
40232	USB1	0,011657272	-0,136	ES
52305	HNRNPA1	0,011969411	0,04	ES
8513	TXNRD1	0,012265479	0,025	ES
326	ETFA	0,012382269	-0,048	ES
65100	SNHG5	0,012382269	0,111	ES
46273	MORF4L2	0,012411955	0,063	ES
48442	ZNF678	0,012928395	-0,696	ES
24728	ZNF680	0,012995393	-0,455	ES
3558	TFDP2	0,012995393	0,097	ES
39192	FDPS	0,012995594	0,032	ES

Event ID	Gene Symbol	FDR	Δψ	AS type	Event ID	Gene Symbol	FDR	Δψ	AS type
3355	PIEZO1	0,013195875	-0,131	ES	5365	CD44	0,022719594	0,206	ES
13012	ACTN1	0,013264348	-0,185	ES	61875	PUS1	0,022719594	0,08	ES
30183	NPRL3	0,01340064	0,303	ES	52478	PEMT	0,02272464	0,061	ES
62219	DLEU2	0,014085898	0,386	ES	10313	RSU1	0,023002338	0,115	ES
66058	MYL6	0,014402685	0,051	ES	52813	SEC31A	0,023184505	-0,19	ES
52769	CNN2	0,015269072	0,156	ES	16551	SP140L	0,023304349	0,182	ES
41133	JARID2	0,015538834	-0,196	ES	28429	AP3S2	0,023304349	-0,437	ES
25405	MECOM	0,015579003	-0,358	ES	7414	CUL9	0,023304349	-0,238	ES
23875	CD46	0,015695603	0,335	ES	28741	NUP62	0,023532765	-0,122	ES
9961	C11orf73	0,015695603	-0,071	ES	34977	RBM39	0,023532765	-0,062	ES
45357	SREK1	0,016049237	-0,246	ES	67316	ABI1	0,023532765	0,134	ES
67136	SPPL2A	0,016049237	0,162	ES	2521	IFT22	0,023655231	-0,213	ES
31533	SLC25A39	0,016357813	0,061	ES	2250	CLSTN1	0,023656119	0,321	ES
42767	SPATS2L	0,016357813	-0,738	ES	62625	DCTN2	0,023710375	-0,044	ES
22439	FAM122B	0,016956412	-0,198	ES	12125	PSMB3	0,023831926	-0,034	ES
64991	DHX35	0,016956412	-0,092	ES	53490	PFKM	0,023831926	0,072	ES
21537	RBM4	0,017217179	0,272	ES	11350	EIF6	0,024349686	0,059	ES
62609	PIGB	0,017217179	0,315	ES	42782	TBC1D17	0,024539349	-0,047	ES
17375	TPM1	0,017777138	0,063	ES	30186	NPRL3	0,025215687	0,195	ES
16257	GOLGA4	0,018411794	0,137	ES	5444	TEP1	0,025215687	-0,343	ES
41193	PCK2	0,018558268	-0,106	ES	64269	PTGES2	0,025557486	-0,066	ES
63675	RBM26	0,018558268	0,165	ES	27810	VCPKMT	0,025870915	-0,205	ES
48940	PRDM8	0,018880103	-0,318	ES	36715	TMEM69	0,026217587	0,057	ES
31485	HLA-J	0,018917793	0,321	ES	62538	IMPDH2	0,026217587	0,037	ES
5938	SUPT20H	0,018917793	0,25	ES	46611	UQCRH	0,026741045	0,245	ES
27455	GTF3A	0,019411845	-0,054	ES	46963	NEK1	0,026741045	-0,181	ES
11347	EIF6	0,019802943	0,064	ES	65102	SNHG5	0,026741045	0,112	ES
53997	LDLR	0,02014152	-0,061	ES	68071	SNHG6	0,026741045	0,029	ES
31379	APH1A	0,020443262	-0,057	ES	33627	DPM1	0,026950868	0,037	ES
3623	COPZ2	0,020540637	0,057	ES	33877	KLF7	0,026950868	-0,035	ES
22110	RAD51AP1	0,020647724	-0,148	ES	17697	CDK11B	0,027005514	0,192	ES
4919	PSMG4	0,020647724	0,131	ES	27193	VPS13B	0,027005514	0,571	ES
26891	BTBD1	0,020783842	-0,046	ES	44808	COA1	0,027620203	0,23	ES
55213	APLP2	0,021703281	0,04	ES	33726	RBCK1	0,02892161	-0,151	ES
18592	FYN	0,022214022	0,043	ES	34262	U2AF1L4	0,02892161	0,106	ES
41419	CDC42BPA	0,022413038	0,25	ES	23160	STAU1	0,02950215	-0,214	ES
41765	TXNL4A	0,022719594	0,086	ES	33730	RBCK1	0,02962746	-0,111	ES
42775	SPATS2L	0,022719594	0,46	ES	60508	RP11-421L21.3	0,029700422	0,385	ES

Event ID	Gene Symbol	FDR	Δψ	AS type
58039	ZDHHC16	0,030194597	0,083	ES
40117	ARID4B	0,03066724	-0,053	ES
27734	MRE11A	0,031104551	-0,147	ES
68072	SNHG6	0,031159833	-0,087	ES
24099	CCDC112	0,031275595	0,251	ES
66836	PHLPP2	0,031275595	0,364	ES
20062	TMEM161B	0,031703576	0,196	ES
66945	ZNF75A	0,031703576	-0,034	ES
21999	FAM49B	0,031726549	-0,145	ES
11982	POFUT2	0,032367318	0,148	ES
2287	FAM193B	0,032367318	0,403	ES
21794	CCNT2	0,032714065	-0,134	ES
23758	ERI3	0,032714065	0,066	ES
58752	TSPAN5	0,033090628	-0,077	ES
49373	MFF	0,033954413	0,16	ES
29321	KIF13A	0,034459319	-0,133	ES
67841	C11orf80	0,035197002	0,213	ES
12904	LRRFIP2	0,03579312	0,163	ES
37635	BCL2L13	0,03579312	0,08	ES
11263	DDX11	0,035951762	0,211	ES
173	PCNP	0,035951762	-0,059	ES
23867	CD46	0,035951762	0,146	ES
38850	WARS	0,035979066	-0,554	ES
67306	PUF60	0,035979066	-0,166	ES
58036	ZDHHC16	0,037581118	0,076	ES
19657	IFT122	0,038333676	-0,161	ES
55982	PCBP2	0,038500207	0,054	ES
52070	KIAA1109	0,038520155	-0,101	ES
31295	NR2C1	0,038827579	0,134	ES
43684	BAZ1A	0,039437671	-0,163	ES
54885	ERGIC3	0,03971388	-0,045	ES
61531	ACSL3	0,040744346	-0,218	ES
8626	ST3GAL3	0,041094674	-0,188	ES
7839	ELP3	0,041141715	-0,083	ES
31034	TMEM126A	0,04141805	-0,138	ES
54426	LARP7	0,041554303	-0,049	ES
27088	CPSF6	0,04183291	-0,24	ES
40752	TMEM241	0,043173058	0,538	ES
4959	TNRC18	0,043173058	0,337	ES
51005	CLTC	0,043173058	0,03	ES
57443	GAB1	0,043173058	-0,194	ES
60038	CHMP1A	0,043173058	-0,054	ES
36055	CANX	0,043211079	-0,097	ES
37637	BCL2L13	0,043471003	0,088	ES
38828	WARS	0,044140481	-0,401	ES
62947	ADRM1	0,045003105	0,087	ES
15815	ZNF721	0,045648426	-0,279	ES
59726	HABP4	0,047068921	-0,249	ES
14287	TCF12	0,047405334	-0,111	ES
315	ETFA	0,047871673	0,302	ES
22902	UBE2C	0,047900237	0,291	ES
29759	ANXA2	0,048286717	0,023	ES
63694	PLAC8	0,048286717	0,319	ES
57847	AC004381.6	0,048440321	0,401	ES
44629	ARSK	0,048686124	0,077	ES
12123	PSMB3	0,049115397	-0,054	ES
21275	ELK1	0,04941949	-0,171	ES
54473	ERRFI1	0,04941949	-0,025	ES
32154	CALM2	0,049724344	-0,128	ES
1621	GTPBP1	0,049757242	0,436	ES
21168	MIOS	0,049757242	-0,226	ES
5270	DUXAP8	5,57E-10	-0,85	A3SS
5837	ACTG1	1,21E-09	-0,034	A3SS
1393	HLA-C	1,20E-08	0,079	A3SS
554	TOR1AIP1	2,85E-07	-0,614	A3SS
4900	SLMO2	0,000335747	0,037	A3SS
6102	MORF4L2	0,000749024	0,228	A3SS
8308	CIART	0,000749024	-0,375	A3SS
8472	STRN4	0,000749024	-0,474	A3SS
4603	RP1-197B17.3	0,002047631	0,646	A3SS
7248	RNF216	0,007009235	-0,246	A3SS
8746	RABGGTA	0,007009235	0,169	A3SS
7798	MPV17	0,011356252	-0,167	A3SS
2482	CSE1L	0,011576042	0,043	A3SS
7072	EIF3B	0,011979017	-0,301	A3SS
2889	FAM122B	0,012034816	-0,148	A3SS

Event ID	Gene Symbol	FDR	Δψ	AS type
2937	LMBR1L	0,0125928	0,513	A3SS
6728	ZNF138	0,0125928	-0,308	A3SS
5537	AGTRAP	0,013183098	-0,345	A3SS
575	SNRPB	0,013183098	-0,095	A3SS
5698	ANKHD1	0,016263437	0,173	A3SS
7725	PSAP	0,016263437	0,029	A3SS
3511	APOL3	0,016668017	0,338	A3SS
5536	AGTRAP	0,016668017	-0,307	A3SS
6316	EIF4G1	0,018703631	-0,293	A3SS
1484	CYB561D1	0,020269087	0,184	A3SS
6225	TMEM214	0,021710833	-0,576	A3SS
3000	WDR75	0,024949482	0,008	A3SS
7377	STXBP4	0,024949482	-0,428	A3SS
5982	SREK1	0,027390675	-0,121	A3SS
1811	SMG7	0,028259638	0,072	A3SS
188	STAG3L5P-PVRIG2P-PILRB	0,028502771	-0,349	A3SS
115	PPP1R12A	0,030036632	0,021	A3SS
5983	SREK1	0,032527426	-0,395	A3SS
761	PRDM5	0,032658324	0,538	A3SS
6104	MORF4L2	0,033624134	0,123	A3SS
7032	SEC31A	0,039833732	-0,174	A3SS
8368	ZNF232	0,048506342	-0,07	A3SS
4443	PTPRG-AS1	0,049277866	-0,226	A3SS
8583	TCTN1	0,049277866	0,366	A3SS
9001	OCIAD2	0,049277866	-0,067	A3SS
5320	PARP2	5,07E-10	-0,683	A3SS
2474	PPIE	1,73E-06	-0,104	A3SS
3103	SNHG8	2,23E-05	0,252	A3SS
1687	PDXK	0,000128943	0,559	A3SS
3139	PRPSAP2	0,001762532	0,482	A3SS
5475	KAT6B	0,001762532	0,546	A3SS
2183	TTC31	0,004609881	0,476	A3SS
5474	KAT6B	0,006470994	0,466	A3SS
3887	UBAP2L	0,007609514	-0,113	A3SS
4175	COPS3	0,007609514	-0,394	A3SS
378	ILKAP	0,010060501	-0,418	A3SS
3108	ZBTB8OS	0,010730847	-0,377	A3SS
5411	SNHG5	0,013463225	0,114	A3SS
527	ZNF653	0,01647713	-0,294	A3SS
547	RPS12	0,024017009	-0,008	A3SS
5019	TMEM256-PLSCR3	0,024384658	0,249	A3SS
4660	OCIAD1	0,02576111	0,066	A3SS
518	WDR91	0,03498053	-0,538	A3SS
5514	MYL6	0,03498053	0,139	A3SS
3926	SETD2	0,048528859	0,345	A3SS
431	NID2	2,38809E-12	-0,805	RI
6398	ACTB	4,37816E-12	-0,031	RI
5480	HNRNPH1	1,88062E-09	-0,053	RI
3260	SRSF7	1,72467E-07	-0,055	RI
64	HLA-A	7,58087E-07	-0,038	RI
5211	RPS2	1,52548E-06	-0,019	RI
4290	YBX3	2,55978E-06	-0,021	RI
4105	COX7C	7,57937E-05	-0,766	RI
1236	POLR1C	8,81338E-05	-0,079	RI
2524	POLE3	0,000111919	-0,115	RI
3354	RBM39	0,000146299	-0,138	RI
2549	PSMC5	0,000269018	-0,013	RI
2834	SMYD2	0,000330034	-0,031	RI
5481	HNRNPH1	0,000364332	-0,027	RI
2296	CSNK1D	0,000383436	-0,077	RI
2342	CD46	0,00042123	0,072	RI
5483	HNRNPH1	0,00042123	-0,041	RI
1138	GNB2L1	0,000618528	-0,018	RI
4152	PLCD4	0,001027662	-0,288	RI
53	ARL16	0,00129482	0,332	RI
5315	RPL17	0,001675716	-0,087	RI
6445	ARAP3	0,001919775	-0,259	RI
2031	IFT80	0,002599721	0,324	RI
1168	TMEM55B	0,003197889	-0,088	RI
3050	XPNPEP1	0,00332968	-0,134	RI
3334	SOD2	0,00332968	-0,15	RI
4904	DDX23	0,003606081	-0,05	RI
4251	GLTSCR2	0,004376086	0,008	RI
4282	PRMT5	0,004507942	-0,021	RI
4292	YBX3	0,006143137	-0,279	RI

