Pancreatic ductal adenocarcinoma is a devastating disease with a poor prognosis regardless of stage. To date the mainstay of therapy for advanced disease has been chemotherapy with little incremental improvements in outcome. Despite extensive research investigating new treatment options the current practices continue to utilise fluorouracil or gemcitabine containing combinations. The need for novel therapeutic approaches is mandated by the ongoing poor survival rates associated with this disease. One such approach may include manipulation of ribosome biogenesis and the nucleolar stress response, which has recently been applied to haematological malignancies such as lymphoma and prostate cancer with promising results. This review will focus on the current therapeutic options for pancreatic ductal adenocarcinoma and the complexities associated with developing novel treatments, with a particular emphasis on the role of the nucleolus as a treatment strategy.

**Key words:** ribosome biogenesis; nucleolar stress; rRNA polymerase I; Pancreatic ductal adenocarcinoma; Chemotherapy

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**Core tip:** This manuscript is a review of the complexities involved in the treatment of advanced pancreatic ductal adenocarcinoma. It details the current approaches to therapy and the disease factors which have impacted on progress thus far. This review identifies the possible role of nucleolar stress as a treatment modality based on recent data from studies of haematological malignancies and some other solid organ cancers and explains the basic science involved in this process.
INTRODUCTION

Advanced pancreatic ductal adenocarcinoma (PDAC) remains a significant cause of mortality accounting for up to 4% of all cancer related deaths world-wide[3]. Of concern, despite treatment, the mortality rates remain high and essentially unchanged over the last two decades[2]. Furthermore, even in the setting of adjuvant therapy, 5 year survival rates remain poor at only 25%[2], while for metastatic disease it is 1%[3]. Such poor statistics are no doubt compounded by the fact that current backbones to treatment, fluorouracil or gemcitabine, have also remained unchanged over the last 10-20 years and continue to form the basis for new combination approaches[4]. As a consequence, there is an ongoing need for the development of improved diagnostic techniques, treatment options and surveillance markers to improve the outcomes for this devastating disease.

In recent years, various studies have focused on trialling new chemotherapeutic drug combinations for the treatment of PDAC. The PRODIGY 4/ACCORD 11 trial[5,6] demonstrated that FOLFIRINOX, a combination of fluorouracil, oxaliplatin and irinotecan, provided superior progression free survival (PFS), overall survival (OS) and response compared with gemcitabine alone for patients with metastatic disease. Unfortunately, however, the drawback was that many patients displayed significant toxicity associated with this multidrug approach including an higher incidence of grade 3 or 4 neutropenia and thrombocytopenia[5].

Two years later, the MPACT trial compared the combination of gemcitabine and nab-paclitaxel against gemcitabine alone in a total of 842 patients and demonstrated a statistically significant improvement in median OS (8.7 mo vs 6.6 mo)[7] and was particularly interesting given that previous investigations comparing gemcitabine combinations against gemcitabine alone for PDAC had been negative. Importantly, this trial did not exclude more elderly patients unlike the ACCORD-11 study which only incorporated patients with a mean age of 61 years[8]. In keeping with PDAC demographics, MPACT included patients with a mean age for men of 71 and 75 for women[8]. Toxicities related to gemcitabine and nab-paclitaxel were overall very similar to those seen with FOLFIRINOX though there is a perception that FOLFIRINOX is best reserved for “fit” patients despite the two regimens never formally being compared.

