Molecular targets for novel drug development in pancreatic cancer

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Introduction

The pancreas is a secretory organ containing both endocrine and exocrine glands. The exocrine glands, which account for 95% of the glands, produce the pancreatic juice containing the digestive enzymes. The endocrine cells are present in the islets of Langerhans. These islets are responsible for the production of the two main metabolic hormones, insulin and glucagon [1]. Pancreatic cancer is the tenth most common cancer representing 2.8% of cancer cases in the USA [2] and 3% in the UK, equally affecting males and females [3]. The most prevalent form of pancreatic cancer is Pancreatic Ductal Adenocarcinoma (PDAC), the malignant transformation of the exocrine duct cells [4]. PDAC, constituting 90% of all pancreatic cancer cases, displays local invasion and distant metastasis during early disease stages. It is a very aggressive cancer type with an overall 5-year survival of only 6% [5,6]. In 2014, 46,420 new cases and 39,590 deaths were recorded in the USA [2], while the annual incidence of PDAC in the UK is approximately 8,700 cases [3]. A major cause of the poor prognosis of PDAC is the frequent metastasis of PDAC cells to the liver, lungs, and other organs.

Unfortunately, as is also the case with many other cancer types, the symptoms of pancreatic cancer are generally non-specific. These include abdominal pain, unexplained weight loss, diarrhoea, constipation, bloating, vomiting, jaundice and malaise [7]. Tumour growth can result in bile duct blockage, which may lead to a build-up of bilirubin, causing jaundice. Tumours can also block the duodenum, causing nausea, vomiting and constipation [8].

To date, surgery is the only chance of cure. Unfortunately, due to the highly non-specific symptoms, at the time of diagnosis the vast majority of patients already have advanced disease and are not...
suited for surgical resection [4]. Even in patients who undergo resection, 80% eventually relapse and succumb to the disease [9,10]. Systemic therapies are largely ineffective, increasing the overall survival by only a few months on average. Consequently, there is an urgent need to develop a better understanding of the molecular background of pancreatic cancer, its heterogeneity and how this may relate to chemotherapy responses.

Review

Risks factors of pancreatic ductal adenocarcinoma

The causes of pancreatic cancer remain largely undetermined. However, environmental factors are believed to be important contributors to the development of pancreatic cancer (Table 1). Tobacco emerged as a prevalent risk factor in PDAC development. Cigarette smokers have an up to 3.6 times greater chance of developing pancreatic cancer [11] and recent figures show that smoking is accountable for 29% of all PDAC cases in the UK [12]. It is believed that carcinogens released from the tobacco smoke are responsible. Due to the fact that only a small proportion of smokers develop PDAC, the assumption is that some individuals are more susceptible due to a less effective protective system against the toxic/carcinogenic molecules in the tobacco smoke. For example, it is believed that mutations or polymorphisms in enzymes of the cytochrome P system (e.g., CYP1A2) and n-Acetyl transferase enzymes affects their activity and ability to detoxify tobacco products and may be an underlying cause of this susceptibility [13].

Table 1. Epidemiology of pancreatic cancer: 1970's versus current data. Table adopted from [61].

<table>
<thead>
<tr>
<th>1975 data</th>
<th>Current data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common in western countries</td>
<td>Still true [5].</td>
</tr>
<tr>
<td>Increasing frequency</td>
<td>Yes, in both males and females [3].</td>
</tr>
<tr>
<td>Increased risk for smokers</td>
<td>Confirmed [12,61].</td>
</tr>
<tr>
<td>Increased risk for diabetics</td>
<td>Type II and type I diabetes increases risk [20].</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>No clear correlation.</td>
</tr>
<tr>
<td>Obesity</td>
<td>20% increased risk of developing pancreatic cancer [16].</td>
</tr>
<tr>
<td>Hereditary pancreatitis</td>
<td>High risk of pancreas cancer in patients with hereditary pancreatitis [15,22,23].</td>
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</tbody>
</table>

Alcohol consumption is another proposed risk factor. Studies revealed that drinking three or more alcohol beverages each day increases the chance of developing pancreatic cancer by 20%. This has been linked to pancreatitis induced by alcohol consumption [14]. In today's culture, a strong relationship exists between alcohol consumption and cigarette smoking making it difficult to determine the individual contribution of alcohol consumption versus tobacco smoke to PDAC development [14]. However, a clear link has been established between excessive alcohol consumption, chronic pancreatitis and development of pancreatic cancer as a number of large-scale studies linked chronic pancreatitis with an increased risk of pancreatic cancer [15].

Obesity is another underlying risk factor. Individuals suffering from obesity have exhibited a 20% greater chance of developing PDAC [16,17]. Further supporting the potential contribution of metabolic disorders to PDAC development, diabetes has been identified as another risk factor [18]. However, the causative link between diabetes and pancreatic cancer remains unclear as development of PDAC itself can lead to diminished insulin production and subsequent diabetes. In patients with diabetes mellitus (DM), the chance of developing PDAC is increased by 40-100% [19]. It has been reported that the majority of PDAC (80-90%) patients have type II diabetes [20], although studies have shown a two-fold increased risk of pancreatic cancer in patients with type I diabetes as well [20]. While environmental factors seem to play a central role in PDAC development, even possibly to a higher extent than in case of some other cancers, the mechanism how they trigger or potentiate the malignant transformation is not well understood.