Event ID	Gene Symbol	FDR	Δψ	AS type
6037	GMPR2	0,006143137	0,072	RI
1179	BAZ2A	0,006145712	-0,138	RI
6382	MRPL28	0,006145712	-0,225	RI
4003	FYCO1	0,006458167	0,237	RI
1775	AHSA2	0,00775104	0,244	RI
2856	BDH2	0,009134778	-0,342	RI
2996	RPS16	0,009134778	-0,038	RI
3540	NFE2L1	0,009134778	0,044	RI
3596	HMGN1	0,009134778	-0,028	RI
4542	APBB3	0,009134778	-0,298	RI
4657	LINC00240	0,009134778	-0,573	RI
6339	DUSP28	0,009134778	-0,35	RI
6622	PSMB4	0,009134778	-0,051	RI
8	TAF1D	0,009134778	0,042	RI
2442	COL7A1	0,010702523	-0,28	RI
727	FAM135A	0,011576654	-0,287	RI
699	ATP5G1	0,012214022	0,378	RI
5408	RGL2	0,017296116	-0,128	RI
976	SKIV2L	0,022917422	0,064	RI
5858	AKR1B1	0,024849441	0,02	RI
4617	CELSR3	0,025252766	-0,442	RI
6567	TMEM91	0,025252766	-0,409	RI
1919	IDI1	0,02686997	-0,029	RI
6134	HSF4	0,027781068	0,29	RI
5149	SAT2	0,028340442	0,099	RI
3079	FBXO9	0,029665887	-0,078	RI
2948	RAD51D	0,029881136	-0,1	RI
5429	HDLBP	0,029881136	-0,156	RI
1121	HLA-C	0,030025671	-0,445	RI
4611	RAD52	0,030025671	0,216	RI
5924	FN1	0,030025671	0,084	RI
4460	IFT88	0,030657995	0,241	RI
5198	EIF3B	0,033781751	-0,031	RI
4360	ACTG1	0,036588901	-0,02	RI
2779	RPL3	0,038943881	0,161	RI
4486	CPSF7	0,038943881	-0,033	RI
4588	EIF4A1	0,04073975	-0,044	RI
6213	TGFB1I1	0,041061894	-0,197	RI
5681	RPS26	0,041833259	-0,01	RI
6044	CYB5A	0,042736083	-0,015	RI
929	DDX5	0,043472732	-0,044	RI
3080	KLHDC2	0,04459965	-0,069	RI
4304	COMMD4	0,045663097	0,385	RI
1553	CCDC14	0,048607976	-0,264	RI
2518	RP1-178F15.5	0,048607976	0,127	RI
3341	NDUFB10	0,049913734	-0,051	RI
5276	SYNE4	0,049913734	0,292	RI

Chapter 6

SF3B1 modulators affect key genes in metastasis and drug influx: a new approach to fight pancreatic cancer chemoresistance

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Abstract

Aim: Because mutations of splicing factor 3B subunit-1 (SF3B1) have been identified in 4% of pancreatic ductal adenocarcinoma (PDAC) patients, we investigated the activity of new potential inhibitors of SF3B1 in combination with gemcitabine, one of the standard drugs, in PDAC cell lines.

Methods: One imidazo[2,1-*b*][1,3,4]thiadiazole derivative (IS1) and three indole derivatives (IS2, IS3 and IS4), selected by virtual screening from an in-house library, were evaluated by the sulforhodamine-B and wound healing assay for their cytotoxic and antimigratory activity in the PDAC cells SUIT-2, Hs766t and Panc05.04, the latter harbouring the SF3B1 mutations. The effects on the splicing pattern of proto-oncogene recepteur d'origine nantais (RON) and the gemcitabine transporter human equilibrative nucleoside transporter-1 (hENT1) were assessed by PCR, while the ability to reduce tumour volume was tested in spheroids of primary PDAC cells.

Results: The potential SF3B1 modulators inhibited PDAC cell proliferation and prompted induction of cell death. All compounds showed an interesting anti-migratory ability, associated with splicing RON/ΔRON shift in SUIT-2 cells after 24 h exposure. Moreover, IS1 and IS4 potentiated the sensitivity to gemcitabine in both conventional 2D monolayer and 3D spheroid cultures, and these results might be explained by the statistically significant increase in hENT1 expression ($P<0.05$ *vs*. untreated control cells), potentially reversing PDAC chemoresistance.

Conclusion: These results support further studies on new SF3B1 inhibitors and the role of RON/hENT1 modulation to develop effective drug combinations against PDAC.

Keywords: Pancreatic ductal adenocarcinoma, gemcitabine, indole derivatives, anti-proliferative activity, anti- migratory activity, SF3B1, RON, hENT1

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers in the world. The survival rate has increased in recent years, and double-digit survival rates are increasingly seen, but epidemiological studies also report a rising incidence [1,2]. Thus, PDAC is projected to become the second leading cause of cancer-related death by 2030 [3]. This grim future has multifactorial causes. There are no tools for prevention, and early diagnosis of PDAC is complicated. Most patients are diagnosed when the tumour has already spread throughout the body due to the lack of early symptoms and specific biomarkers [4,5]. The treatment options for PDAC are also relatively limited. The only current curative treatment at the moment is surgical resection, which is possible in only 20% of patients. Moreover, this treatment has a high complication rate and recurrence is often seen [6]. The standard of care treatment is chemotherapy, using polychemotherapy regimens or gemcitabine monotherapy [7]. Gemcitabine, approved by the Food and Drug Administration in 1996, was the standard of care in the treatment of locally advanced and metastatic PDAC for over two decades. A better efficacy was found for various chemotherapy combinations such as FOLFIRINOX [5-fluorouracil, folinic acid (leucovorin), irinotecan, oxaliplatin] and gemcitabine plus nab-paclitaxel (GEM-NAB, Abraxane®) [8].

Most PDAC cases are characterised by inherent or acquired chemoresistance. This resistant behaviour is determined by multiple cellular-autonomous factors, such as reduced expression of key drug transporters, and/or by different components of the tumour microenvironment (TME) [9,10].

Recent studies suggest that alternative splicing (AS) deregulation plays a pivotal role in tumorigenesis and cancer drug resistance [11,12]. Aberrant splicing has been shown to occur in genes involved in drug metabolism, including transporters responsible for drug uptake. In this regard, a well-known example of aberrant splicing is the exon 13 skipping in the *SLC29A1* gene (solute carrier family 29 member 1) which encodes the human equilibrative nucleoside transporter-1 (hENT1) [13]. This splicing alteration is due to an intronic mutation which leads to a reduction in the expression and uptake of another cytidine analogue, cytarabine [11,14].

Drug resistance is also associated with alterations in genes that regulate apoptosis, often generating proteins with antagonistic functions (e.g., BCL-X and MCL-1) or migration (e.g., RON), favouring invasion and metastasis. Noteworthy, the pre-mRNA splicing process is also involved in the regulation of the DNA damage repair, influencing with high probability the

resistance to therapy [10,11,14,15].

Additionally, aberrations directly affect splicing regulation, and it has recently been demonstrated that somatic mutations of splicing factor genes are common in not only hematopoietic neoplasms but also solid tumours including PDAC [16]. The splicing factor 3B subunit-1 (SF3B1), which is involved in the branch site recognition during the pre-mRNA splicing process, is the most frequently mutated RNA splicing factor gene in cancer, and mutations in the HEAT domain of the *SF3B1* gene have been detected in 4% of PDAC patients [12].

Against this background, and given the central role of AS in cancer, targeting this process is considered a potential therapeutic approach. Pre-clinical studies have shown potential in the modulation of splicing in cancer cells via small molecules targeting SF3B1 [11], namely pladienolide B (PB), spliceostatin A and herboxidiene, which interfere with the splicing modulation [17]. Two synthetic analogues of PB, E7107 and H3B-8800 (orally available small molecule), are the only SF3B1 modulators in clinical trials [18]. Of note, one patient with acinar pancreatic carcinoma and hepatic metastases had a confirmed partial response lasting eight months during the phase I trial testing E7107, but severe ophthalmologic disturbances halted further clinical development of this drug [19].

As mentioned above, splicing modulation represents an innovative and interesting therapeutic strategy in the fight against cancer.

Preclinical studies revealed that low-dose splicing modulators are synergistic in combination with conventional anticancer agents [20,21]. We previously demonstrated that modulation of splicing in cancer cells was an effective therapy in an *in vivo* model, both as a monotherapy with direct inhibitors of SF3B1 and in combination with other anticancer agents, with acceptable toxicity [11]. This combination could expand the therapeutic window of the splicing modulators.

Therefore, investigations on new molecules that could target aberrant splicing in PDAC are warranted. In the present study, we performed structural computational studies and virtual screening of compounds available in an in-house molecular library, and we selected some indole derivatives to evaluate their antitumour activity in appropriate preclinical models of

PDAC and their potential to fight molecular mechanisms underlying PDAC chemoresistance. In particular, we used the epithelial and mesenchymal cells SUIT-2 and Hs766t, as well as Panc05.04 cells, carrying the *SF3B1* mutations p.Q699H and p.K700E.

Remarkably, heterocyclic compounds play a pivotal role in the field of drug design because the insertion of a heterocyclic moiety into a molecule can modulate drug properties such as potency and selectivity through bioisosterism, lipophilicity, polarity and aqueous solubility [22]. Among heterocyclic compounds, indoles have been investigated extensively due to their interesting versatility [23]. Many natural and synthetic derivatives of indoles have indeed shown a wide spectrum of pharmacological properties including antibacterial [24-27], antifungal [28,29], anti-inflammatory [30], antihistamine [31], anticholinesterase, antioxidant [32], anti-diabetic [33], antiviral [34] and anticancer activities [35,36].
The promising results obtained in our previous studies concerning the anticancer properties of imidazo[2,1-*b*][1,3,4]thiadiazole and indole scaffolds [25,37-40] prompted us to explore the biological activity of the selected compounds alone and in combination with gemcitabine. Gemcitabine still represents the cornerstone of PDAC treatment and in preclinical models of peritoneal mesothelioma we observed that our imidazo[2,1-*b*][1,3,4]thiadiazole derivatives potentiated its antiproliferative effects [40]. Since these results were associated with increased expression of hENT1, which plays a key role in the uptake and cytotoxicity of gemcitabine [41], in the present study, we also focused on the effect of AS on hENT1 expression in order to bypass one of the most important mechanism involved in the resistance to gemcitabine.

METHODS

Ligand preparation and protein preparation

Both ligands to be screened and co-crystallised ligands within the Protein Data Bank (PDB) structures were optimised using EpiK tool to energetically minimise their structure and generate protomers and tautomers at pH 7.4 ± 0.5 [42,43].

The 3D structures of the SF3B complex were downloaded from the PDB [44] and imported into the Schrödinger suite to optimise the structure by using the “Protein preparation” tool [42]. The bond orders for untemplated residues were assigned and hydrogens were added to the structure. Water molecules beyond 5.0 Å from any of the HET groups, including ions, were deleted. Finally, PROPKA [45] was run under pH 7.0 to optimise side chain states.

Pharmacophore creation and screening

LigandScout [46,47] software was employed to create the pharmacophore model and find the common feature between the two PDB structures by using the common pharmacophore map for virtual screening. The pharmacophore model was created, using the PDB coordinate of the ligand-protein complex (PDB IDs: 5ZYA and 6EN4). Starting from the two pharmacophore maps, only the common features were retained to be used for further studies. In the screening module, the "pharmacophore fit-score" was used as scoring function and "match all query features" was chosen as screening mode. The selected retrieval mode was "get best matching conformation".

Docking

The docking grid was generated using Glide software [48]. The scaling factor was set at 1.0 Å with a partial charge cut-off of 0.25, and the co-crystalised ligand was chosen as grid centroid. Molecular docking was carried out using Glide software [48] by Schrödinger (release 2018-4). The van der Waals radii scaling factor for ligands to be screened was set as 0.8, with a partial charge cut off by 0.15. The ligands were considered as flexible, and Epik state penalties were considered as docking score. The in-house compounds library was screened in standard precision mode. Molecules were then ranked based on the docking score.

Drugs and chemicals

The imidazo[2,1-*b*][1,3,4]thiadiazole derivative IS1 and the indole derivatives IS2, IS3 and IS4 were synthesised at the Department of Pharmacy, University of Palermo, Italy, following the synthetic procedures previously described [39,49]. The compounds were dissolved in dimethyl sulfoxide (DMSO), as reported previously [41]. Gemcitabine was kindly provided by Eli Lilly Corporation (Indianapolis, IN, USA) and dissolved in sterile water. Cell medium and newborn calf serum (NBCS) were from Gibco (Gaithersburg, MD, USA), while penicillin (50 IU mL^{-1}) and streptomycin (50 µg mL^{-1}) were from Lonza (Switzerland). Insulin-transferrin-selenium 100× was from Gibco (Grand Island, NY, USA) and PB was purchased from Cayman Chemical (Ann Arbor, MI, USA). All other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Cell lines and culture conditions

The PDAC cell lines SUIT-2 and Hs766t were purchased from the American Type Culture Collection (ATCC, Manassas, VA), while the Panc05.04 cell line was a generous gift from Dr Eric Eldering (Department of Experimental Immunology, AMC, The Netherlands). SUIT-2 is a mesenchymal tumour cell line derived from a metastatic liver tumour of human pancreatic carcinoma. It produces and releases two tumour markers, carcinoembryonic antigen and carbohydrate antigen 19-9 (CA19-9), in culture *in vitro* and in nude mice *in vivo* [50]. Hs766t is an epithelial cell line isolated by R. Owens *et al*. [51] in 1973 from a pancreatic carcinoma metastatic to a lymph node (ATCC® catalogue number HTB-134™). Panc05.04 is a pancreatic adenocarcinoma epithelial cell line derived, in 1995, from a primary tumour resected from the head of the pancreas of a woman with PDAC (ATCC® catalogue number CRL-2557™). The PDAC-3 primary culture cells were obtained from patients undergoing pancreatoduodenectomy, as described previously [52].