More recently, the NAPOLI-1 study compared the effect of nanoliposomal irinotecan with or without fluorouracil and folinic acid as well as the combination of fluorouracil and folinic acid alone in patients with metastatic disease who progressed following a gemcitabine based approach[9]. In its nanoliposomal form, irinotecan has improved drug stability allowing its active metabolite, SN-38, to remain in circulation longer than free irinotecan, ultimately prolonging intratumoral levels of the active drug[10,11]. The median OS was significantly improved for those receiving nanoliposomal irinotecan, fluorouracil plus folinic acid (6.1 mo vs 4.2 mo) compared with patients who did not receive nanoliposomal irinotecan. Similarly, the PFS noted for those receiving combination therapy with nanoliposomal irinotecan was significantly improved compared to fluorouracil and folinic acid alone at 3.1 mo vs 1.5 mo. Differences between nanoliposomal irinotecan monotherapy and fluorouracil with folinic acid alone were not statistically significant[9]. Based on this study and others the FDA has approved the use of nanoliposomal irinotecan with fluorouracil and folinic acid for treatment of metastatic PDAC refractory to gemcitabine based therapy. Interestingly the toxicities seen with this newer regimen were relatively similar to those seen with FOLFIRINOX and gemcitabine with nab-paclitaxel including grade 3 or 4 toxicities in such domains as neutropenia, diarrhoea, vomiting and fatigue.

While new chemotherapy combinations have shown promise in the clinical setting, PDAC remains a difficult disease to manage due to multiple factors including patient age, medical co-morbidities and cancer related symptoms. As is often the case in advanced/metastatic malignancies, the art of medicine relies on achieving a balance between therapeutic gain, drug induced side effects and quality of life. To this end, novel approaches that are non, or at least minimally genotoxic are of particular interest. The potential advantages observed with targeted therapies in reducing systemic side effects and improving outcomes for other malignancies have gained interest in the realm of PDAC and will be briefly discussed in this review with a particular focus on the emerging role of nucleolar stress pathway and ribosome biogenesis as a potential treatment option given its promising results in the management of haematological malignancies and prostate cancer[12-14].

DISEASE COMPLEXITIES

When evaluating the role of novel therapies, it is important to understand potential interactions with the disease in question, specifically focusing on disease factors that could impact on drug delivery, tolerability or dosing for example. PDAC is associated with multiple complexities which have precluded the development of novel approaches and therefore require due consideration.

Patient factors

diagnosis.
with either locally advanced or metastatic disease at their first consultation\textsuperscript{15}. Due its anatomical location the symptoms associated with PDAC tend to occur insidiously therefore contributing to the often delayed time to investigation and subsequent diagnosis\textsuperscript{16}. Such patient factors have important implications on treatment decisions which may in part explain the limited use of FOLFIRINOX in many patients despite its improved median OS, PFS and objective response\textsuperscript{5}.

Given the advanced age and stage at diagnosis the traditional focus on clinical outcomes and survival for interventional studies of PDAC management have shifted with increasingly more importance being placed on patient reported outcomes such as pain management and appetite. This is of particular importance for advanced PDAC given its poor prognosis\textsuperscript{17}. Without acknowledging the impact the treatment may have on patient reported outcomes, therapeutic advancements may be futile, thus developing drugs that are minimally toxic would be beneficial, yet to date, therapeutic advancements have continued to cause very similar side effect profiles to one another.

\textbf{Genetic basis of PDAC}

Inherited predispositions to PDAC account for 5\%-10\% of cases\textsuperscript{18}. In ongoing studies through the Australasian Pancreatic Cancer Genome Initiative, Humphris et al\textsuperscript{19} described the manifestations of inherited PDAC as occurring in 3 distinct settings, hereditary tumour predisposition syndromes, hereditary pancreaticitis and familial pancreatic cancers which were further defined as occurring in a kindred in whom at least 2 first degree relatives have PDAC without diagnostic criteria for an inherited cancer syndrome\textsuperscript{19,20}. Interestingly some of the genes identified in hereditary forms of PDAC affect pathways involved in DNA repair such as BRCA1/2 and PALB2 which have been noted in more recent detailed sequencing/mutational studies presented by Waddell et al\textsuperscript{21} and Bailey et al\textsuperscript{22} in two separate comprehensive analyses. While inherited forms of cancer are of interest the vast majority of genetic/metabolic pathway abnormalities are not inherited and form the basis of most PDAC cases.