Studies are beginning to identify familial links in pancreatic cancer. For example, Permut-Wey and Egan have shown that there is almost a 2-fold increased risk in developing PDAC when there is a family history of the disease [21]. Another familial linkage associated with the onset of pancreatic cancer is inherited pancreatitis, a rare autosomal disease. Its symptoms are similar to those of chronic pancreatitis [22,23]. Other germ-line diseases have also been linked to small subsets of PDAC, the best known of these are listed in Table 2.

Current treatment methods

Surgeons would consider tumours of an exocrine origin to be ‘resectable’ if the cancer has not metastasized beyond the pancreas [7]. Resectable tumours are usually removed by the ‘Whipple’ procedure (pancreatoduodenectomy) or by a distal resection, 80% eventually relapse and succumb to the disease [9,10]. Systemic therapies are largely ineffective, increasing the overall survival by only a few months on average. Consequently, there is an urgent need to develop a better understanding of the molecular background of pancreatic cancer, its heterogeneity and how this may relate to chemotherapy responses.

Table 2. Diseases associated with the onset of PDAC. Table adopted from [61].

<table>
<thead>
<tr>
<th>Disease</th>
<th>Chromosome</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li-Fraumeni syndrome</td>
<td>17p13.1</td>
<td>Defective p53. Modest increased risk of pancreatic cancer [62].</td>
</tr>
<tr>
<td>Cystic Fibrosis</td>
<td>7q31</td>
<td>Increased risk of pancreatic tumours [63].</td>
</tr>
<tr>
<td>Peutz-Jeghers syndrome</td>
<td>19p</td>
<td>Mutation may contribute to both sporadic and inherited disease [64].</td>
</tr>
<tr>
<td>Familial adenomatous polyposis</td>
<td>5q12–21</td>
<td>Mutation found in pancreas cancers [64].</td>
</tr>
<tr>
<td>Hereditary non-polyposis colon cancer (HNPPCC)</td>
<td>2, 3</td>
<td>Risk of developing pancreatic cancer.</td>
</tr>
</tbody>
</table>
pancreatectomy [7]. Staging of PDAC is however a difficult task, which may explain why the cancer may return after surgery. As a result chemotherapy drugs, such as gemcitabine (Table 3), are used as an adjuvant therapy with the aim to eradicate potential disseminated tumour cells [24]. Unfortunately, the majority of patients receive little or no benefit from current chemotherapies mainly because most of the cancer cells are either intrinsically chemoresistant or they become resistant during therapy. In addition, extrinsic mechanisms such as the tumour stroma have been discussed as a driver of resistance to chemotherapy in PDAC [25].

Therefore, it is of great interest to identify and characterize what molecules promote drug resistance with the hope to inhibit these molecules as novel targeted therapy options.

Locally advanced pancreatic cancers are difficult to treat with surgery. The combination of chemotherapy and radiation (chemoradiation) is often used to help reduce the tumour size [7]. However, this is an intensive form of treatment; the combination could result in severe side effects for the patient. Metastatic PDAC is also difficult to tackle with surgery. Chemotherapy drugs such as gemcitabine, albumin-bound paclitaxel nanoparticles (Abraxane) or the combination of four chemotherapy drugs, known as FOLFIRINOX (Folinic Acid, Fluorouracil, Irinotecan Hydrochloride, Oxaliplatin) are the standards of care for metastatic disease [24] (Table 3). The intensity of this treatment, once again, can result in severe side effects for the patients, including fever, diarrhoea, vomiting, exhaustion and neutropenia [24].

Recent findings in the US have shown that by reducing the administration of Abraxane to every other week, the side effects of the treatments could be reduced without compromising the efficacy of the treatment [26]. However, the side effects of chemotherapy are still significant. Moreover, these treatments have limited efficacy, often only improving the quality of life of patients for a period of time rather than curing the disease itself. Thus, understanding the molecular drivers of PDAC progression is crucial as this would allow identification of alternative treatment strategies.

**Molecular alterations in pancreatic cancer emerge as novel therapeutic targets**

Recent advancements have determined that there are specific genetic alterations associated with the development of pancreatic cancer. This has led to the identification of several novel therapeutic targets that are currently under investigation. The use of molecularly targeted therapies has shown promise in improving outcomes for patients with pancreatic cancer.