PDAC-3, SUIT-2 and Hs766t cells were cultured in RPMI-1640 (Roswell Park Memorial Institute 1640) and DMEM (Dulbecco's Modified Eagle's Medium) medium, respectively, supplemented with 10% NBCS and 1% penicillin/streptomycin. Panc05.04 cells were cultured in RPMI-1640 supplemented with 20% NBCS, 1% penicillin/streptomycin and insulin-transferrin-selenium 100×. The cells were kept in a humidified atmosphere of 5% CO_2 and 95% air at 37°C and harvested with trypsin-EDTA (Ethylenediaminetetraacetic acid) in their exponential phase cultures. The cells were tested for mycoplasma monthly using the MycoAlert Mycoplasma Detection Kit (Westburg, Leusden, The Netherlands).

Evaluation of cell growth inhibition by the sulforhodamine-B assay

In vitro chemosensitivity was assessed with the sulforhodamine-B (SRB) assay, as reported previously [53,54]. The SUIT-2 and Hs766t cells were seeded in triplicate in 96-well flat bottom plates at their optimal seeding concentration of $3\text{-}5\times10^3$ cells in 100 µL/well for both cell lines. They were incubated overnight at 37°C with 5% CO_2 to ensure cells adhesion creating a confluent monolayer. Cells were treated in triplicate with 100 µL of drugs dissolved in DMSO at different concentrations in the nano- and micro-molar range and incubated at 37°C with 5% CO_2 for 72 h. Thereafter, the cells were fixed with 25 µL of cold 50% trichloroacetic acid for at least 60 min at 4°C. Then, the medium was removed, and the plates were gently washed five times with tap water, dried at room temperature overnight and stained with 50 µL of 0.4% (w/v) SRB solution in 1% acetic acid for 15 min at room temperature (RT). The plates were gently washed four times with 1% acetic acid and dried at RT for a

minimum of 6 h. After adding 150 µL of tris(hydroxymethyl)aminomethane solution, the plates were gently mixed for 2-3 min at 350-400 rpm on a plate shaker. The optical density (OD) was spectrophotometrically read at wavelengths of 490 and 540 nm on a plate reader (BioTek Instruments Inc., Winooski, VT). Cell growth inhibition was calculated as the percentage of drug treated cells *vs*. vehicle-treated cells ("untreated cells or control") OD (corrected for OD before drug addiction, "Day 0"). The 50% inhibitory concentration of cell growth (IC_{50}) was calculated by non-linear least squares curve fitting (GraphPad PRISM, Intuitive Software for Science, San Diego, CA).

Since gemcitabine is commonly used (in monotherapy or within polychemotherapy regimens) for the treatment of PDAC patients and our previous studies in preclinical models of mesothelioma showed that thiadiazole derivatives potentiated gemcitabine effects [40], we evaluated the cytotoxic activity of the most promising compounds (IS1 and IS4) in combination with gemcitabine. For these studies, we used the above-described SRB assay exposing cells to IC_{50} values of the experimental compounds, added to IC_{25} values of gemcitabine, for 72 h, as described previously [40].

Evaluation of cell death by trypan blue assay

The *in vitro* sensitivity to the most promising compounds (IS1 and IS4) was also assessed for the PDAC cell line Panc05.04 carrying two endogenous SF3B1 mutations: p.Q699H and p.K700E. Of note, these cells have a duplication time above 36 h and are therefore less suitable for the assessment of cytotoxic activity in 96-well plates with the SRB assay. Therefore, we used a trypan blue assay, as described below. The Panc05.04 cells were seeded in a 6-well flat bottom plate in a volume of 1 mL at the density of 2×10^4 cells/well. They were incubated overnight at 37°C with 5% CO_2 to create a confluent monolayer and treated with 1 mL of drug dissolved in DMSO at concentrations ranging from 0.1 to 10 µM. After 96 h of treatment, the old medium was removed and the cells were washed twice with phosphate-buffered saline (PBS). Cells were harvested with trypsin-EDTA and incubated for 15 min at 37°C with 5% CO_2. After the addition of the new medium, the cells were resuspended and 10 µL of the cell suspension was harvested into a sterile Eppendorf. Noteworthy, only dead cells are coloured, since healthy living cells exclude trypan blue and are not coloured in this assay. Specifically, trypan blue is unable to penetrate the intact cell membrane of living cells. On the contrary, dead cells have a peculiar blue colour due to the absorption of the dye that crosses the compromised cell membrane. Trypan blue (10 µL) and 10 µL of the mixture for each

Eppendorf were transferred to a cell counting slide. The percentage of viable cells *vs*. non-viable cells was determined using the LUNA II™ Automated Cell Counter according to the manufacturer's protocol (Westburg, Leusden, The Netherlands).

Analysis of cell migration by wound-healing assay

The anti-migratory activity was determined with the *in vitro* scratch wound-healing assay. SUIT-2 cells were seeded in 96-well flat bottom plates, at the optimal density of 5×10^4 cells/well in 100 µL and incubated for 24 h. The scratch was performed with a 96-pin scratcher on confluent cell monolayers. After the removal of detached cells, the plate was washed twice with 200 µL of PBS and 100 µL of medium was added to all the wells. Thereafter, the experimental wells were treated with 100 µL of the drugs at concentrations of $4\times IC_{50}$ and an additional 100 µL of the medium was added to the control wells. Images were taken immediately after scratching procedure, as well as 8 and 24 h after the exposure of the drugs by phase-contrast microscopy using the Leica DMI300B microscope (Leica Microsystems, Eindhoven, the Netherlands). The results were analysed with Scratch Assay 6.2 software (Digital Cell Imaging Labs, Keerbergen, Belgium), as described previously [53].

PCR assay to evaluate SF3B1 and hENT1

Real-time quantitative reverse transcription PCR (qRT-PCR) was performed to evaluate the gene expression of *SF3B1* and *hENT1* in the PDAC cell lines, using *GUSB* and *GAPDH* as housekeeping genes. The cells were seeded at $3\text{-}5\times10^3$ in a 6-well flat bottom plate with 2 mL medium per well and incubated with gemcitabine (IC_{50}) for 24 h. Thereafter, the medium was collected and cells were washed using 2.5 mL PBS. Trypsin-EDTA was then added, and, after 5 min incubation the detached cells were resuspended in the previously collected medium and centrifuged at 1500 rpm for 5 min. The pellets were either stored at -80°C or used immediately for RNA extraction, using the RN-easy RNA isolation kit (Qiagen) following the manufacturer's instructions. One microgram of RNA was then used to synthesise complementary DNA (cDNA) in a volume of 20 µL of sterilised dH_2O (Versilene® Fresenius, Fresenius Kabi France) for each sample, as described previously [55]. The resulting cDNA was amplified by quantitative-PCR using specific primers for SF3B1 and GUSB with the LightCycler® 480 Real-Time PCR System (Roche, Rotkreuz, Switzerland). The mRNA expression of hENT1 was evaluated using the specific kits for hENT1 and GAPDH with the ABIPRISM-7500 instrument (Applied Biosystems, Foster City, CA), as described previously [41].

To visualise the splicing modulation induced by the potential SF3B1 inhibitors on RON, we performed an end-point PCR assay followed by agarose gel electrophoresis. The SUIT-2 cells were seeded in 6-well flat bottom plates and incubated for 24 h with 20 μM of the two most promising compounds in 2 mL medium. RNA isolation and cDNA synthesis were performed according to the protocol described above. The primers for RON were designed considering the exons of this gene, as follows: Exon 10_ Forward (5'-CCT GAA TAT GTG GTC CGA GAC CCC CAG-3'); Exon12_ Reverse (5'-CTA GCT GCT TCC TCC GCC ACC AGT A-3'). PCR was performed as described previously [55], at the annealing temperature of 62°C.

Analysis of antitumour activity in multicellular spheroids of primary cells

PDAC-3 spheroids were established seeding 20000 cells/mL in DMEM/F12 + GlutaMAX-I (1:1), in 24-well ultra-low attachment plates (Corning, NY, USA) according to manufacturers' protocol. Spheroids were generated for 3-7 days, and then harvested for growth inhibition studies in 96-well plates. After checking their growth rate and stability, the spheroids were treated at concentrations of $4\times IC_{50}$ of gemcitabine, IS4 and their combination for 72 h. The cytotoxic effects were evaluated by measuring the size of spheroids compared to untreated controls, as described previously [38].

Statistical analysis

All experiments were performed in triplicate and repeated at least twice. The percentages of cell migration were calculated taking into account at least nine scratches. Data were expressed as mean values ± SEM and analysed by Student's *t*-test or one-way ANOVA. The cut-off level of significance was $P < 0.05$.

RESULTS

Selection of potential SF3B1 inhibitors

To explore the binding mode and prioritise putative active compounds, preliminary computational studies were performed using the crystallographic structures of SF3B1 selected from the PDB. The crystallographic structure of two SF3B1 protein ensembles (PDB ID: 6EN4 [56] and PDB ID: 5ZYA [57]) in complex with PB and its analogue E7107 were selected as a starting point for computational studies to build a structure-based pharmacophore and docking model [56]. The interaction map of the two protein-ligand complexes was compared as a guide for the crucial interactions to be accounted in our investigations. From

the structural analysis of the two compounds compared, the common residues of the protein complex interacting with PB and E7107 were: V1078, V1110, V1114 and L1066 of the subunit SF3B1 and R38 and Y36 in the PHF5A subunit [57]. Starting from the two crystal structures, a pharmacophore map was created using LigandScout v.4.4 [46,47], and geometrically common features were selected, thus removing two distal features. As a result, six common pharmacophoric features were found and the common pharmacophore was created [**Figure 1**]. The common pharmacophore was then used for virtual screening studies to identify the molecular scaffolds of interest using both our in-house molecular library and commercially available molecular libraries.

According to the binding mode with the amino acid residues of the common pharmacophore, the most suitable molecules were selected and then their structures were carefully analysed. It was then found that most of them showed a common feature: an indole group and a nearby amide group. Docking studies were performed on the same crystallographic structures using Glide 2018-4 [48] to have a consensus mode of selection. Structure-based pharmacophore and docking exploit different algorithms; thus, we decided to see which molecules of our in-house library were prioritised by the two techniques adopted. As shown in **Figure 2**, Tyr36, Arg38, Arg1074, Arg1075 and Leu1066 residues were found to be important for the protein-ligand complex stabilisation. From these analyses, four compounds were prioritised in terms of interactions and theoretical binding energy.

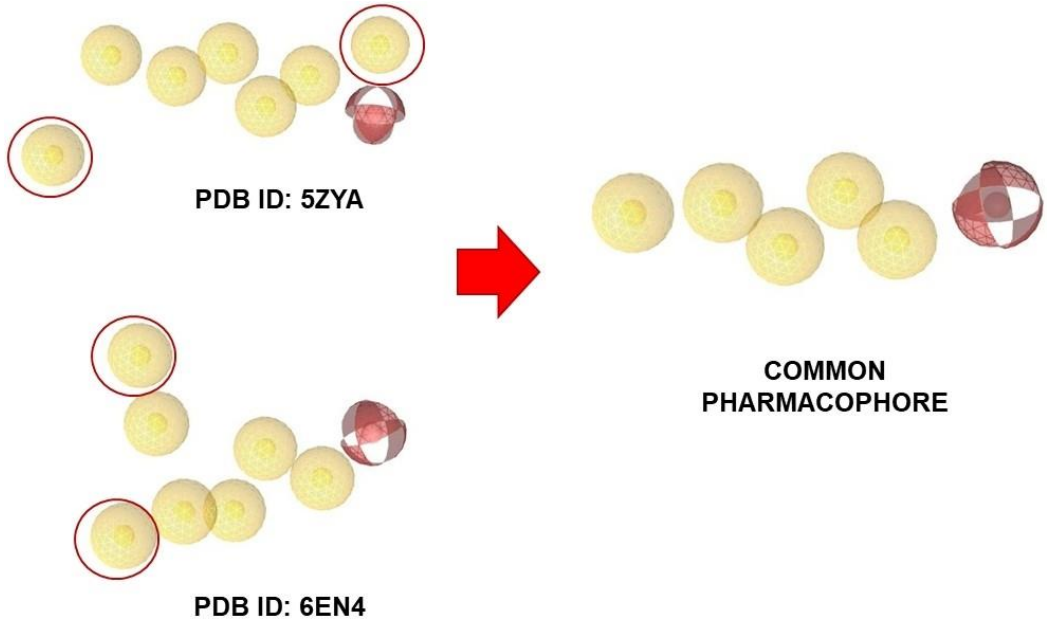


Figure 1. Creation of the common pharmacophore. The common pharmacophore was created starting from the interaction map of the two protein-ligand complexes interacting with PB and E7107, using

LigandScout v.4.4. Geometrically common features were selected and six common pharmacophoric features were found.

Drug sensitivity

The *in vitro* sensitivity to the potential SF3B1 modulators {splicing inhibitors IS1, IS2, IS3 and IS4 [**Figure 3**]} was evaluated for the PDAC cells SUIT-2 and Hs766t. These cells were selected because they are representative of PDAC mesenchymal and epithelial phenotype [25].

