Combating pancreatic cancer with PI3K pathway inhibitors in the era of personalised medicine

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ABSTRACT
Pancreatic ductal adenocarcinoma (PDAC) is among the most deadly solid tumours. This is due to a generally late-stage diagnosis of a primarily treatment-refractory disease. Several large-scale sequencing and mass spectrometry approaches have identified key drivers of this disease and in doing so highlighted the vast heterogeneity of lower frequency mutations that make clinical trials of targeted agents in unsel ected patients increasingly futile. There is a clear need for improved biomarkers to guide effective targeted therapies, with biomarker-driven clinical trials for personalised medicine becoming increasingly common in several cancers. Interestingly, many of the aberrant signalling pathways in PDAC rely on downstream signal transduction through the mitogen-activated protein kinase and phosphoinositide 3-kinase (PI3K) pathways, which has led to the development of several approaches to target these key regulators, primarily as combination therapies. The following review discusses the trend of PDAC therapy towards molecular subtyping for biomarker-driven personalised therapies, highlighting the key pathways under investigation and their relationship to the PI3K pathway.

INTRODUCTION
Accounting for ~95% of pancreatic cancers, pancreatic ductal adenocarcinoma (PDAC) has a very poor overall 5-year survival of 8% and is predicted to be the second leading cause of cancer-related deaths in the developed world by 2030.1-3 This has only marginally improved since the introduction of gemcitabine in 1995.4,5 Surgery remains the only curative treatment and is often applied with adjuvant chemotherapy, but as few as 10%-15% of patients are eligible at initial diagnosis.6-8 Most patients with PDAC have few or non-specific symptoms as the tumour develops, and this means that a large proportion are diagnosed at a late stage, already presenting with locally advanced or metastatic disease.9 For those patients that are not immediately eligible for resection, neoadjuvant chemotherapy can be given to reduce borderline tumours prior to resection.10 Recent clinical trials aimed at improving response to chemotherapy have demonstrated improved survival with patients treated with either a combination of gemcitabine and nab-paclitaxel or FOLFIRINOX (folinic acid, 5-fluorouracil, irinotecan, and oxaliplatin).12-16 However, patient tolerability may be limited with such aggressive treatment regimens.16 While improvements in surgical techniques and chemotherapy regimens are providing modest improvements in survival, there is a clear need to better understand this aggressive disease to facilitate both earlier diagnosis and elucidate new targets for combination therapies.

PDAC progression model
The most widely accepted model for PDAC development is the progression model, in which PDAC originates from preinvasive pancreatic intraepithelial neoplasms (PanINs), which occur in distinct pathological stages, namely PanIN-1A, PanIN-1B, PanIN-2 and PanIN-3.17-19 In the early stages (ie, PanIN-1A and PanIN-1B or low-grade), a mucinous epithelium replaces the typically cuboidal morphology of normal pancreatic ducts, with a low level of dysplasia.19,22 Yet, recent work has suggested that pancreatic repair after injury, by the process of acinar-to-ductal metaplasia, may also be involved in PDAC initiation.23-25 As these PanINs progress (ie, to PanIN-2 and PanIN-3 or intermediate-grade and high-grade, respectively), dysplasia increases and is detectable as nuclear irregularity, loss of cell polarity and an increase in intraluminal apoptotic debris.19,22 This progression towards an invasive carcinoma has been shown to occur in parallel with increased proliferation and mutational burden from early preinvasive PanIN stages to metastatic PDAC (figure 1).19,26 Importantly, a mechanism underlying the switch from PanIN to metastatic PDAC remains unclear, but new genetically engineered mice that model multistep carcinogenesis may support the widely accepted stepwise mutational model, where some have suggested that catastrophic genomic events may instead trigger the transformation from preneoplastic lesions.27-29 A similar progression model has been proposed for intraductal papillary mucinous neoplasms (IPMNs), which are generally benign, but progress to an invasive carcinoma in up to 25% of cases.30-32 Both IPMNs and mucinous cystic neoplasms are radiologically detectable as macroscopic lesions and are classified according to the Sendai guidelines.33 They are typically distinguished from PDAC at a macroscopic level by mucoid contents and have distinct zonal subtypes at a microscopic level.34,35 Indeed, the mucoid expression itself has been used to subtype IPMNs according to whether the gene expression is gastric or intestinal, which clearly distinguished aggressive disease as the intestinal subtype.35 Attempts have been made to classify PanINs in terms of their mutational burden. Initially, evidence
of telomere shortening and mutations in KRAS were found to occur very early in PanIN progression. This excluded KRAS as a potential marker for PDAC progression but highlights the general classification as the earliest initiator mutation in PDAC, occurring in ~95% of PDAC cases. Progression through to PanIN-2 and PanIN-3 typically includes additional mutations in TP53, SMAD4 and/or CDKN2A, but the vast molecular heterogeneity of this disease precludes any single mutation as essential for PDAC development. With this in mind, several large-scale sequencing and mass spectrometry approaches have been implemented to subtype the disease based on these molecular characteristics. Early work stratified PDAC according to an activated stromal index, which classified patients according to the ratio of alpha smooth muscle actin (immunohistochemical (IHC) staining) and collagen (stained with the collagen-specific Aniline blue). Such an index informs primarily on stromal targeting and alone is not sufficient to guide therapies aimed at complete tumour regression. Indeed, a second study took the opposite approach and removed the stroma by laser microdissection from the PDAC samples, prior to microarray analysis and subtyping of PDAC based on multivariate analysis of transcriptional profiles, namely classical, exocrine-like and quasimesenchymal (QM; table 1, see column ‘Collisson’). Such an approach allowed the authors to identify neoplastic epithelial-specific gene expression and to identify pathways involved in PDAC progression. This approach also motivated metabolite profiling within these subtypes, where classical tumours were shown to be lipogenic, while QM tumours were glycolytic. With clear subtype-specific metabolic targets, new avenues for combination therapies within a personalised setting are an obvious progression to improve patient responses. Additionally, increasing evidence for the importance of the stroma in disease progression means assessment of either the tumour or stroma in isolation is likely to be too simplistic to provide any lasting improvements in patient survival.
Recent advances in basic science