### Table 3. Current available treatments for pancreatic cancer [65-67].

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Comments</th>
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<tbody>
<tr>
<td>Surgery</td>
<td>Procedure to remove pancreatic tumours.</td>
</tr>
<tr>
<td></td>
<td>• Whipple procedure.</td>
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<tr>
<td></td>
<td>• Total pancreatectomy.</td>
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<tr>
<td></td>
<td>• Distal pancreatectomy.</td>
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<tr>
<td></td>
<td>Types of palliative surgery to remove symptoms and improve quality of life.</td>
</tr>
<tr>
<td></td>
<td>• Surgical biliary bypass.</td>
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<tr>
<td></td>
<td>• Endoscopic stent placement.</td>
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<tr>
<td></td>
<td>• Gastric bypass.</td>
</tr>
<tr>
<td>Radiation therapy</td>
<td>External radiation.</td>
</tr>
<tr>
<td></td>
<td>Internal radiation utilised sealed radioactive substances in close proximity to the tumour.</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>Food and Drug Administration (FDA) approved drugs.</td>
</tr>
<tr>
<td></td>
<td>• Abraxane (Paclitaxel Albumin-stabilized Nanoparticle Formulation).</td>
</tr>
<tr>
<td></td>
<td>• Adrucil (Fluorouracil).</td>
</tr>
<tr>
<td></td>
<td>• Afinitor (Everolimus).</td>
</tr>
<tr>
<td></td>
<td>• Efudex (Fluorouracil).</td>
</tr>
<tr>
<td></td>
<td>• Erlotinib Hydrochloride.</td>
</tr>
<tr>
<td></td>
<td>• Everolimus.</td>
</tr>
<tr>
<td></td>
<td>• Fluoroplex (Fluorouracil).</td>
</tr>
<tr>
<td></td>
<td>• Fluorouracil.</td>
</tr>
<tr>
<td></td>
<td>• Gemcitabine Hydrochloride.</td>
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<tr>
<td></td>
<td>• Gemzar (Gemcitabine Hydrochloride).</td>
</tr>
<tr>
<td></td>
<td>• Mitomycin C.</td>
</tr>
<tr>
<td></td>
<td>• Mitozytrex (Mitomycin C).</td>
</tr>
<tr>
<td></td>
<td>• Mutamycin (Mitomycin C).</td>
</tr>
<tr>
<td></td>
<td>• Paclitaxel Albumin-stabilized Nanoparticle Formulation.</td>
</tr>
<tr>
<td></td>
<td>• Sunitinib Malate.</td>
</tr>
<tr>
<td></td>
<td>• Sutent (Sunitinib Malate).</td>
</tr>
<tr>
<td></td>
<td>• Tarceva (Erlotinib Hydrochloride).</td>
</tr>
<tr>
<td>Combinational chemotherapy</td>
<td>• FOLFIRINOX (Folinic Acid, Fluorouracil, Irinotecan Hydrochloride, Oxaliplatin).</td>
</tr>
<tr>
<td></td>
<td>• GEMCITABINE-CISPLATIN.</td>
</tr>
<tr>
<td></td>
<td>• GEMCITABINE-OXALIPLATIN.</td>
</tr>
<tr>
<td></td>
<td>• OFF (Oxaliplatin, Fluorouracil, Folinic Acid).</td>
</tr>
<tr>
<td>Chemoradiation</td>
<td>Combinations of chemotherapy and radiation therapy to increase the effects of both.</td>
</tr>
</tbody>
</table>
tumorigenesis and pathogenesis of pancreatic cancer. These include activation of oncogenes, inactivation of tumour suppressor genes and overexpression/overactivation of proliferation pathway drivers. The discovery of the genetic alterations in PDAC provides a window of opportunity for the identification of molecular targets for treatment. Due to the central role of NF-κB and KRAS in driving pancreatic cancer, the following section discusses the feasibility of targeting these molecules and the current status of research.

**KRAS mutations in PDAC**

Kirsten rat sarcoma viral oncogene homolog (KRAS) is one of the best characterised oncogenes as a key mediator of growth factor signal transduction [27]. Oncogenic mutations of KRAS are the most common mutations detected in PDAC [28]. The KRAS protein is a GTPase protein, acting as molecular switch for RAF activation and consequent activation of the mitogen activated protein kinase cascade inducing cell division [28]. Oncogenic mutation of KRAS usually results in a single amino acid substitution that leads to reduced GTPase activity, extending the time period RAS stays active after stimulation, affecting mitogen activated protein kinase (MAPK) signalling and consequent uncontrolled growth of cells [28].

Mutation of glycine at position 12 to aspartate (KRASG12D) is the most common KRAS mutation in pancreatic cancer [28]. These mutations are primary events in PDAC, with mutated KRAS is detected in 90% of low-grade pancreatic intraepithelial neoplasia lesions (PanIN) [29].

Interestingly, when tissues from healthy individuals and cancer patients were compared, oncogenic KRAS was detected in a significant number of healthy tissues, including the pancreas, colon and lungs [28]. Additionally, only a small fraction mutated KRAS-expressing cells of KRASG12D-expressing mice develop cancers, implying that KRAS alone is not sufficient to initiate carcinogenesis and other co-operating genetic or epigenetic factors that lead to elevated KRAS activity are required launch fully fetched malignant transformation in KRAS mutated cells. Studies that support this hypothesis have shown that upstream KRAS signals, such as epidermal growth factor and inflammatory stimuli, may be critical for carcinogenesis [30].

**Targeting mutated KRAS in pancreatic cancer**

The activation of oncogenic KRAS appears to be one of the preliminary events detected in pancreatic cancers. This discovery makes KRAS a desired molecular target for the treatment of pancreatic cancer [31]. However the direct targeting of KRAS was not successful to date [32]. As an alternative approach, targeting downstream mediators of RAS-signalling are emerging with most of them being in pre-clinical development stage. These approaches are detailed below.