A pre-screening cytotoxicity SRB assay was initially performed using concentrations of 0.1, 1 and 10 μM. Notably, all compounds showed concentration-dependent inhibition of proliferation; thus, we expanded our studies using at least eight different concentrations (from 125 nM to 16 μM) to define more accurate IC_{50} values. Compounds IS1 and IS4 showed the highest sensitivity in both preclinical models [**Figure 4A** and **B**]. In particular, the Hs766t cells were most sensitive to both compounds, with IC_{50}s of 2.4 and 2.7 μM after exposure to IS4 and IS1, respectively. In contrast, the SUIT-2 cells were least sensitive, with IC_{50}s ranging from 4.5 to 7.5 μM. Considering the interesting results of antiproliferative activity *in vitro*, we selected the most promising compounds (IS1 and IS4) for the analysis of migration inhibition and the modulation of the splicing of RON, an overexpressed gene in PDAC.

Induction of cell death in cells harbouring SF3B1 mutations

Heterozygous mutations in the splicing factor *SF3B1* have been found to particularly occur in haematological malignancies, but more recently they have also been detected in several solid tumours including PDAC [41] with a frequency of 4% [58-60]. Previous studies have shown that *SF3B1* mutations are concentrated in the sequence encoding the HEAT repeat domains with major hotspots including p.R625, p.K666 and p.K700E [60-62]. Interestingly, the latter mutation is carried by the Panc05.04 cell line together with p.Q699H [63]. Therefore, we used this model to perform further studies with the IS1 and IS4 compounds. Notably, the mutations of SF3B1 do not affect SF3B1 gene expression, which is similar to the other PDAC cells, as assessed by PCR (data not shown).

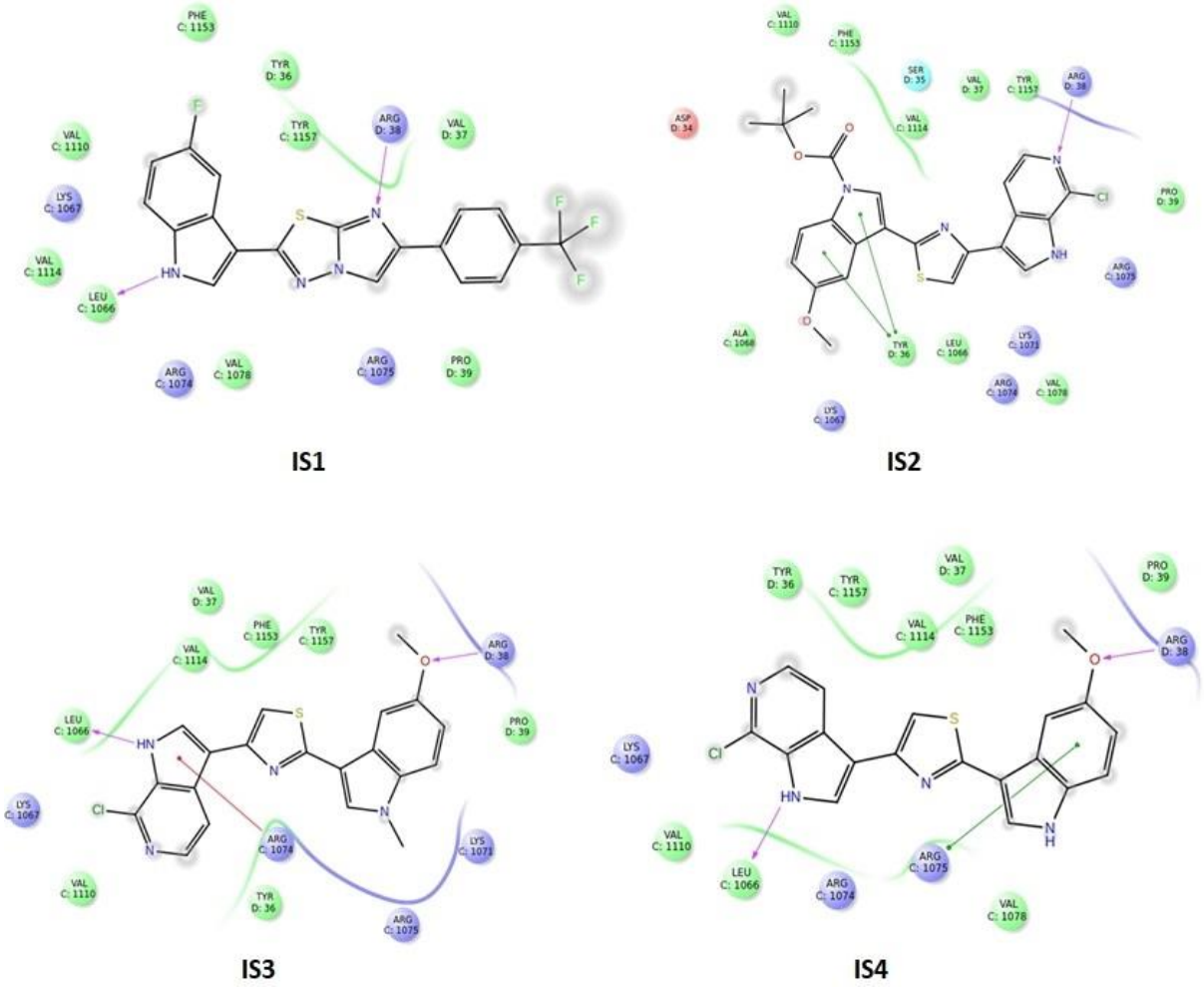


Figure 2. Structure-based pharmacophore and docking were used to prioritise molecules. Tyr36, Arg38, Arg1074, Arg1075 and Leu1066 residues were found to be important for the protein-ligand complex stabilisation. From these analyses, four compounds were prioritised in terms of interactions and theoretical binding energy.

Panc05.04 cells have a relatively long doubling time (46 h) compared to most ATCC cell lines. To achieve reliable results, these cells were exposed for 96 h to compounds IS1 and IS4, at five different concentrations in the micromolar range (from 0.1 to 10 µM). Remarkably, both drugs induced cell death, ranging from 52% to 63% at a concentration of 1 µM [**Figure 4C**]. However, since these Panc05.04 cells are the only known PDAC cells harbouring a mutation in SF3B1, we could not draw conclusions on whether they are more sensitive to potential SF3B1 inhibitors.

Anti-migratory activity and modulation of RON splicing pattern

The metastatic potential is one of the hallmarks of PDAC, and it is closely related to the grim

prognosis of this disease. Currently, the key mechanisms underlying this process are poorly understood, although it has been shown that several factors govern the metastatic process, including cell migration and invasion [5]. The promising results on the antiproliferative activity prompted us to also investigate the anti-migratory effect of our potential SF3B1 modulators by the wound healing assay, which was performed on SUIT-2 cells. These cells were selected because of their ability to form monolayers at optimal cell confluence within 24 h. A concentration of $4\times IC_{50}$ was used for each compound because of the shorter drug exposure time compared to growth inhibition studies, which lasted 72 h, and because it was able to slightly reduce migration already after 8 h exposure compared to untreated cells (set at 100%). However, IS1 and IS4 significantly inhibited the migration rate of SUIT-2 cells after 24 h of drug exposure [**Figure 5A**], with percentages of migration rates below 40% and 10% for IS1 and IS4, respectively.

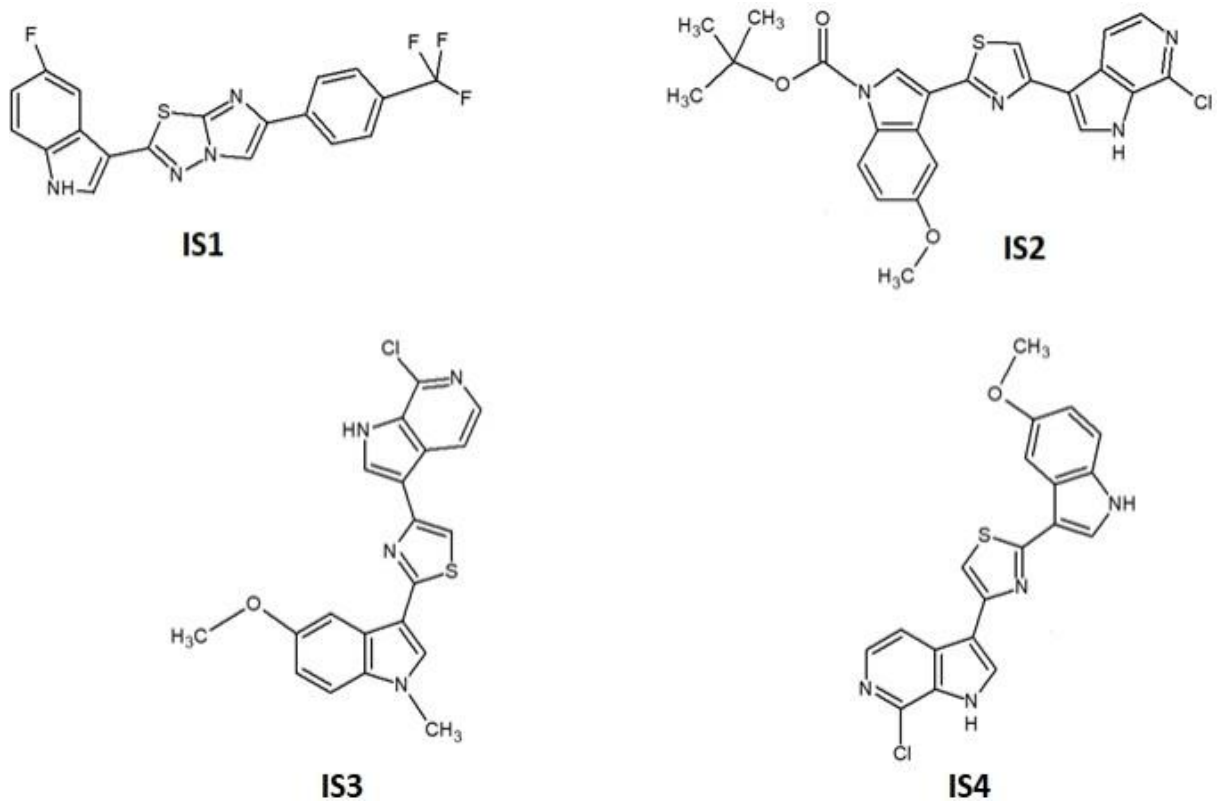


Figure 3. Chemical structures of compounds IS1, IS2, IS3 and IS4. The synthesis of compound IS1 is described in [39], while the descriptions of compounds IS2, IS3 and IS4 can be found in [49].

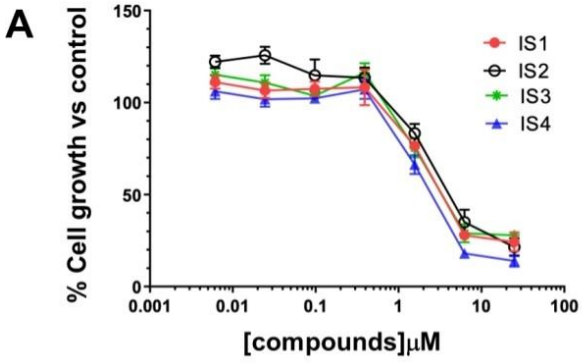
Remarkably, this effect was associated with the mis-splicing of RON, which is a tyrosine kinase receptor belonging to the c-MET kinase family. This gene is overexpressed in PDAC and promotes cell migration, invasion and apoptotic resistance [64,65]. Of note, RON

commonly undergoes AS resulting in different shorter ΔRON spliced variants [66]. The PDAC SUIT-2 cells express the truncated variant ΔRON, which plays a pivotal role in tumour cell motility due to the constantly activated kinase function [65]. A 24 h exposure to IS1 and IS4 caused intron retention in RON transcript and decrease in transcript abundance, probably due to nonsense-mediated decay [**Figure 5B**].

Synergistic interaction with gemcitabine is associated with an increase of hENT1 mRNA expression

Gemcitabine is a pyrimidine analogue (2',2'-difluoro-2'-deoxycytidine, dFdC; Gemzar®) widely prescribed to treat a variety of solid tumours [67]. It has been used for decades as the first-line treatment for metastatic PDAC, and it is still commonly used for PDAC patients in combination with nab-paclitaxel or as monotherapy in patients who are unfit for combination regimens, as mentioned above [7,9].

Our previous data show that some compounds from a series of new imidazo[2,1-*b*][1,3,4]thiadiazole derivatives potentiated the antiproliferative effects of gemcitabine in peritoneal mesothelioma cells [40]. However, different splicing aberrations have previously been shown to enhance the activity of proliferative and glycolytic signalling associated to gemcitabine resistance [68-70].



B

	IC_{50} (μM) ± SEM Cell Lines	
Compound	SUIT-2	Hs766t
IS1	5.8 ± 0.5	2.7 ± 0.7
IS2	6.2 ± 0.6	6.1 ± 1.1
IS3	7.5 ± 0.3	2.9 ± 0.2
IS4	4.5 ± 0.2	2.4 ± 0.2

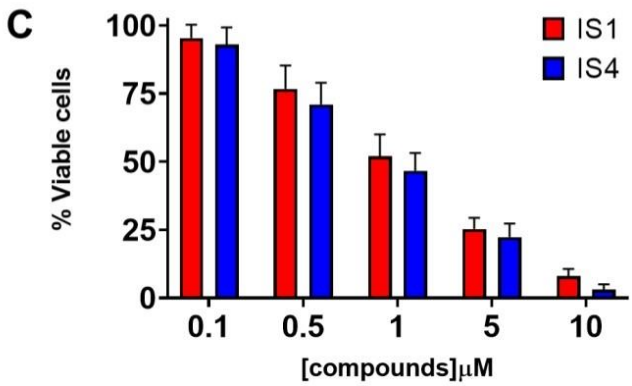


Figure 4. Antiproliferative activity and induction of cell death by IS1 and IS4 in PDAC cells. **(A)** Representative growth inhibition curves of PDAC cells Hs766t treated for 72 h with the compounds IS1, IS2, IS3 and IS4. Points, mean values; bars, Standard Error of the Mean (SEM). **(B)** Table summarising the IC_{50} values of the IS1, IS2, IS3 and IS4 compounds against the PDAC cells SUIT-2 and Hs766t. The values are reported as means ± SEM of three separate experiments. IS1 and IS4 had the lowest IC_{50} values and statistical analyses showed significant differences of these compounds compared to both IS2 and IS3. Thus, we selected these compounds for the following studies. **(C)** Representative bar graph of trypan blue exclusion assay, showing the percentage of viable Panc05.04 cells after treatment for 96 h and the compound IS1 and IS4 at five different concentrations (0.1, 0.5, 1, 5 and 10 μM). Columns, mean values; bars, SEM.