Four major driver genes, \textit{KRAS}, \textit{TP53}, \textit{CDKN2A} and \textit{SMAD4} have been identified in the development of PDAC\textsuperscript{22}, each sharing common oncogenic signalling pathways. \textit{KRAS} mutations occur in > 90\% of tumours and may represent the underlying insult to numerous subsequent events contributing to disease development. This mutation is widely accepted as a requirement for “reprogramming” pancreatic cell metabolism to facilitate the acidic environment needed for extracellular matrix breakdown and tumour invasion common to PDAC\textsuperscript{23,24}.

Of interest, the complex genetic and metabolic pathways associated with PDAC have identified various interactions and sites which can be utilised for therapeutic means. Among these include certain growth factor receptors such as epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor which have important roles in the RAS/RAF pathway as outlined in Figure 1. Furthermore it is now clear that drugs such as nab-paclitaxel have therapeutic actions against some of these pathways either directly or downstream accounting for their efficacy.

Recent data published in Nature by Waddell et al\textsuperscript{21} reported the complex mutational landscape of PDAC, identifying multiple point mutations and structural variations in key genes. This data confirmed that \textit{KRAS} abnormalities were almost ubiquitous while \textit{TP53} lesions were noted in 74\% of samples, closely followed by \textit{CDKN2A} lesions (35\%) and \textit{SMAD4} abnormalities (31\%). Through their analysis, PDAC was sub classified into 4 subtypes based on the distribution of events such that samples fell into either stable, scattered, unstable or locally arranged groupings. Of interest, the same study established a relationship between mutational load and abnormalities affecting DNA maintenance genes, specifically \textit{BRCA}\textsubscript{2}\textsuperscript{25} and \textit{PALB2}\textsuperscript{26}. It is not surprising that PDAC associated with either \textit{PALB2} or \textit{BRCA2} mutations should behave in a similar fashion given their roles in DNA damage and repair. \textit{PALB2} binds and co-localizes with \textit{BRCA2} in order to facilitate double stranded DNA damage. In cases where PDAC harbours \textit{BRCA2} mutations there is good evidence for improved response to platinum containing therapies unlike other forms of PDAC\textsuperscript{21}. For \textit{PALB2} mutant disease there have been a number of reports noting improved outcomes in response to platinum based therapies or mitomycin C when compared with gemcitabine\textsuperscript{27,28} and there is potential for targeted treatment with poly ADP ribose polymerase (PARP) inhibitors especially in the setting of \textit{BRCA2} as seen in breast and ovarian cancers.

Further to the comprehensive studies of Waddell et al\textsuperscript{21}, Bailey et al\textsuperscript{22} identified aggregates of point mutations in core molecular pathways affecting cellular functions including DNA damage and repair pathways, cell cycle regulation, transforming growth factor beta (TGFβ) signalling, chromatin regulation and axonal guidance. Based on the expression of 32 recurrently mutated genes found to aggregate into ten distinct pathways. From this analysis four subtypes were identified, comprising of, squamous, pancreatic progenitor, immunogenic and aberrantly differentiated endocrine and exocrine categories\textsuperscript{22}. Similar to Waddell et al\textsuperscript{21} the implications of these findings may potentially identify opportunities for therapeutic development. While these data have provided valuable insights into the complex molecular basis of PDAC many studies have previously attempted to manipulate some of these genes and/or pathways at various levels already and will be briefly discussed here.

Given the near ubiquitous nature of \textit{KRAS} mutations in PDAC, targeting this gene and its associated pathways has been an area of interest however thus far this has not translated to clinically significant outcomes. In an
erlotinib in conjunction with gemcitabine, however this was only in the order of two weeks survival benefit and little mention of the related quality of life impact secondary to treatment was reported [34].