**KRAS degradation by a programmed ubiquitin ligase (E3)**

Forced degradation of KRAS by the ubiquitin-proteasome pathway (UPP) supplies an alternative method of KRAS targeting for pancreatic cancer. The UPP is composed of the proteasome complex and a ubiquitinating enzyme family [33]. These include the ubiquitin-activating enzymes (E1), the ubiquitin-conjugating enzymes (E2) and the ubiquitin ligases (E3). E3 is the enzyme that recognises the target substrate, in this case KRAS, and tags it with a poly-ubiquitin tail where the ubiquitin units are connected after each other through their lysine 48 (K48) residue [33]. The work by Ma and colleagues helped to develop an engineered U-box based chimeric E3 ligase, known as RC-U, which is able to trigger the degradation of KRAS through the UPP. The specificity of the ubiquitin ligase (E3) is a key factor in targeting the KRAS oncogene. RAF1 (v-raf-1 murine leukaemia viral oncogene homolog 1) is an essential downstream effector of RAS, specifically activated by KRAS [34]. Studies have shown that the RAS-binding domain (RBD) and the cysteine-rich domain (CRD) of RAF1 interacts with KRAS in vivo [34] and thus were chosen as the interaction domain of the engineered E3 ligase. The E3 ligase, used by Ma and colleagues, had a RBD+CRDRAF1-U-Box (RC-U) structure [34].

It was shown that ectopic expression of RC-U in mutated KRAS expressing PANC-1 cells and MIA PaCa-2 cells reduced the levels of KRAS protein [34]. To confirm if the decreased level of KRAS protein was a result of the presence of the RC-U E3 ligase, an ubiquitination assay was conducted. The test established that KRAS was in fact polyubiquitinated in the presence of RC-U [34]. Moreover, ectopic expression of RC-U significantly reduced PANC-1 xenograft growth in nude mice [34]. Immunohistochemistry confirmed that the decrease in tumour size was associated with KRAS degradation, with the control groups exhibiting stronger KRAS expression than the RC-U expressing group [34].

The UPP system has a high specificity and has the possibility to eliminate oncogenic KRAS. With recent results arising about the alternative pathway to the UPP system that involves ornithine decarboxylase (ODC) and antizyme (AZ), the engineered targeted KRAS oncogene degradation may be a highly efficient treatment option for PDAC. [34]. While these results are promising, the mechanism of delivery and development of the RC-U engineered protein into a therapeutic may be challenging.

**Targeting KRAS using RNA interference**

Silencing of KRAS has proven to be effective in down-regulating the proliferation of pancreatic cells. The development of a controlled delivery system could enable the treatment of pancreatic cancer using siRNA technology [35].

Technology developed by Khvalevsky and colleagues gave rise to new hope with targeted drug delivery systems. A delivery system known as Local Drug EluteR (LODER) was developed [36]. This 'gradual-release' polymer matrix was utilised for the treatment of solid tumours in murine models. This siG12D LODER was developed on the basis that most KRAS ‘gain-of-function’ mutations are of a replacement of the glycine (G) at position 12 to aspartate (D) [36]. The siG12D LODER is a small, biodegradable
polymeric matrix which encapsulates the KRASG12D siRNA drug. Its design features take into consideration the requirements for slow, steady drug release and the resistance of the LODER against rapid excretion [36].

The functionality of the LODER was assessed in PANC-1 cells, containing KRASG12D and which also expressed luciferase [36]. Mice were injected with the PANC-1-Luc in the pancreas. The mice had a laparotomy and two LODERs were inserted into the tumour masses. By measuring luciferase activity, it was found that the siG12D LODER reduced tumour growth in comparison to the controls [36].

Immunohistochemical detection of KRAS confirmed that siG12D LODER reduced KRAS expression [36]. Utilising a LODER defeats many of the obstacles other approaches were facing. The tumour could be targeted where a stable, local release of the drug could be established [36]. This could be a potential mechanism for the specific targeting of pancreatic tumours.

Targeting scaffold-CRAF kinase interaction with RAS-ERK pathway

High activity of ERKI/2 detected in PDAC results from mutant KRAS [35]. Therefore, the targeting of RAS-downstream kinases, such as RAF and MEK (activators of ERK), are currently being tested as therapeutic targets [35]. Yet, obtaining an inhibitor, which is specific to the kinases, proves difficult due to the similar catalytic domain structure that many kinases share [35]. Luan and colleagues identified prohibitin (PHB), a scaffold protein, which is required for the interaction between RAF and RAS [35]. This interaction is essential for the activation of RAF and the downstream activation of the ERK pathway [35]. Another study by Polier and colleagues identified that the binding of Rocaglamide (RocA), a naturally occurring compound, to PHB impairs the interaction between cRAF (RAFI) and PHB, and thus blocking ERKI/2 activation in leukaemia cells [37].