Therefore, we evaluated whether the combinations with the compounds IS1 and IS4 at their IC_{50} would increase sensitivity to gemcitabine of SUIT-2 and Hs766t cells. The combination of both compounds IS1 and IS4 with gemcitabine at IC_{25} levels led to a significant reduction in cell growth compared to untreated cells, below 20% and 12%, respectively [**Figure 6A**] [71,72]. These values were well below the theoretical achievable growth inhibition of the combinations and can therefore be considered as a synergistic effect.

Because of its hydrophilic nature, gemcitabine requires facilitated or active transport for cellular uptake, which is mediated by membrane nucleoside transporters, including the human concentrative nucleoside transporter-3 and hENT1. The latter has been evaluated in several preclinical and clinical studies as a potential determinant of gemcitabine efficacy in PDAC [9].

Previously, our imidazo[2,1-*b*][1,3,4]thiadiazole compounds in combination with gemcitabine significantly increased the expression of hENT1, suggesting its potential role in increasing the activity of gemcitabine [40]. These promising results prompted us to adopt the same strategy to investigate potential molecular mechanisms underlying the reduced activity of gemcitabine in combination with IS1 and IS4. Therefore, we measured the modulation of the gene expression of hENT1. Both compounds, also in this case, increased hENT1 expression significantly [**Figure 6B**], supporting the role of these new compounds in reversing a key mechanism of resistance to gemcitabine.

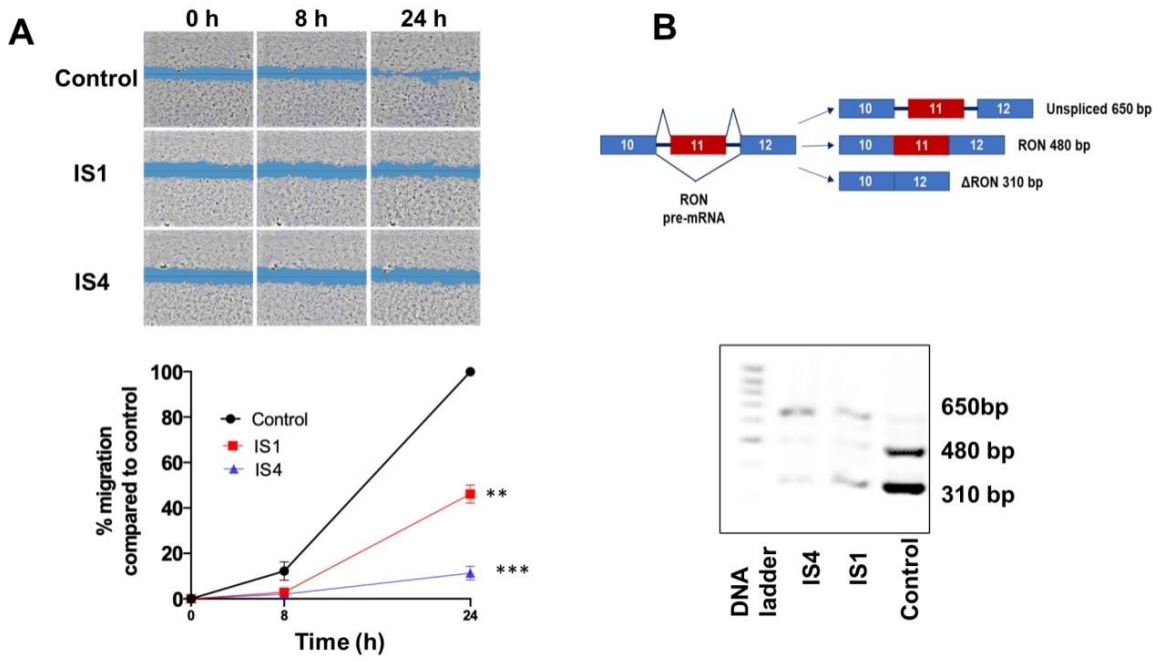


Figure 5. Inhibition of migration and modulation of RON splicing by compounds IS1 and IS4 in SUIT-2 cells. **(A)** (Top) Representative pictures of scratch areas in untreated (control) and treated cells during the wound healing assay. Original magnification 5×. (Bottom) Percentage of migration over time (0, 8 and 24 h) of SUIT-2 cells treated with the compounds IS1 and IS4 at concentrations of 4×IC_{50}. $^{**}P$ < 0.01, $^{***}P$ < 0.005. **(B)** (Top) Schemes of RON pre-mRNA variant structures with predicted PCR products are shown on the left. (Bottom) Representative picture of PCR splicing pattern assessment of RON in SUIT-2 cells after 24 h of drug exposure. Original magnification 5×.

The combination of gemcitabine and IS4 reduced spheroids of PDAC primary cultures

The sensitivity to anticancer drugs, including gemcitabine, in two-dimensional monolayer cell culture models is typically different from three-dimensional (3D) culture models. Thus, to determine whether IS4 would enhance the efficacy of gemcitabine in 3D systems, we tested these drugs in spheroids of PDAC3 cells [**Figure 7A**]. We transferred in each well of 96-well plates spheroids that were approximately 500 μm in diameter. These growing spheroids were exposed to gemcitabine, IS4 and their combination for 72 h. The growth of these spheroids was slightly inhibited by gemcitabine and IS4, while the combination remarkably increased their disintegration, and they were significantly reduced in size compared to the untreated spheroids as well as to spheroids exposed to gemcitabine-alone [**Figure 7B**].

DISCUSSION

In this paper, we demonstrate that in PDAC cells inhibition of splicing can help to fight the

typical resistant behaviour of these tumours to standard chemotherapeutic drugs, such as gemcitabine, most likely by reducing cell aggressiveness/invasiveness and increasing the expression of the limiting uptake transporter hENT1. The treatment of patients with gemcitabine alone gives a moderate effect, and any improvement of this effect would increase the prospects of PDAC patients. Only 15%-20% of all PDAC patients qualify for curative resection followed by adjuvant chemotherapy, often including gemcitabine [5], and treatment options for most PDAC patients are limited. Thus, there is a clear need for new therapeutic approaches targeting key determinants of PDAC aggressive behaviour and reversing or bypassing resistance to existing therapies [10].

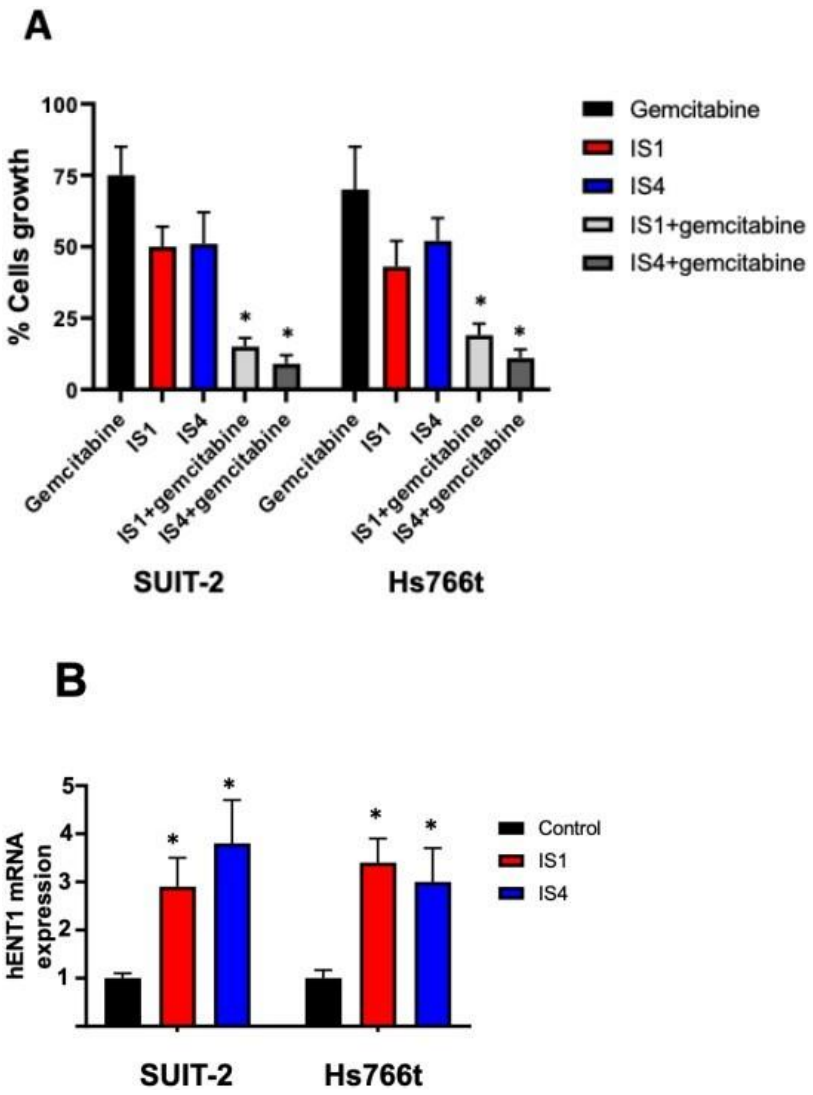


Figure 6. Increase of gemcitabine sensitivity. **(A)** Effect on growth of SUIT-2 and Hs766t cells of the combination of gemcitabine at its IC_{25}, with the compounds IS1 and IS4 at their IC_{50}. The observed values were lower than the theoretical values. **(B)** Modulation of hENT1 mRNA levels. Expression was determined with quantitative-PCR by normalisation with the GAPDH housekeeping gene, as

described in the methods. Since we previously demonstrated that hENT1 protein levels correlated with hENT mRNA expression, we did not include hENT1 protein expression [71,72]. Columns, mean values obtained from triplicate experiments; bars, SEM; **P* < 0.05.

Recent genomic studies have shown that heterozygous mutations in the splicing factor SF3B1 frequently occur in several tumours and prompt cancer progression through the activation of cryptic splice sites in multiple genes [11]. Most SF3B1 mutations have been detected in haematological malignancies, but PDAC is among the solid tumours harbouring these mutations in more than 3% of cases [12,59]. Moreover, PDAC has high levels of expression of SF3B1, and recent studies have demonstrated a positive correlation between expression levels of wildtype (WT) SF3B1 and tumour malignancy [11,62], further supporting the search for drugs targeting this key spliceosomal factor.

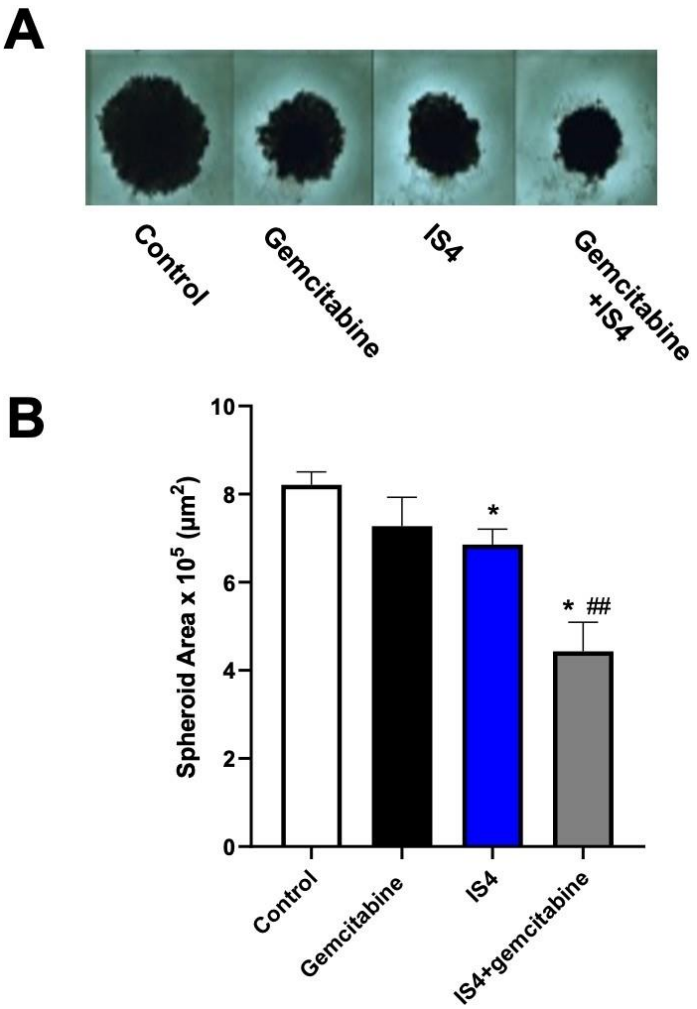


Figure 7. Increase of gemcitabine sensitivity in 3D models. **(A)** Representative images of PDAC-3 spheroids untreated (control) or treated with gemcitabine, IS4 or their combination (original

magnification, 40×). **(B)** Effects of gemcitabine, IS4 or their combination on the areas of PDAC-3 spheroids after 72 h exposure. Columns, mean values obtained from triplicate experiments; bars, SEM; $^{*}P < 0.05$ *vs*. control, $^{\#\#}P < 0.01$ *vs*. gemcitabine.