The JAK/STAT pathway has also been implicated as a regulator in the development of PDAC via its role in activating signalling cascades and gene transcription. Stimulated by oxidative stress, this pathway ultimately induces the production of inflammatory cytokines as well as cell proliferation, malignant transformation and inhibition of apoptosis in the pancreas [37,38]. This association with inflammation has prompted trials of the JAK-1/2 inhibitor ruxolotinib in combination with capecitabine for patients with metastatic PDAC after failure of first line therapy if patients expressed elevated inflammatory markers as assessed by C-reactive protein [39]. In this phase II randomised trial, 127 patients were treated with either ruxolotinib and capecitabine or capecitabine and placebo. However, interim analyses failed to demonstrate sufficient efficacy with ruxolotinib combination therapy and further investigations of this drug has been suspended [40].

Together this information provides us with a raft of data for potential therapeutic targets, whether via drivers, alterations in signalling pathways or effort to target as many known mutations/pathways as possible various studies have focused on novel therapies in combination with known chemotherapeutics in the hopes that treatment outcomes improve.

Selumetinib, an orally bioavailable selective MEK1/2 inhibitor showed promise in preclinical studies but failed to demonstrate a survival advantage in the second line setting when compared with the oral equivalent to fluorouracil, capecitabine [29]. Similarly, attempts at targeting other known PDAC genes/pathways including P13K/Akt/mTOR have been clinically disappointing [30,31].

Her2/neu amplification is well characterised in a number of malignancies which have shown response when used as druggable targets in breast and gastric malignancies for example [32-34]. In PDAC, Her2/neu is amplified in up to 45% of cases [35], particularly in the advanced setting. Unfortunately, while initial pre-clinical studies indicated a potential role for Her-2 directed therapy specifically trastuzumab as a monotherapy or in combination with gemcitabine [36] it did not prove clinically advantageous. Similarly, investigations combining trastuzumab with capecitabine were disappointing [37]. In a related fashion EGFR which is known to co-express with Her2 has also been investigated with statistical gains noted for the EGFR inhibitor
susceptibility genes. For example in the case of PALB2 mutation-associated disease there are multiple reports suggesting improved outcomes in response to platinum based therapy or mitomycin C when compared with gemcitabine[27,28] suggesting that a more comprehensive understanding of the genetic complexity of PDAC will assist in treatment decisions. Thus understanding the similarities and differences between the “poor” and “exceptional responders” may provide biomarkers to identify patients who might benefit from these treatments and improve outcomes[41].

Stromal microenvironment and drug delivery

PDAC is characterised by the surrounding cells, specifically activated fibroblasts, myofibroblasts and pancreatic stellate cells which contribute to the composition of the surrounding matrix, elements such as hyaluronan, growth factors (e.g., TGF-β1) and secreted protein acidic rich in cysteine (SPARC)[42,43]. These result in a unique stromal microenvironment which may not only promote tumour initiation and progression but also create a barrier to drug delivery thus rendering PDAC relatively chemoresistant. Consequently, much effort has focused on ways to deplete or manipulate the stromal microenvironment and improve therapeutic outcomes.

SPARC is a glycoprotein believed to be involved in cancer development via its modulation of cell proliferation, progression, angiogenesis, migration, metastasis and apoptosis[44]. It’s normal role in cellular functions is thought to be multifactorial with effects on cell dispersion and chemosensitization as well as induction of apoptosis but also has antiangiogenic properties[45-48]. While the intricacies of SPARC and cancer are yet to be fully elucidated its potential to increase the invasive capacity of malignant cells and possible association with poor prognosis is recognised[49]. Of interest, SPARC methylation leading to pathogenesis correlates with both tobacco smoking and alcohol consumption, which are known associated modifiable risk factors for PDAC[49].

Interestingly gemcitabine has been reported to alter SPARC expression in a dose dependent manner in cell lines[50] however, there is also evidence to suggest that SPARC overexpression enhances PDAC cell chemosensitivity to gemcitabine[51]. In fact, SPARC may actually assist in the delivery of nab-paclitaxel to the tumour due to its affinity for albumin[52]. Nab-paclitaxel is formulated with human albumin at concentrations that closely resemble physiological albumin levels. This feature seems to enable nab-paclitaxel to penetrate the stromal environment and reach the tumour more efficiently[53]. Despite these studies however the role for SPARC within the stromal micro-environment and its implications on therapy remain controversial. For example data presented by Hidalgo et al[54] reported no clear association between SPARC levels and treatment efficacy with combination therapy using gemcitabine and nab-paclitaxel or gemcitabine alone in metastatic PDAC.