It was verified that PHB was abundantly present in pancreatic cancer cells and silencing of PHB blocked EGF-induced phosphorylation of ERK in PDAC cell lines suggesting that PHB is required for the activation of ERKI/2 in pancreatic cancer cells [35]. When the cells were treated with RocA, the phosphorylation of cRAF and PHB in the membrane fraction was reduced [35]. The anti-tumour activity of RocA was further tested in severe immunodeficient mice with orthotopic AsPC-1 tumour cell xenografts [35]. The mice were treated daily with RocA via intraperitoneal injection. The mice treated with the RocA exhibited a significant decrease in tumour growth compared to the vehicle-treated mice [35].

Targeting the PHB-CRAF interaction emerges as a new potential mechanism for the inhibition of oncogenic KRAS, an alternative to the current unsuccessful direct inhibition of KRAS. RocA arises as a new potential approach, overcoming the lack of specificity than can be associated with generic kinase inhibitors.

Aberrant Nuclear Factor-κB pathway

Nuclear factor kappa B (NF-κB) is activated in over 70% of human pancreatic cancer cell lines [38]. NF-κB represents a family of transcription factors essential for regulation of the transcription of genes responsible for cellular functions such as apoptosis, inflammation and oncogenesis [39,40]. The NF-κB family consists of five members. These are as follows; cellular reticuloendotheliosis viral oncogene homolog (c-Rel), RelB, RelA (p65), p59 (coded by NF-κB1 gene and its precursor is p105, which undergoes limited proteolysis to produce the functional, p50 fragment) and p52 (coded by the NF-κB2 gene whose precursor is p100) [39]. These proteins all share a common amino-terminal Rel homology domain (RHD), essential for dimerization, DNA binding and inhibitor kappa B protein (IκB) interaction [39].

κB, RelA and RelB also possess a transcription activation domain (TAD) required for induction of gene expression. p50 and p52 lack this domain and are only functional when associated to a TAD-containing family member [39].

There are two distinct pathways of NF-κB (Figure 1). The canonical pathway leads to the activation of the p50/p65 dimer and is typically activated in response to Tumour Necrosis Factor (TNF) [39]. When TNF binds to its receptors, it activates the IκB kinase (IKK) complex consisting of IκKα, IκKβ and NEMO. The complex phosphorylates inhibitor kappa B (IκB) leading to its ubiquitination and consequent degradation of and release of NF-κB from the inhibitory interaction with IκB [41]. The non-canonical pathway is activated by other TNF cytokines, such as CD40 and it is dependent on NF-κB inducing kinase (NIK) activation, as opposed to the IKK complex. NIK phosphorylates p100, which initiates its processing to p52. This results in the accumulation of RelB/p52 and transcription of NF-κB target genes [41]. The main difference between the two NF-κB activation pathways is that the canonical pathway triggers a quick, transient NF-κB response, while the non-canonical pathway leads to a slower, maintained NF-κB signal [42].

Similarly to other tumours, the constitutive activation of NF-κB in pancreatic cancer is not primarily determined by mutations of genes involved in its regulation, but rather by pro-inflammatory cytokines, such as TNF and IL-1α released by tumour-infiltrating immune cells. Studies have demonstrated that IL-1α drives the activation of NF-κB in metastatic pancreatic cancer cell lines, which in turn induces expression and secretion of IL-1α from the tumour cells, thus establishing a positive feedback loop for constitutive NF-κB activation [43,44].

Interestingly, Bang and colleagues have shown that oncogenic KRAS promotes constitutive NF-κB activation by inducing glycogen synthase kinase 3a (GSK-3a). GSK-3a binds to the IKK-activating protein complex, consisting of transforming growth factor-beta activated kinase 1 (TAK1) and TAK1-binding protein (TAB), and stabilises it, thus enhancing canonical NF-κB activation. GSK-3a can also enhance IκKα and NIK activity through which it also drives the non-canonical NF-κB pathway [45]. Upon constitutive activation, NF-κB can inhibit pro-apoptotic signalling pathways.

Targeting the NF-κB pathway in pancreatic cancer

The non-canonical pathway of NF-κB is often active in pancreatic
Figure 1: The canonical and the non-canonical NF-κB pathways.

(A) The canonical NF-κB pathway. Binding of TNF to TNFR1 triggers the recruitment of an adaptor protein complex consisting of TRADD, TRAF2, cIAP1, cIAP2 and RIP1. cIAPs dimerise inducing their E3 ligase activity. cIAPs add non-degradative (K11, K63) ubiquitin chains onto receptor interacting protein kinase 1 (RIP1), themselves and other binding partners. RIP1 ubiquitination initiates the binding of two kinase complexes, the IκB kinase complex and the TAK1-TAB2/3 complex, and another ubiquitinating protein complex, called LUBAC. The LUBAC complex contains two regulatory subunits: SHANK-associated RH domain interactor (SHARPIN) and heme-oxidized IRP2 ubiquitin ligase 1 homolog (HOIL-1), and the catalytic subunit HOIL-1-interacting protein (HOIP). LUBAC adds a linear ubiquitin chain to NEMO contributing to its activation. Ubiquitination of RIP1 brings the IKK complex into proximity of the TAK1 kinase complex, leading to phosphorylation and activation of IKKB. IKKB phosphorylation triggers IκBa phosphorylation and subsequent proteasomal degradation and freeing the p65 and RelA NF-κB subunits, their translocation to the nucleus and initiation of gene expression. (B) The non-canonical NF-κB pathway. In resting conditions, cIAP1/2, TRAF2 and TRAF3 form a complex with NIK, the key regulator of non-canonical NF-κB signalling. In the complex, NIK is brought to close proximity with cIAPs and it becomes ubiquitinated with K48, degradative linkages that target it for proteasomal degradation. Activation of TNF superfamily receptors such as CD40 leads to the recruitment of the cIAP/TRAF2/TRAF3 complex to the receptor where the substrate specificity of IAPs change; instead of targeting NIK, they ubiquitinate themselves, leading to their degradation and release of NIK allowing its accumulation. NIK phosphorylates IKKa dimers, p100 is phosphorylated and its partial proteasomal degradation yields the truncated and active p52 fragment. The p52 fragment dimerises with RelB and translocates to the nucleus to drive NF-κB dependent gene expression.