In the present study, we evaluated for the first time four potential spliceosome inhibitors {one imidazo [2,1-*b*][1,3,4]thiadiazole derivative (IS1) and three indole derivatives (IS2, IS3 and IS4)}, which were selected by virtual screening from an in-house molecular library in order to investigate their potential efficacy against PDAC cells. Similar approaches have allowed identifying several splicing modulators other than SF3B1 inhibitors in different high-throughput screens, which are currently undergoing further evaluation in preclinical studies, as reviewed previously [73,74].

The emerging potential SF3B1 modulators IS1 and IS4 were able to inhibit cell proliferation in SUIT-2 and Hs766t cells, displaying IC_{50} values ranging from 2.4 to 5.8 µM. Remarkable growth inhibition was also observed in Panc05.04 cells, harbouring SF3B1 mutations. This is in agreement with previous findings, showing that E7107 substantially reduced leukaemia cell burden in an isogenic mouse model carrying an Srsf2 P95H mutation as well as in PDX models from patients harbouring SRSF2 mutations compared to WT models [75].

The IC_{50} values observed after treatment with our most promising compounds were however higher than what has been reported for PB and E7107 in different preclinical models of solid tumours, such as mesothelioma, where IC_{50} values of these SF3B1 modulators are in the nanomolar range [54]. However, this might mitigate adverse events, which limited the clinical development of E7107 [19]. Similarly, the excellent results of splice-switching oligonucleotides and RNA interference *in vitro* are extremely difficult to translate to the clinical setting due to limited stability in plasma and intracellular uptake [76].

In the present study, we also evaluated the modulation of the gene expression of hENT1. It has been reported repeatedly that high hENT1 levels are correlated with increased gemcitabine cytotoxicity and prolonged disease-free status and overall-survival in patients receiving gemcitabine adjuvant chemotherapy [41], including a PCR on laser-microdissected tissues study in which Giovannetti *et al*. [77] reported an overall survival of 25.7 and 8.5 months in PDAC patients with high and low levels of hENT1, respectively. Of note, the expression and activity of hENT1 is affected by multiple molecular mechanisms. In particular, it is worth

mentioning that the TME of PDAC influences the expression of hENT1 causing PDAC gemcitabine chemoresistance. In fact, various components of the extracellular matrix limit the availability of oxygen (hypoxia), hindering the transport of gemcitabine via hENT1 [41]. Of note, several polymorphisms may affect the gene expression of hENT1, and therefore the efficacy of gemcitabine. Specifically, Myers and collaborators showed that individuals with CAG and CGC haplotypes exhibited significantly higher hENT1 expression than individuals with the normal CGG haplotype [78]. Other mechanisms affecting hENT1 expression include epigenetic modulation and microRNA [41], and recent studies have shown interesting interrelationships between miRNA and splicing factors in PDAC [79].

Remarkably, the IS1 and IS4 compounds potentiated the activity of gemcitabine. In previous studies, after SF3B1 and PHF5A knockdown, leukaemia cells became highly sensitive to mitomycin C, suggesting that a combination of splicing modulation with DNA damaging agents could achieve synergistic effects [80]. However, we might also hypothesise that this effect is caused by the positive modulation of hENT1 mRNA expression, for which a low expression has been associated with gemcitabine resistance in different cancer cell types [41]. Thus, our data suggest that splicing inhibition can reverse resistance to gemcitabine.

In addition, using a 3D culture model (e.g., spheroids) of primary cell culture that mimics the 3D organisation of PDAC tumour cells *in vivo* [81], we showed that the antitumour activity of gemcitabine was significantly increased by the simultaneous addition of IS4.

Finally, the IS1 and IS4 compounds were also able to induce a splicing shift from RON and ΔRON after 24 h from the start of treatment, which might at least in part explain the strong anti-migratory ability of IS1 and IS4 in SUIT-2 cells. Of note, RON and cMET are important indicators of prognosis in PDAC, and previous studies have shown the synergistic interaction of inhibitors of these protein kinases with gemcitabine [81,82], further providing new means to predict clinical outcome and targets for more effective therapies against PDAC.

Other markers should be evaluated in the future. However, another splice variant evaluated in previous studies, MCL-1 (myeloid cell leukemia 1) [58], did not show an aberrant splicing pattern when evaluated using IS4 and not even with PB as reference splicing inhibitor. Therefore, we did not proceed with this marker in view of our potential SF3B1 modulators.

Novel compounds targeting pivotal splicing factors, such as SF3B1, could have relevant antitumour activity, and, in the present study, we identified four potential SF3B1 inhibitors, selected from an in-house library, that showed cytotoxic and antimigratory activity in PDAC cells and potentiated the antitumour effects of gemcitabine. Our studies supported the role of RON and hENT1 modulation as molecular mechanisms to be further exploited for the characterisation of these new therapeutic approaches, other than for prognostic purposes [1].

In conclusion, our novel findings prompt further analysis of the selectivity and toxicity of our potential SF3B1 inhibitors, as well as the role of the modulation of RON and hENT1 for further studies in appropriate preclinical models, including *in vivo* models and new model systems [83], in order to guide the rational development of new drug combinations that could reverse chemoresistance of PDAC.

DECLARATIONS

Acknowledgments

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Writing - original draft preparation: Randazzo O, Cascioferro SM, Pecoraro C
Writing - review and editing: Peters GJ, Giovannetti E
Supervision: Diana P, Giovannetti E
Funding acquisition: Diana P, Giovannetti E
All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Chapter 7

Discussion and conclusions

Discussion

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers worldwide, its incidence increased in recent decades and is expected to continue to rise. The limited knowledge of tumor biology as well as the absence of symptoms in the early stages of PDAC and the lack of specific and sensitive biomarkers strongly contribute to the grim prognosis of the disease. Therefore, early detection of pancreatic cancer is essential in order to optimize treatment options and improve patient outcome [1]. In particular, biomarkers represent a valuable tool for providing PDAC patients with personalized anticancer therapy. Despite the great scientific efforts during the last decades, the ideal biomarker has not yet been identified but a careful and detailed analysis of the human Equilibrative Nucleoside Transporter-1 (hENT1) could represent a keystone for the treatment of PDAC. hENT1 has been proposed as a potential biomarker to predict the effect of the anticancer drug gemcitabine in PDAC, as it is the main transporter involved in the intracellular uptake of this drug that has been the mainstay of PDAC treatment for more than twenty-five years [2,3].

Chapter 2 provides an in-depth commentary on the controversial results regarding the predictive value of hENT1 and discusses the various molecular and pharmacological factors that influence its expression and activity. Several clinical studies reported the association between high levels of hENT1 expression and a statistically significantly longer overall survival of PDAC patients using qRT-PCR and immunohistochemistry (IHC) as an alternative methodology. In particular, the most comprehensive multimodal analysis of hENT1 status performed by Raffenne and collaborators highlighted the predictive value of hENT1 expression only when assessed using the anti-hENT1 10D7G2 mouse clone and not the anti-hENT1 SP120 rabbit clone. However, although controversial data was sometimes obtained, possibly due to the different antibodies used, overall the findings using proper methodology supported the role of hENT1 as a predictive biomarker for the efficacy of gemcitabine in resected PDAC patients undergoing gemcitabine-based adjuvant chemotherapy.

The urgent need for reliable prognostic biomarkers is also found in mesothelioma which, just like pancreatic cancer, remains a clinical challenge and a global health problem. Specifically, malignant pleural mesothelioma (MPM) is a rare disease that develops in the pleural cavity and comprises 70-90% of all cases of malignant mesothelioma. It is associated with chronic inflammation induced by occupational exposure to asbestos and is characterized by a long latency period (20-40 years) between exposure and disease development which delays the diagnosis contributing to a poor prognosis of 12 months of median survival [4,5].

The intracellular focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase overexpressed in various tumor types, including PDAC and MPM, and has emerged as a potential therapeutic target for both tumors [6]. To this end, we previously observed a promising antitumor activity of a new class of imidazo[2,1-*b*][1,3,4]thiadiazole compounds in PDAC models and revealed their ability to inhibit FAK phosphorylation in this tumor [7,8].

Since FAK also emerged as a target of DMPM (Diffuse malignant peritoneal mesothelioma), in **Chapter 3** we investigated the *in vitro* cytotoxic activity of ten imidazo[2,1-*b*][1,3,4]thiadiazole compounds (**Table 1**) on two human DMPM primary cell cultures, MesoII and STO cells by the SRB (Sulforhodamine B) assay. Four out of ten compounds (**1**, **2**, **7** and **8**) inhibited cellular growth with a 50% growth inhibition (IC_{50}) between 0.59 and 5.9 μM in both cell lines after 72 hours of drug exposure, although with lower values in the STO cells. The IC_{50} value of the other eight compounds was >10 μM in both cell lines. Compounds **1** and **2** were the most promising with an IC_{50} value between 0.59 and 2.81 μM in both cell lines; therefore they were explored in more detail using the spheroids of mesothelioma cells as three-dimensional (3D) model. A significant reduction was observed in the size of the MesoII and STO spheroids treated with compounds **1** and **2** at concentrations of 5xIC_{50} value. In particular, we found approximately 2-fold change reduction after 17 days of treatment compared to the untreated spheroids. Further promising results were observed in the assessment of the anti-migratory activity by the wound healing assay of the compounds **1** and **2** in STO cells. In fact, after 20 hours of drug exposure at concentrations of 5xIC_{50} value, a reduction in the migration rate of 25.8% and 20%, respectively, of compounds **1** and **2** was observed. The ELISA assay in STO cells also confirmed the ability of compounds **1** and **2** to significantly inhibit the phosphorylation of FAK at the tyrosine residue 397. Furthermore, the combination of both compounds **1** and **2** resulted in a significant reduction in cell growth and an increased mRNA expression of the hENT1 which is one of the main transporters of gemcitabine within the cells. These results suggested a correlation between the new class of imidazothiadiazole compounds that potentiated the antiproliferative activity of gemcitabine and their role in the modulation of FAK and hENT1.

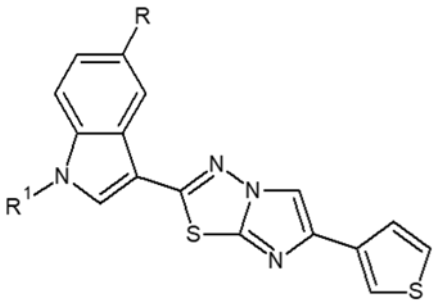

Table 1. Imidazo[2,1-*b*][1,3,4]thiadiazole synthesized.

Compounds	R	R^1
1	H	H
2	H	CH_3
3	Br	CH_3
4	Cl	H
5	Cl	CH_3
6	F	CH_3
7	Br	H
8	F	H
9	OCH_3	H
10	OCH_3	CH_3

MicroRNA (miRNAs) may become therapeutic biomarkers for the early diagnosis of PDAC since there is limited availability of screening modalities for this disease [9]. Splicing inhibitors have only recently received attention as new anticancer drugs and emerging therapeutic strategy for PDAC [10].

Chapter 4 extensively discussed the possibility of a relationship between miRNAs and the splicing deregulation, which represent two key epigenetic processes that strongly influence the pathogenesis of PDAC. While the clinical relevance of miRNAs (tissue and blood-derived) in PDAC is known, the splicing deregulation represents a new emerging therapeutic approach especially in the carcinogenesis of solid tumors. Several studies suggest the importance of splicing deregulation due to the mutation of splicing factors in various types of tumors, including PDAC. The interaction between the relevant splicing factors in PDAC and their associated miRNAs is reviewed in **Chapter 4**, together with the bioinformatics tools that allow to deepen the knowledge of the biological effects of the interaction between the splicing process and the miRNAs, as well as the predictive models for target prediction.

Although further studies are still needed in this research field, it appears possible to exploit the intricate relationship between miRNAs and the splicing process as a future strategy in the management and treatment of PDAC.

As previously discussed, splicing deregulation is an important feature of the pathogenesis of PDAC as well as a new hallmark of this disease [11]. Although SF3B1 splicing modulators have shown promise in many solid and hematological malignancies in both *in vitro* and *in vivo* models, they have never been tested in PDAC. To this end, in **Chapter 5** we describe for the first time the expression levels of *SF3B1* in PDAC cells and tissues using differential splicing analysis RNA sequencing-based. The analysis was perfomed on transcriptomic data from 5 primary PDAC cell cultures (PDAC-1, -2, -3, -4 and -5) and 2 immortalized normal ductal epithelial cell lines (HPDE and HPNE), from which 340 genes out of 420 were widely involved in the regulation of pre-mRNA splicing process.

The antiproliferative activity of SF3B1 modulators (E7107 and pladienolide B) in primary PDAC cells was confirmed by SRB assay after 72 hours of treatment with increasing drug concentrations, while the effect of both SF3B1 modulators on cell migration was evaluated by wound healing assay in the two most resistant cell lines, namely PDAC-1 and -3. After 16, 20 and 24 hours of incubation with 10 and 30 nM of both drugs, inhibition of cell migration was clearly observed. Therefore, the splicing profile for PDAC-1 and -3 primary cells was also evaluated by RT-PCR. After 24 hours of incubation with 2.5 and 25 nM of E7107, an alteration of the splicing (intron retention) of the proto-oncogene RON occurred.

Studies on PDAC orthotopic mouse models then confirmed the ability of E7107 to inhibit tumor growth *in vivo* without appreciable toxicity and the expression of *SF3B1* in tissue microarrays (TMA) was significantly correlated to OS (overall survival) and PFS (progression-free survival). In conclusion, our encouraging results propose splicing as a new target in PDAC and in particular the splicing factor SF3B1 as a potential prognostic biomarker of the disease, although further validation studies are absolutely necessary.