Physical microdissection approaches rely on IHC to inform stromal activation state and also limit the application of patient subtyping by molecular approaches due to a low sample throughput and smaller sample volume. As large datasets become increasingly common, new analytical approaches improve the readouts incurred. A more recent approach to PDAC subtyping involved virtual microdissection of large microarray datasets, facilitating molecular subtyping of both the tumour and the stroma. Using multivariate analysis to distinguish tumour and stromal components, the tumour was split into a classical and more aggressive basal-like subtype, and the stroma was classified into activated or normal subtypes (table 1, see column ‘Moffit’). This additional stromal subtyping was also recently applied to PDAC patient-derived xenograft (PDX) tumours, whereby tumours classified as basal or classical were shown to have an ‘echo’ in the mouse stroma. They further demonstrated the power of their classifications through inhibition of cholesterol uptake in subtyped PDX models, where basal tumours were highly sensitive to inhibition, but classical tumours were shown to have higher NPC1L1 expression and may require a greater concentration of inhibitor to achieve an equivalent growth inhibition.

Further subtyping was recently performed on a 328 primary patient PDAC cohort using expression analysis from RNAseq (96 patients) and microarrays (232 patients). This study included samples with invasive IPMN-associated PDACs and some metastatic tumours and, in contrast to the previous studies, applied macrodissection to excise areas of nonmalignant tissue, maintaining the stromal component in each sample. Tumour purity could then be inferred in terms of stromal and immune infiltration based on the Estimation of STromal and Immune cells in Malignant Tumor tissues using Expression data approach. Beyond purity assessment, this approach facilitated assessment of GPs associated with microenvironmental factors, such as hypoxia, ECM deposition and activated immune pathways. The microenvironmental influence on cancer progression is an essential consideration for emerging therapies, where immune cells, cancer-associated fibroblasts and ECM components are regularly associated with cancer progression (figure 1). Inclusion of this stromal contribution, as well as the large breadth of patient samples, allowed the authors to reclassify PDAC into four distinct subtypes (summarised in table 1 (see column ‘Bailey’). This is particularly important in light of the high attrition rates for lead compounds currently experienced by the pharmaceutical industry, where more detailed molecular analysis prior to treatment is expected to improve both patient and trial outcomes (figure 2). The goal of this molecular phenotyping is to establish trials, such as IMPaCT, PRECISION-Panc, SHIVA, or biomarker-driven

Figure 2  Adaptable drug development pipeline, demonstrating the progression of lead compounds through target validation, lead compound identification and optimisation, then preclinical validation. The necessary addition to this process is the identification of biomarkers to guide both lead compound development and later stratification in phase II/III clinical trials. These processes may be iterated to improve on-target efficacy, solubility and biomarkers. After safety and tolerability is confirmed in phase I clinical trials, biomarker-driven phase II/III may reduce the high attrition rates of lead compounds if appropriate patient stratification can demonstrate beneficial response in the assessed subsets of patients. These biomarkers may also provide opportunities for retrospective analysis and later iteration into clinical trials. PI3K, phosphoinositide 3-kinase.

Table 1  Molecular subtyping of patients with pancreatic cancer

| Approach                  | Cohort                                      | Tumour/stromal contribution | Tumour subtypes | Stromal subtypes |
|---------------------------|---------------------------------------------|----------------------------|-----------------|-----------------|
| Collision                 | 63 primary resected PDAC                    | Microdissection            | Classical       | Not assessed    |
| Moffit                    | 145 primary resected and 61 metastatic PDAC | Multivariate analysis (virtual microdissection) | Classical | Activated       |
| Bailey                    | 96 RNAseq and 242 microarray primary patient samples | Macrodissection | Pancreatic progenitor Immunogenic | ESTIMATE |

This pancreatic cancer subtype table is adapted from refs 40–42. ADEX, Aberrantly Differentiated Endocrine eXocrine; ESTIMATE, Estimation of STromal and Immune cells in MA lignant Tumor tissues using Expression data; PDAC, pancreatic ductal adenocarcinoma; QM, quasimesenchymal.
patient-selective assessment of PI3K pathway inhibitors to push PDAC survival beyond the current standard of care.