Growth factors such as TGF-β1 are produced by cells within the stromal microenvironment. Interestingly TGF-β1 levels correlate with tumour metastases and progression as well as poorer patient outcomes[55]. The specific role for TGF-β1 in this case is not clear but may involve regulation of cell cycle arrest, apoptosis, immune response and/or wound healing. Activated TGF-β1 signalling is mediated via SMADs, a known driver of PDAC, which is also reported to correlate with worse prognosis or disseminated disease[56,57].

Intriguingly TGF-β1 has been reported as a tumour suppressor in early stages of malignancy but a promoter in established disease[58] further emphasising the complex nature of PDAC.

Despite the steady accumulation of knowledge from studies into genetic and molecular pathways, complex stromal characteristics and better drug delivery remains an ongoing issue further prompting investigations of novel approaches such as nucleolar stress pathways and ribosome biogenesis.

RIBOSOME BIOGENESIS

Ribosome biogenesis is a highly coordinated process which takes place within a dynamic compartment of the nucleus termed the nucleolus. Long before the functional role of the nucleolus was established, Pianese[59] noted that malignant cells were often characterised by enlarged, abnormal nucleoli. Since then, numerous studies have correlated morphological changes of the nucleol with malignant disease.

It is now clear that the morphological changes affecting nucleoli in malignant cells reflects hyper-activated transcription of ribosomal RNA (rRNA) genes by RNA polymerase I (Pol I) yielding the 47S rRNA precursor which is rapidly processed into mature 18, 5.8 and 28S rRNAs[60] while the SS rRNA precursor is transcribed by RNA polymerase III (Pol III). These RNA’s together with ribosomal proteins (RPs) transcribed by RNA polymerase II (Pol II) are essential for ribosome assembly, central to the synthesis of cellular proteins[61,62]. Co-ordination of all three polymerases is necessary for the development of a functional mammalian ribosome (80S) composed of a small (40S) and large (60S) subunit[63,64].

During active cell division and proliferation ribosome biogenesis increases so as to maintain elevated cellular demands. It is not surprising that this process is a major consumer of cellular energy requiring vigilant regulation[65,66]. However, ribosome biogenesis is not only regulated by RNA polymerases, as depicted in Figure 2, but also involves a number of tumour suppressors or oncoproteins[66] including c-myc, considered a “master regulator” of protein synthesis[67]. Conversely p53 has an important place in inhibiting ribosome biogenesis[68] and promoting cell cycle arrest, senescence or apoptosis.
In normal cells, levels of p53 protein are typically low, kept in check by various mechanisms including MDM2, an E3 ubiquitin ligase, as shown in Figure 3, allowing cell division and proliferation.

Nucleolar stress
Disruptions in ribosome synthesis lead to nucleolar stress which in turn results in nucleolar disruption and the release of RP's i.e., RPL5 and RPL1. These RPs are able to bind to MDM2 and interrupt its interaction with p53. Similarly, the product of the CDKN2A gene, p14ARF, is also able to exert effects on MDM2. Ultimately, these pathways lead to stabilisation of p53 allowing it to act on various transcriptional targets and induce apoptosis, senescence and/or cell cycle arrest depicted in Figure 3. Importantly one of the transcriptional targets for p53 is the MDM2 gene setting up an autologous feedback loop to maintain genomic and cellular homeostasis keeping the process of cell death and proliferation in check.

When the above mentioned homeostatic mechanisms are impaired however, ribosome biogenesis becomes dysregulated and the balance between cell growth and arrest is compromised leading to unchecked cellular proliferation and malignant transformation. Activation of the nucleolar stress pathway may therefore provide a therapeutic strategy against aggressive malignancies.