Since alterations of the splicing process are recurrent in several human solid tumors as well as in hematopoietic malignancies, various compounds with splicing inhibitor activity have recently been designed [12]. In particular, in 2007 the identification of two cytotoxic natural products (FR901464 and pladienolide B) that inhibit the pre-mRNA splicing by binding to the SF3B (spliceosome factor 3B) complex, paved the way for the development of small molecule splicing modulators targeting SF3B1 that represent a potentially promising strategy in cancer treatment [13–15].

Since the mutation of the splicing factor SF3B1 was detected in 4% of PDAC patients [16–18], in **Chapter 6** we investigated the activity of new potential SF3B1 modulators. Preliminary computational studies were performed in order to create a common pharmacophore, derived from the structural analysis of two cristallographic structures of SF3B1 (PDB IDs: 5ZYA and 6EN4) in complex with pladienolide B (PB) and E7107, which were selected from the Protein Data Bank (PDB). Starting from the interaction map of the two protein-ligand complexes that interact with PB and E7107, the common pharmacophoric features were selected, namely the following amino acid residues: V1078, V1110, V1114, L1066 of the SF3B1 subunit and R38 and Y36 of the PHF5A subunit.

The common pharmacophore was then used for virtual screening studies of in-house molecular library and commercially available molecular libraries, in order to identify the molecular scaffolds of interest. Based on the binding mode with the amino acid residues of the common pharmacophore, four potential SF3B1 modulators - one imidazo[2,1-*b*][1,3,4]thiadiazole (**IS1**) derivative and three indole derivatives (**IS2, IS3, IS4**) - were selected (**Figure 1**).

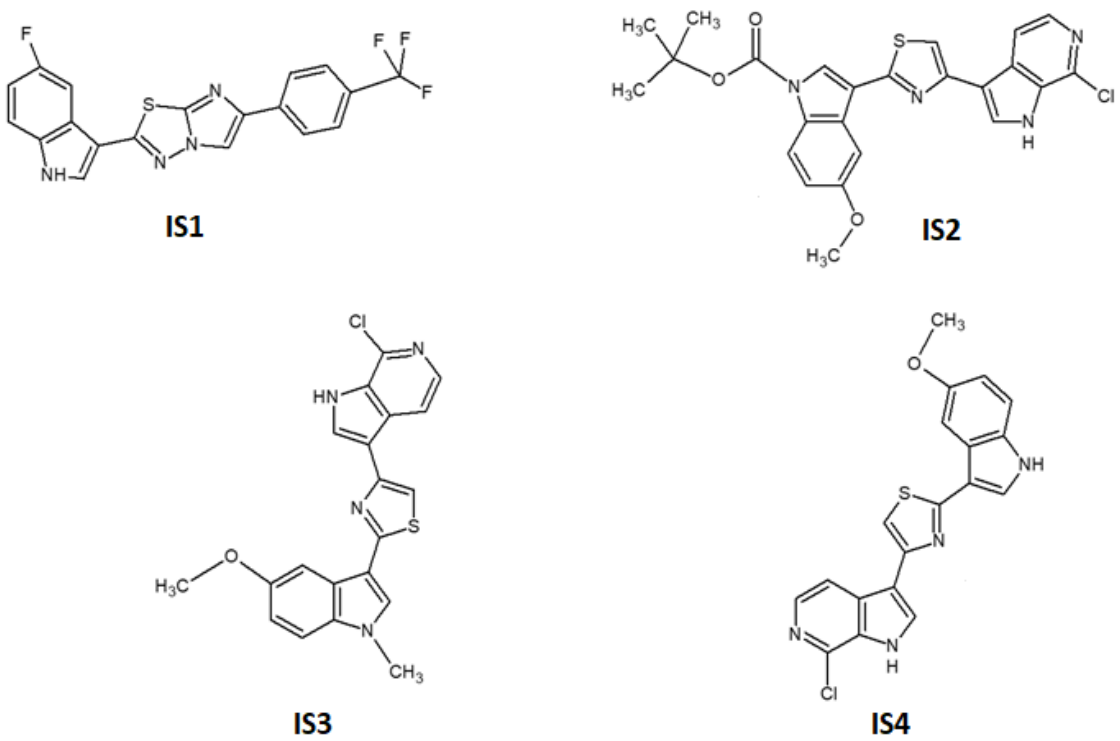

Figure 1. Chemical structures of potential SF3B1 modulators.

In order to prioritise molecules in terms of interactions and theoretical binding energy, docking studies then highlighted the importance of Tyr36, Arg38, Arg1074, Arg1075 and Leu1066 residues in the stabilization of the protein-ligand complex.

We then evaluated the *in vitro* cytotoxic activity of the potential SF3B1 modulators in PDAC SUIT-2 and Hs766t cells by SRB assay. Two out of four compounds (**IS1** and **IS4**) were more promising. In particular, the Hs766t cells after 72 hours of drug exposure were more sensitive to compounds **IS1** and **IS4** with IC_{50} values of 2.7 and 2.4 µM, respectively. Conversely, SUIT-2 cells were less sensitive with IC_{50} values between 4.5 and 7.5 µM. Furthermore, as the PDAC epithelial cell line Panc05.04 harbors the *SF3B1* mutations p.Q699H and p.K700E, we performed further in-depth studies with compounds **IS1** and **IS4**. Therefore, the induction of cell death in Panc05.04 cells was evaluated by trypan blue exclusion test. After 96 hours of drug exposure at 5 different concentrations (0.1, 0.5, 1, 5 and 10 µM), both drugs induced cell death ranging from 52% to 63% at a concentration of 1 µM.

The anti-migratory activity of potential SF3B1 modulators on SUIT-2 cells by wound-healing assay has shown promising results. After 24 hours of drug exposure at concentrations of $4xIC_{50}$ value, migration rates below 40% and 10% for compounds **IS1** and **IS4**, respectively, were observed. Furthermore, the anti-migratory ability of compounds **IS1** and **IS4** could be partially confirmed by the splicing shift from RON to ΔRON in SUIT-2 cells after 24 hours of drug exposure. The combination of both compounds (**IS1** and **IS4**) at their IC_{50} with gemcitabine at its IC_{25} resulted in a significant reduction in cell growth of SUIT-2 and Hs766t cells with values below 20% and 12% compared to untreated cells and in a significant increase of hENT1 mRNA expression ($p<0.05$ vs. untreated control cells), supporting the role of these compounds in reversing a key mechanism of resistance to gemcitabine. Finally, the increased sensitivity to gemcitabine was confirmed in the 3D culture model (spheroids) of PDAC-3 cells. We treated the spheroids with gemcitabine, compound **IS4** and their combination for 72 hours during which we observed their shrinkage. In particular, the growth of the spheroids was significantly inhibited by the combination. Therefore, a statistically significant reduction in the size of the spheroids treated with the drug combination was observed compared to untreated controls as well as to spheroids exposed to gemcitabine-alone.

Conclusions

The highly poor prognosis and the growing incidence of pancreatic cancer led to a considerable interest in the scientific community. Unfortunately, despite countless efforts, we are only at the beginning of understanding the complex cell biology of this tumor. Current anticancer therapies are mainly limited by the development of drug resistance resulting in poor or even no response to treatment. Therefore, there is an urgent need for new therapeutic strategies that target the key determinants of PDAC.

Given the central role of alternative splicing (AS) in cancer, targeting this process is considered as a potential novel therapeutic approach.

In fact, the subject of this dissertation is the search for new therapeutic strategies for pancreatic cancer and aims to implement a Drug Discovery process for the rational design and synthesis of molecules active in the modulation of pathways related to the regulation of pre-mRNA splicing process. It is the result of a joint PhD between the University of Palermo, Italy, and the Department of Medical Oncology, VU University Medical Center, Amsterdam, the Netherlands, which combines medicinal chemistry and translational cancer research.

Chemically, the Thesis aims at the design and synthesis of potential SF3B1 modulators, an over-expressed and/or mutated protein in hematologic malignancies and some solid tumors, including pancreatic cancer (4%), and involved in the splicing regulation.

Based on the interesting anticancer properties recently described for the imidazothiadiazole nucleus as a scaffold for the development of pharmacologically active derivatives, we were encouraged to continue with the same approach. Therefore, a preliminary computational study prompted us to perform virtual screening of an in-house library and commercially available molecular libraries, in order to identify the molecular scaffold of interest and then select the most promising molecules that were subsequently biologically tested *in vitro* on pancreatic tumor cell lines.

Furthermore, the discovery over the years of multiple splicing modulators targeting SF3B1, prompted us to test them for the first time in PDAC as well.

However, since biomarkers represent a valuable tool for providing PDAC patients with personalized existing antitumor therapy, the role of the human Equilibrative Nucleoside Transporter-1 (hENT1) as a potential biomarker in PDAC is extensively covered and explored in the Thesis.

Finally, the need for reliable biomarkers and the promising antitumor activity previously observed for imidazothiadiazole compounds has paved the way for further *in vitro* biological studies concerning a new class of imidazothiadiazole compounds.

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Chapter 8

English summary

Riassunto in Italiano

English summary

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and lethal cancers with a highly poor prognosis and an increasing incidence. To date, PDAC is the seventh most common cancer in the world and is expected to become the third most frequent cause of cancer-related deaths by 2025.

PDAC is highly resistant to current therapies (surgery, chemotherapy, alone or in combination with radiotherapy, and palliative care) and is generally diagnosed at an advanced stage due to the absence of early symptoms, offering patients an overall 5-year survival rate around 7%.

Particularly aggressive biology, the tendency to rapid metastatic spread and resistance to treatment represent highly unfavorable conditions for the fight against PDAC.

Although improvements have been achieved in the diagnosis, prognosis and treatment of this tumor, the need to find diagnostic biomarkers with high sensitivity and specificity remains.

Furthermore, the design of targeted and specialized therapies in combination with conventional treatments is undoubtedly essential in the improvement of cancer therapy.

Therefore, this Thesis aims at the design and synthesis of new anticancer compounds potentially active on *SF3B1* (Splicing Factor 3B subunit 1), one of the numerous genes involved in the RNA splicing process that has been identified as being frequently mutated in various malignant tumors. Since there is an urgent need to find new effective therapeutic strategies for the treatment of patients with PDAC, we have decided to evaluate the antitumor activity of these compounds on preclinical models of PDAC.

In **Chapter 2** we have provided a broad overview of the status of the transmembrane protein hENT-1 (human Equilibrative Nucleoside Transporter-1), involved in the absorption of the anticancer drug gemcitabine which for more than twenty-five years has been the main therapy for PDAC in both pallative and adjuvant fields. Several studies have shown controversial results regarding the predictive value of hENT-1 for gemcitabine activity in PDAC. Therefore, new analyzes with larger patient cohorts, the use of standardized methodologies, and a better understanding of the molecular mechanisms that influence the expression and activity of this nucleoside transporter, should help in the identification of patient subgroups that may benefit of specific treatments.

In **Chapter 3** we evaluated the cytotoxic activity of ten imidazo[2,1-*b*][1,3,4]thiadiazole compounds in two primary cell lines of diffuse malignant peritoneal mesothelioma (DMPM),

Meso II and STO. The promising antiproliferative and antimigration activity of two out of ten compounds has been associated with their ability to reduce focal adhesion kinase (FAK) phosphorylation, which is a potential target for mesothelioma treatment. The enhancement of the antiproliferative activity of gemcitabine and the increase of the mRNA expression of hENT-1 further confirmed the potential of this new class of compounds and paved the way for the rational development of new drug combinations for DMPM.

In **Chapter 4** we reviewed the importance of two epigenetic processes involved in the progression of PDAC, namely the deregulation of microRNAs (miRNAs) and mRNA splicing. Some splicing factors interact with specific miRNAs and facilitate or inhibit their expression. Therefore, we have described the correlation between splicing and miRNAs, as well as novel bioinformatics tools to predict the effect of splicing modulation towards miRNA profiles and predictive target models, in order to provide innovative and effective therapeutic approaches in the treatment of the complex biology of the disease.

In **Chapter 5** we tested for the first time two modulators (pladienolide B and E7107) of the key splicing factor SF3B1 in cell lines (primary and immortalized normal) of PDAC, in order to identify new potential therapeutic targets for the treatment of this grim disease. Promising results in both *in vitro* and *in vivo* models confirmed that SF3B1 represents a potential prognostic factor and an attractive therapeutic target for patients with PDAC. Furthermore, its modulation influences the proliferation, migration and alternative splicing of key oncogenes.

In **Chapter 6** we examined the activity of four novel potential SF3B1 inhibitors - one imidazo[2,1-*b*][1,3,4]thiadiazole derivative and three indole derivatives - selected by virtual screening from an in-house molecular library, in PDAC cell lines. The promising cytotoxic and antimigration activity in PDAC cells, as well as the potentiation of the anticancer effects of gemcitabine and the modulation of RON (recepteur d'origine nantais) and hENT-1, have shown that inhibition of splicing may represent a new approach in the fight against the PDAC chemoresistance.

Riassunto in Italiano

L'adenocarcinoma duttale pancreatico (PDAC) è una delle neoplasie più aggressive e letali con una prognosi altamente infausta e una incidenza in aumento. Ad oggi, il PDAC è il settimo cancro più comune al mondo e si prevede che diventerà la terza causa più frequente di decessi correlati al cancro entro il 2025.

Il PDAC è altamente resistente alle attuali terapie (chirurgia, chemioterapia, da sola o in combinazione con la radioterapia, e cure palliative) e generalmente viene diagnosticato in uno stadio avanzato a causa dell'assenza di sintomi precoci, offrendo ai pazienti una sopravvivenza globale a 5 anni intorno al 7%.

La biologia particolarmente aggressiva, la tendenza ad una rapida diffusione metastatica e la resistenza al trattamento rappresentano condizioni altamente sfavorevoli per la lotta contro il PDAC.