cancers, and has been linked to mediating chemoresistance and metastasis [39]. For example, NIK, the key IkB-phosphorylating enzyme in the non-canonical NF-κB pathway has a high expression levels in pancreatic cancer [39]. Its inhibition, in theory, should prevent pancreatic cancer growth. To date, the inhibition of NIK has not been overly successful. Problems with the inhibitors proposed for NIK included a lack of specificity or ineffectiveness in vivo [46]. A prime example of an unsuccessful inhibitor of NIK is ‘pyrazolo[4,3-c]isoquinoline’. This inhibitor was not specific to the inhibition of NIK. The canonical pathway of NF-κB was also affected [47]. As a result, to date, there is increasing focus on the targeting of binding partners associated with NIK as detailed below.

Upregulation of TRAF2 expression
NIK is the key activator of the alternative NF-κB pathway [48]. NIK, when associated with IKKa, stimulates the non-canonical NF-κB pathway by inducing the conversion of NF-κB2 (p100) to p52 leading to the formation of the p52/RelB NF-κB heterodimer [48]. TRAF3 (TNF receptor-associated factor 3) binds NIK when there is no stimulus, recruiting NIK to the TRAF2/cIAP1/cIAP2 complex [48]. Upon CD40 and other TNF receptor engagement, essential for the mediation of several immune and inflammation responses, the activated receptor recruits...
the TRAF2/TRAF3/cIAP1/cIAP2 complex. When bound to the receptor, cIAPs ubiquitinate TRAF3, instead of NIK, leading to its degradation and release of NIK, now free to activate IKKα and p100 processing (Figure 2) [48].

NIK expression and non-canonical NF-κB activity in pancreatic cancer can be driven by GSK-3 induced by oncogenic KRAS or, as a recent study highlighted may be the cause of proteolytic degradation of the adaptor protein, TRAF2 [45].

TRAF2 downregulation is tightly associated with NIK accumulation in high-grade adenocarcinomas. According to the study of the Storz laboratory of 55 primary PDAC tissues, 83% of the grade 3 adenocarcinomas showed low TRAF2 expression with high NIK expression and high phosphorylated NIK levels (on threonine 559 of NIK). None of the 10 healthy pancreatic tissues showed this association [48]. Further studies showed that ectopic expression of TRAF2 in PANC-1 PDAC cell line with normally very low TRAF2 expression and constitutive NF-κB activity reduced NIK expression, confirming the connection between TRAF2 deficiency and overactivation of the non-canonical NF-κB pathway [48]. These results highlight that by targeting this pathway, the proliferation of pancreatic cancer cells could be decreased which may offer a new approach for treatment. A potential therapeutic strategy could be the inhibition of cIAP1 and cIAP2, the two ubiquitin ligases associated with TRAF2, which ubiquitinate NIK in the absence of a stimulus and degrade TRAF2 and/or TRAF3 upon inflammatory cytokine stimulus [48].

It has to be noted that TRAF2 and IAPs have a dual role in the canonical versus non-canonical NF-κB pathways, whereby in the canonical pathway TRAF2 and IAPs participate in non-degradative ubiquitination reactions necessary for the assembly of the NF-κB-activating complex, while they participate in NIK degradation in the non-canonical pathway (Figure 1). Thus the outcome of IAP inhibition on the balance and dynamics of NF-κB activity is difficult to predict.

### Inhibition of TAK1

Research conducted by Bang and colleagues has led to the discovery of two downstream effectors as potential targets for the inhibition of oncogenic KRAS. These are two kinases called TGF-β–activated kinase 1 (TAK1) and glycogen synthase kinase 3 (GSK-3). Intriguingly, as described previous, these two kinases also play an important role in the NF-κB pathway through which they can enhance mutated KRAS-driven oncogenesis [45].