While p53 dependent nucleolar stress is well understood, more recent research by Quin et al. has identified p53 independent mechanisms involving activation of ATM/ATR signalling which occurs without the induction of global DNA damage. Evidently, ribosome biogenesis is a highly complex and finely balanced process involving multiple key effectors. Disruption of any of these components can mediate the nucleolar stress pathway and have detrimental effects on cell growth and proliferation. It is this feature along with the potential for reduced global DNA damage that makes targeting dysregulated ribosome biogenesis an attractive therapeutic option.

Therapeutic targeting of dysregulated ribosome biogenesis
Many regulators of ribosome biogenesis are also involved in malignant diseases including PDAC. For example, activation of RAS or PI3K pathways known to be involved in PDAC are critical for coordinating protein synthesising capacity (ribosome number) required for maintaining cellular growth and proliferation. Other genes and their products, such as c-myc and p53 also have significant roles in ribosome biogenesis and malignancy when overexpressed or mutated. Attempts at targeting these various genes and pathways have been disappointing in the realm of PDAC but tackling dysregulated ribosome biogenesis have shown more promise.

Figure 2 Pathways involved in ribosome biogenesis. The first rate limiting step in ribosome biogenesis is the transcription of the rRNA genes by Pol I which forms a multiprotein transcription complex at the rDNA promoter. Green arrows indicate up stream regulators which exert positive effects on the Pol I transcription complex including multiple pathways such as PI3K/AKT/mTOR, RAS/RAF/MEK and Myc which functions as the “master regulator” for cell growth. The mature rRNAs together with ribosomal proteins assemble into the 40 and 60S ribosomal subunits, which then form the functional 80S ribosome. Transcription of the rDNA repeat is negatively influenced by p53 (as shown by the red line) to ensure that cell growth/ proliferation is tightly regulated.
Pivotal studies conducted by Bywater et al.[12], provided proof of principal for the benefits of targeting dysregulated ribosome biogenesis as demonstrated by CX5461, a novel small molecule Pol I inhibitor, investigated in the Eµ-Myc mouse model of Burkitt's lymphoma. In this model, cells are highly proliferative and have elevated RNA levels, including rRNA, which correlates with accelerated Pol I transcription and cell growth. Pharmacological inhibition of Pol I transcription with CX5461 induced nucleolar stress leading to an increase in p53 levels resulting in apoptotic cell death of malignant cells. Most compelling however, CX5461 did not kill non-malignant B cells representing a valuable therapeutic option in the management of susceptible malignancies[12].

In addition to the Bywater study, Devlin et al.[13] demonstrated that combination therapy with CX5461 and AKT-mTOR inhibitors worked synergistically leading to significantly extend survival in lymphoma bearing mice[61]. Importantly, the acute lymphoblastic leukaemia cells were more sensitive to rRNA synthesis inhibition compared with normal bone marrow cells[74] further emphasising the potential reduction in global toxicity underlying Pol I inhibition. Similarly, reduced global toxicity was noted by Hannan et al.[75] review of lymphoma models in response to Pol I inhibition. In xenograft mouse models of human MIA PaCa-2 cells, treatment with CX5461 demonstrated significant tumour growth inhibition deemed at least comparable to xenograft models treated with gemcitabine and again shows promise.

CONCLUSION

Given the promising result for Pol I inhibition in the treatment of haematological malignancies and emerging evidence of efficacy in various solid organ cancers the potential for targeting dysregulated ribosome biogenesis and manipulating the nucleolar stress pathway is tantalising, particularly for advanced PDAC given the slow progress in its treatment options so far. Whether this will be in the setting of combinations with already established chemotherapeutics or in more novel ways remains to be seen but the results from other malignancies are encouraging. Of particular note is the...
potential for less toxicity to healthy cells which thus far has been a major limitation in improving treatment outcomes for PDAC.

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