Sebbene siano stati ottenuti miglioramenti nella diagnosi, nella prognosi e nel trattamento di questo tumore, rimane la necessità di trovare biomarcatori diagnostici con un'alta sensibilità e specificità.

Inoltre, la progettazione di terapie mirate e specializzate in combinazione con i trattamenti convenzionali è indubbiamente essenziale nel miglioramento della terapia del cancro.

Pertanto, questa Tesi mira alla progettazione e sintesi di nuovi composti antitumorali potenzialmente attivi su *SF3B1* (Fattore di Splicing 3B subunità 1), uno dei numerosi geni coinvolti nel processo di splicing dell'RNA che è stato identificato come frequentemente mutato in vari tumori maligni. Poiché c'è un urgente bisogno di trovare nuove strategie terapeutiche efficaci per il trattamento dei pazienti affetti da PDAC, abbiamo deciso di valutare l'attività antitumorale di tali composti su modelli preclinici di PDAC.

Nel **Capitolo 2** abbiamo fornito un'ampia panoramica sullo status della proteina transmembrana hENT-1 (Trasportatore Nucleosidico Equilibrativo umano-1), coinvolta nell'assorbimento del farmaco antitumorale gemcitabina che da più di venticinque anni rappresenta la terapia principale per il PDAC sia in ambito palliativo che adiuvante. Diversi studi hanno mostrato risultati controversi riguardo il valore predittivo di hENT-1 per l'attività della gemcitabina nel PDAC. Pertanto, nuove analisi con coorti più ampie di pazienti, l'uso di metodologie standardizzate e una migliore conoscenza dei meccanismi molecolari che influenzano l'espressione e l'attività di tale trasportatore nucleosidico, dovrebbero aiutare nell'identificazione di sottogruppi di pazienti che possono beneficiare di trattamenti specifici.

Nel **Capitolo 3** abbiamo valutato l'attività citotossica di dieci composti imidazo[2,1-*b*][1,3,4]tiadiazolici in due linee cellulari primarie di mesotelioma peritoneale maligno diffuso (DMPM), Meso II e STO. La promettente attività antiproliferativa e antimigratoria di due composti su dieci è stata associata alla loro capacità di ridurre la fosforilazione della chinasi di adesione focale (FAK), un potenziale bersaglio per il trattamento del mesotelioma. Il potenziamento dell'attività antiproliferativa della gemcitabina e l'aumento della espressione dell'mRNA di hENT-1, hanno ulteriormente confermato il potenziale di questa nuova classe di composti e hanno spianato la strada per lo sviluppo razionale di nuove combinazioni di farmaci per il DMPM.

Nel **Capitolo 4** abbiamo rivisto l'importanza di due processi epigenetici coinvolti nella progressione del PDAC, ovvero la deregolazione dei microRNA (miRNA) e lo splicing dell'mRNA. Alcuni fattori di splicing interagiscono con miRNA specifici e facilitano o inibiscono la loro espressione. Pertanto, abbiamo descritto la correlazione tra splicing e miRNA, nonché nuovi strumenti di bioinformatica per prevedere l'effetto della modulazione dello splicing verso profili di miRNA e modelli predittivi del target, al fine di fornire approcci terapeutici innovativi ed efficaci nel trattamento della complessa biologia della malattia.

Nel **Capitolo 5** abbiamo testato per la prima volta due modulatori (pladienolide B ed E7107) del fattore chiave di splicing SF3B1 in linee cellulari (primarie e normali immortalizzate) di PDAC, al fine di identificare nuovi potenziali bersagli terapeutici per il trattamento di questa tragica malattia. I risultati promettenti in modelli sia *in vitro* che *in vivo* hanno confermato che SF3B1 rappresenta un potenziale fattore prognostico e un bersaglio terapeutico interessante per i pazienti con PDAC. Inoltre, la sua modulazione influenza la proliferazione, la migrazione e lo splicing alternativo di oncogeni chiave.

Nel **Capitolo 6** abbiamo esaminato l'attività di quattro nuovi potenziali inibitori di SF3B1 - un derivato imidazo[2,1-*b*][1,3,4]tiadiazolico e tre derivati indolici - selezionati tramite screening virtuale da una libreria molecolare interna, in linee cellulari di PDAC. La promettente attività citotossica e antimigratoria nelle cellule PDAC, così come il potenziamento degli effetti antitumorali della gemcitabina e la modulazione di RON (recepteur d'origine nantais) ed hENT-1, hanno dimostrato che l'inibizione dello splicing può rappresentare un nuovo approccio nella lotta alla chemioresistenza del PDAC.

Curriculum vitae

List of publications

Personal information

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Education

November 2018 - November 2021: PhD Student (joint PhD) in Pharmaceutical/Medicinal Chemistry and Oncological Research. Title of the dissertation: *Design of SF3B1 subunit modulators of the SF3B spliceosome complex*

Institutions involved in the research PhD project:

- Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Palermo, Italy.
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  Co-promotor: Dr. Stella Maria Cascioferro
- Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands.
  Promotor: Dr. Elisa Giovannetti
  Co-promotor: Prof. Dr. Godefridus J. Peters

June 2018: Qualification examination for the profession of Pharmacist, University of Palermo, Palermo, Italy.

April 2018 - May 2018: Voluntary research collaborator in organic synthesis of molecules with potential antitumor activity, Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Palermo, Italy.

March 2018: Master's degree in Pharmacy, Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Palermo, Italy.

Vote: 110/110 *cum laude*. Thesis Title: *Design and synthesis of new 4-chinazolinone derivatives potential inhibitors of dihydrofolate reductase*

Promotor: Dr. Maria Valeria Raimondi.

Other experiences

February 2020 – October 2020: tutor of students during their Thesis at the Department of Medical Oncology, Cancer Center Amsterdam (CCA), Amsterdam UMC, VU University Medical Center (VUmc), Amsterdam, The Netherlands.

November 2018 – September 2019: tutor of bachelor's students during their experimental Thesis in Medicinal Chemistry at the University of Palermo, Palermo, Italy.

May 2017 - January 2018: Practical and professional curricular internship. *Main activities and responsibilities:* professional order and deontology; technical-administrative management of the pharmacy; purchase, storage and dispensation of drugs; stability and good preservation of drugs; procedures for the management of expired or revoked drugs; economic-fiscal management; information and health education of the population through the distribution aimed at the correct use of drugs and prevention.

Awards

The top 10 review articles of 2019 in the Medicinal Chemistry section of *Molecules:*
DHFR Inhibitors: Reading the Past for Discovering Novel Anticancer Agents
Maria Valeria Raimondi*, **Ornella Randazzo***, Mery La Franca, Giampaolo Barone, Elisa Vignoni, Daniela Rossi, Simona Collina
* These authors contributed equally
Molecules. 2019 Mar 22;24(6):1140. doi: 10.3390/molecules24061140.

List of publications

- **SF3B1 modulators affect key genes in metastasis and drug influx: a new approach to fight pancreatic cancer chemoresistance**
  Ornella Randazzo*, Stella M. Cascioferro*, Camilla Pecoraro*, Widad Ait Iddouch, Amir Avan, Barbara Parrino, Daniela Carbone, Ugo Perricone, Godefridus J. Peters, Patrizia Diana, Elisa Giovannetti
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  Cancer Drug Resist 2021;4:904-922. doi: 10.20517/cdr.2021.61.

- **Interrelationship between miRNA and splicing factors in pancreatic ductal adenocarcinoma**

  I Gede Putu Supadmanaba*, Giulia Mantini*, **Ornella Randazzo***, Mjriam Capula, Ittai B. Muller, Stella M. Cascioferro, Patrizia Diana, Godefridus J. Peters and Elisa Giovannetti

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  Epigenetics. 2021 May 30;1-24. doi: 10.1080/15592294.2021.1916697.

- **"Open Sesame?": Biomarker Status of the Human Equilibrative Nucleoside Transporter-1 and Molecular Mechanisms Influencing its Expression and Activity in the Uptake and Cytotoxicity of Gemcitabine in Pancreatic Cancer**

  Ornella Randazzo*, Filippo Papini*, Giulia Mantini, Alessandro Gregori, Barbara Parrino, Daniel S. K. Liu, Stella M. Cascioferro, Daniela Carbone, Godefridus J. Peters, Adam E. Frampton, Ingrid Garajova** and Elisa Giovannetti**

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  Cancers (Basel). 2020 Oct 31;12(11):3206. doi: 10.3390/cancers12113206.

- **New Imidazo[2,1-*b*][1,3,4]Thiadiazole Derivatives Inhibit FAK Phosphorylation and Potentiate the Antiproliferative Effects of Gemcitabine Through Modulation of the Human Equilibrative Nucleoside Transporter-1 in Peritoneal Mesothelioma**

  Giovanna Li Petri*, Camilla Pecoraro*, **Ornella Randazzo***, Silvia Zoppi, Stella M. Cascioferro, Barbara Parrino, Daniela Carbone, Btissame El Hassouni, Andrea Cavazzoni, Nadia Zaffaroni, Girolamo Cirrincione, Patrizia Diana, Godefridus J. Peters and Elisa Giovannetti

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  Anticancer Res. 2020 Sep;40(9):4913-4919. doi: 10.21873/anticanres.14494.

- **The role of developmental signaling pathways in non-small cell lung carcinoma**

  Darko Durovski, **Ornella Randazzo**, Godefridus J. Peters and Elisa Giovannetti

  Journal of Molecular and Clinical Medicine. 2019, 2(2):41-53. doi:10.31083/j.jmcm.2019.02.151.

- **DHFR Inhibitors: Reading the Past for Discovering Novel Anticancer Agents**

Maria Valeria Raimondi*, **Ornella Randazzo***, Mery La Franca, Giampaolo Barone, Elisa Vignoni, Daniela Rossi, Simona Collina

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Molecules. 2019 Mar 22;24(6):1140. doi: 10.3390/molecules24061140.

In preparation

- **Exploring splicing modulation as a novel strategy against pancreatic cancer**

  Rocco Sciarrillo*, **Ornella Randazzo*,** Giulia Mantini*, Btissame El Hassouni, I Gede Putu Supadmanaba, Tonny Lagerweij, Tom Würdinger, Godefridus J. Peters, Carla F.M. Molthoff, Gerrit Jansen, Gertjan J.L. Kaspers, Jacqueline Cloos, Elisa Giovannetti

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Conference abstracts and poster presentations

- **Splicing modulation as novel strategy against pancreatic cancer**

  Ornella Randazzo, I Gede Putu Supadmanaba, Rocco Sciarrillo, Btissame El Hassouni, Giulia Mantini, Stella M. Cascioferro, Patrizia Diana, Godefridus J. Peters, Elisa Giovannetti

  In: XLIV National Congress of AISP (Italian Association for the Study of the Pancreas), Verona, Italy, 17-19/09/2020.

- **NOTCH2 gene amplification is associated with prognosis and supports novel therapeutic strategies for malignant pleural mesothelioma**

  Ornella Randazzo*, Camilla Pecoraro*, Maria Gemelli, Amir Avan, Stella M. Cascioferro, Patrizia Diana, Paolo A. Zucali, Elisa Giovannetti

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  In: 3rd International Conference "Cancer Stem Cells: Impact on Treatment", Seefeld, Tyrol, Austria, 13-17/12/2020.

- **Synthesis of an isoster of 3,5-dimethyl-6-phenyl-8-(trifluoromethyl)-5,6-dihydropyrazole[3,4-*f*][1,2,3,5]tetrazepin-4(3H)-one (CF3-TZP) with potential biological activity**

Ornella Randazzo, Giovanna Li Petri, Fabiana Plescia, Benedetta Maggio, Giuseppe Daidone, Demetrio Raffa, Mery La Franca, Roberta Listro, Agnese Ribaudo, Giampaolo Barone, Maria Valeria Raimondi

In: Proceedings of the Congress of the Italian Chemical Society - Joint Congress of the Sicily and Calabria Sections 2018. p.60, Catania, Italy, 9-10/02/2018.

- **Synthesis and biological evaluation of new nitro-derivatives pyrrolomycin D analogue active on Pseudomonas aeruginosa**

  Agnese Ribaudo, Maria Grazia Cusimano, Fabiana Plescia, Benedetta Maggio, Giuseppe Daidone, Demetrio Raffa, Mery La Franca, Giovanna Li Petri, **Ornella Randazzo**, Roberta Listro, Giampaolo Barone, Domenico Schillaci, Maria Valeria Raimondi

  In: Proceedings of the Congress of the Italian Chemical Society - Joint Congress of the Sicily and Calabria Sections 2018. p.61, Catania, Italy, 9-10/02/2018.

- **Design and synthesis of new 4-quinazolinone derivatives potential inhibitors of dihydrofolate reductase**

  Mery La Franca, **Ornella Randazzo**, Fabiana Plescia, Benedetta Maggio, Giuseppe Daidone, Demetrio Raffa, Giovanna Li Petri, Roberta Listro, Agnese Ribaudo, Giampaolo Barone, Maria Valeria Raimondi

  In: Proceedings of the Congress of the Italian Chemical Society - Joint Congress of the Sicily and Calabria Sections 2018. p.62, Catania, Italy, 9-10/02/2018.

- **Pyrrolomycins which inhibit Sortase A in infections caused by Gram-positive bacteria**

  Roberta Listro, Maria Grazia Cusimano, Fabiana Plescia, Benedetta Maggio, Giuseppe Daidone, Demetrio Raffa, Mery La Franca, Giovanna Li Petri, Agnese Ribaudo, **Ornella Randazzo**, Giampaolo Barone, Domenico Schillaci, Maria Valeria Raimondi

  In: Proceedings of the Congress of the Italian Chemical Society - Joint Congress of the Sicily and Calabria Sections 2018. p.63, Catania, Italy, 9-10/02/2018.