Transforming growth factor β activated kinase-1 (TAK1) is a mitogen-activated kinase kinase kinase (MAP3K). When TAK1

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**Figure 2. Mechanism for maintenance of NIK in pancreatic cancer cells.**

In normal pancreatic cells, the TRAF2/TRAF3/cIAP1/cIAP2 complex degrades NIK via ubiquitination. In PDAC, TRAF2 degrades via ubiquitination, preventing the complex formation, allowing for accumulation of NIK. NIK activates IKKα by phosphorylating it. Once active, IKKα phosphorylates p100 that initiates its ubiquitination and limited proteolysis in the proteasome generating the p52 fragment. p52 forms a heterodimer with RelB to drive the expression of NF-κB target genes that promote cancer cell proliferation and disease progression. (=:K48 linkage between ubiquitin units).
binds to its partner proteins TAB1-4 (TGF-β activated kinase binding protein 1-4) it undergoes autophosphorylation and becomes active. Once active, TAK1 activates NF-κB by phosphorylating and thus activating IKKβ.

While targeting of NF-κB itself is not feasible due to its broad range of activities and its central role in the immune system, inhibition of TAK1 may offer a safer strategy to inhibit constitutive NF-κB activity in PDAC [45].

To establish the role of TAK1 in the activity in pancreatic cancer, Bang and colleagues assessed the significance of the TAK1-TAB1 complex and the effects of KRAS on the complex in pancreatic cancer cells [45]. They showed that TAK1-TAB1 complex formation was low in normal human pancreatic ductal epithelial cells (HPDE6) [45] and knockdown of KRAS in KRAS-G12V-transformed HPDE6 cells (HPDEKR+) impaired TAK1-TAB1 complex formation. In connection with a study conducted by Melisi and colleagues [49], Bang and colleagues highlighted the importance of TAK1 in NF-κB regulation in cancer cells and that formation of the TAK1-TAB1 complex was driven by oncogenic KRAS activity [45].

The effects of the first generation TAK1 inhibitor, 5Z-7 oxozeaenol, were analysed in pancreatic cancer cells expressing mutant KRAS [45]. The results were consistent with the hypothesis of Melisi [49]. Upon treatment with the TAK1 inhibitor, there was a significant decrease in the proliferation of the MIA PaCa-2 pancreatic cancer cell line [45]. The 5Z-7-oxozeaenol inhibitor triggered cell cycle arrest in the G2/M phase of PANC-1 pancreatic cells indicating a role of TAK1 in cell cycle progression [45]. Collectively, the data suggests that the activation of TAK1 is driven by oncogenic KRAS and it is involved in cell cycle progression [45]. Consequently, TAK1 emerges as a molecular target for the treatment of pancreatic cancer. By inhibiting TAK1, cell-cycle arrest presumably results in decreased cell proliferation [45,49]. The development of an inhibitor, specific to the inhibition of TAK1 in pancreatic cancer, could prove advantageous in the treatment of pancreatic cancer.

**Inhibition of GSK-3**

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase with two isoforms: GSK-3α and GSK-3β. This kinase plays a key role in cell cycle progression and apoptosis. GSK-3α promotes constitutive IKK and NF-κB activity in pancreatic cancer by targeting TAK1 [31]. To determine if GSK-3 is required to preserve TAK1-TAB interactions, the effect of GSK-3 inhibition was tested in MIA PaCa-2 cells [45]. It appeared that inhibiting the activity of GSK-3α, but not GSK-3β, decreased TAK1 activity, preventing the formation of the TAK1-TAB1 complex [45]. In vivo mouse xenograft studies confirmed these results. Treatment of xenograft carrying mice with the GSK inhibitor AR-A014418 reduced tumour growth [45]. It appeared that upon inhibiting GSK-3, the expression of proliferation promoting genes, such as c-Myc and cIAP2, were reduced [45]. These studies showed that GSK-3α plays a role in mediating the effect of oncogenic KRAS through the regulation and stabilisation of TAK1-TAB1 and thus emerges as a potential therapeutic target [45].

**Other genetic alterations in pancreatic cancer**

Epidermal growth factor receptor (EGFR) is encoded by the c-ERBB-1 proto-oncogene. In a healthy pancreas, it is only expressed in the islets of Langerhans. Overexpression of the ERBB-1 gene is present in approximately 85% of pancreatic cell lines in PDAC due to extensive gene transcription [31,50]. Pancreatic cancer progresses through a series of genetic alterations. KRAS mutations and EGFR gene amplification are the primary genetic alterations to occur, but these mutations are usually followed by mutations in tumour suppressor genes, such as p16. The inactivation of the TP53, SMAD4 (DPC4) and BRCA2 genes are genetic alterations that occur in the late stages of pancreatic cancer [31].

In pancreatic cancer, inactivation of tumour suppressor genes (TSGs) has been shown to contribute to growth advantage [51,52]. Loss of TSGs results from abnormalities such as allelic deletion, mutations and chromosomal recombination [51,52]. The most frequent TSG mutation in PDAC is of p16, which is inactivated in 95% of pancreatic cancers by mechanisms such as homozygous deletion and loss of function mutation [53,54]. p16 (cyclin-dependent kinase inhibitor 2A/CDKN2A) plays an important regulatory role at the G1-S phase checkpoint in the cell cycle by binding to cyclin-dependent kinases [54]. TP53 (tumour protein p53) has many important cellular functions including cell cycle control, stimulation of apoptosis and DNA repair [51]. The loss of p16 and TP53 functionality encourages cell survival and division of cells with damaged DNA, facilitating the development of pancreatic cancer [51,54]. Another common TSG mutation in PDAC is of DPC4 (SMAD4). Loss of DPC4 (deleted in pancreatic carcinoma, locus 4) typically occurs by homozygous deletion and mutation. Mutations of DPC4 are associated with poor prognosis and metastasis of pancreatic cancer [54,55].

BRCA2 (breast cancer 2, early onset) is involved in homologous recombination and thus its role is fundamental in the repair of double-strand breaks during cell cycle division [56]. Biallelic inactivation of BRCA2 occurs in approximately 7-10% of pancreatic cancer. BRCA mutations are unlikely to be initiators of pancreatic cancer, during the ontogenesis of PDAC other genetic alterations occur before the inactivation of BRCA2 [56].

**Targeting the other genetic alterations**

As mentioned above, EGFR (HER1) is often overexpressed in pancreatic tumours. This overexpression of EGFR has been successfully targeted by the HER1/EGFR tyrosine-kinase inhibitor, erlotinib. Erlotinib used in combination with gemcitabine improved progression-free survival of patients with advanced pancreatic cancer. The effects were modest but significant with the one year survival rate increasing from 17% with gemcitabine alone to 23% with the combination of erlotinib and gemcitabine [57].

BRCA2 also emerges as a potential molecular target for the treatment of pancreatic cancer. BRCA2-mutated tumour cells,
being impaired in homologous recombination DNA repair, rely on alternative DNA repair pathways for survival. Inhibition of these pathways, especially blocking the activity of the poly ADP ribose polymerase (PARP) enzyme, induces synthetic lethality in BRCA-mutant tumour cells. PARP inhibition results in the transformation of single-stranded breaks to double-stranded breaks, however in the absence of homologous recombination repair, these lesions become cytotoxic, presenting an opportunity for therapeutic intervention [58].

The synthetic lethal interaction between BRCA and PARP has been further supported by the work of O’Reilly and colleagues who showed that the BRCA2 mutation is a biomarker predicting the efficacy of genotoxic drugs for BRCA2 mutation-carrying PDAC patients [59]. The phase Ib lead-in dose-finalisation study showed a high activity of Veliparib, a PARP1 and PARP2 inhibitor, in combination with cisplatin and gemcitabine in patients with BRCA2-mutated pancreatic adenocarcinoma. This triple combination is now currently in phase II trial in BRCA/PALB2-mutated patients with stage III/IV disease [60].

Conclusions
Pancreatic cancer is resistant to traditional forms of cancer treatments due to the high abundance of molecular alterations. The development of therapies to target these mutations could help increase patient response to the traditional treatments such as chemotherapy and radiation. Targeting of the NF-κB pathway and oncogenic KRAS only represents two of the many potential targets for the treatment of pancreatic cancer. KRAS is so far an undruggable protein and thus alternative approaches aim to block the signalling either upstream or downstream of RAS. A very promising upstream target was the farnesyl transferases that create the lipid anchor of KRAS for membrane localisation. In preclinical models, the farnesyl transferase inhibitors showed great potency; however in clinical studies, their activity was far less than anticipated. The approaches to target the downstream segment of RAS signalling still hold promise. A significant amount of data exists to support the evidence that both NF-κB and KRAS are essential in transformation to pancreatic cancer by proliferation and resistance to apoptosis. However, one must consider that the targeting of the KRAS and NF-κB by the processes described above are only in pre-clinical preformed on mice models. The response of mice to the pre-clinical study to the treatment may differ in human trials. Limited understanding of the molecular pathogenesis of pancreatic cancer contributes to previous failed attempts at targeting this disease. Extensive clinical testing is required, but the discovery of these molecular targets proposes new directions in the treatment of this pathogenic disease. The use of these novel molecular targeted drugs in combination with primary treatments, such as gemcitabine, could prove beneficial in achieving a more effective treatment for PDAC.

List of abbreviations
PDAC: Pancreatic Ductal Adenocarcinoma

KRAS: Kirsten rat sarcoma viral oncogene homolog
MAPK: Mitogen-activated protein kinase
NF-κB: Nuclear Factor kappa B
TNF: Tumour Necrosis Factor
IkB: Inhibitor kappa B
IKK: Inhibitor kappa B kinase
NIK: NF-κB inducing kinase
IL-1α: Interleukin-1 alpha
TAK1: Transforming growth-factor beta activated kinase
TAK1-binding protein
EGFR: Epidermal growth factor receptor
TP53: Tumor protein p53
SMAD4: SMAD family member 4
BRCA2: Breast Cancer Type 2 Susceptibility Gene
TRAF2/3: TNF receptor-associated factor 2 and 3
cIAP1/2: Cellular inhibitor of apoptosis 1 and 2
GSK-3: Glycogen synthase kinase
UPP: Ubiquitin-proteasome pathway
RAF1 (cRAF): v-raf-1 murine leukaemia viral oncogene homolog
LODER: Local Drug EluteR
ERK: Extracellular signal-regulated kinase
MAPK: Mitogen-activated protein kinase

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
EO’R and ES collected the information, and prepared the manuscript draft. CS and ES were responsible for conceptual framework and revisions of the manuscript.

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