**THE PHOSPHOINOSITIDE 3-KINASE (PI3K) PATHWAY**

A broad range of cancer types, including pancreatic cancer, have been candidates for targeting of the PI3K pathway, due to amplification, mutation or loss of key regulators. The PI3K pathway mediates transduction of signals from both extracellular and intracellular sources, including growth factors and nutrients, leading to downstream signalling involved in cancer growth, survival and progression (figure 1). The pathway is also essential for many cancer-associated activities, including endothelial cell sprouting for angiogenesis, macrophage transcriptional reprogramming, T cell differentiation and homeostasis and fibroblast-supported chemoresistance (figure 1).

Collectively, this suggests that application of PI3K pathway inhibitors as a PDAC therapy may provide an opportunity for dual targeting of cancer cells and the deregulated cancer-associated stromal components. PDAC is regularly associated with increased Akt activity, which has been identified in ~60% of PDAC samples, with amplification of the AKT2 oncogene occurring in 10%–20% of PDAC cases. Akt is a key effector of the PI3K pathway, downstream of both PI3K and receptor tyrosine kinases (RTKs; table 2). Furthermore, PDAC tumours have been shown to bear an activating mutation in PIK3CA and/or loss of the tumour suppressor PTEN in ~4% and 25%–70% of cases, respectively.

Interestingly, patients with low PTEN expression have a much higher incidence of recurrence or metastasis, compared with those with high PTEN. Furthermore, it has been shown that PDAC patients with high PI3K pathway activity show a significantly poorer survival than those with low activation of this pathway.

Several signalling pathways are known to converge on the MAPK and PI3K pathways as effectors of cellular response within the cell. For example, in ~95% of cases, pancreatic cancer is driven by activating mutations in KRAS, which in turn activates PI3K signalling through the p110α subunit, along with another pathway component PDK1, indicating that a large proportion of patients could benefit from effective targeting of this pathway.

Further detection of mutations in PIK3CA can be predictive for improved patient response in preclinical models of PDAC and in patients with breast cancer stratified according to detection of mutations in circulating cell-free DNA. Given the varied roles of different PI3K isoforms in both the tumour and associated stromal cells, isoform-specific inhibitors provide isolated targeting of oncogenic signalling and allow redundancy to alleviate off-target side effects in healthy tissues (table 2; reviewed in refs 80 81). Notably, a PI3Kα-specific inhibitor has shown promising efficacy in combination with an EGFRi in PDAC with high EGFR and Akt phosphorylation.

Interestingly, PIK3CA mutations in breast cancer have also been linked with Akt-independent tumour progression through SGK3 and highlight the importance of all levels of this key signalling cascade. Similarly, isoform-specific PI3Kβ inhibition extended PDAC survival beyond mTORC1/2 targeting alone, and in other cancers, inhibition of PI3Kβ and PI3Kδ has shown antimetastatic effects and suggests a role of PI3K in tumour metastatic dissemination. Furthermore, isoform-specific inhibition of PI3K in cancer-associated immune cells was shown to downregulate their tolerance to PDAC, which improved the activity of T cells against the cancer. Collectively, we see strong evidence accumulating for the efficacy of upstream isoform-specific targeting of PI3K in emerging PDAC combination therapies.
### Table 2  List of PI3K pathway inhibitors currently undergoing clinical development for pancreatic cancer

| Target | Inhibitor | Phase | Status | Patients | Combination | NIH number | Reference(s) |
|--------|-----------|-------|--------|----------|-------------|------------|--------------|
| Akt inhibitors | MK2206 | I | Completed | AdvST/MST (~9% pancreatic cancer) | Monotherapy | NCT00670488 | 262 263 |
| | I | Completed | AdvST/MST | Selumitinib (MEKi) | NCT01021748 |
| | I | Completed | PDAC (PTEN loss) | Monotherapy | NCT00848718 |
| | I | Completed | Pancreatic cancer | Dinacilib (CDKi) | NCT01783171 |
| | II | Completed | Pancreatic cancer | Selumitinib (MEKi) versus mFOFOX6 | NCT01658943 |
| | Afuresertib (GSK2110183) | I | Completed | AdvST (21% pancreatic cancer) | Trametinib (MEKi) | NCT01476137 |
| | II | Ongoing | AdvST | NCT01531894 |
| | Upertorib (GSK2141795) | I | Completed | Pancreatic cancer | Trametinib (MEKi) | NCT01138085 |
| | I | Completed | AdvST | NCT00920257 |
| | Oleandrin (PBI-05204) | I | Completed | AdvST (6% pancreatic cancer) | NCT02048384 |
| | II | Ongoing | Metastatic pancreatic cancer | NCT02329717 |
| | Oleandrin (PBI-05204) | I | Completed | Pancreatic cancer | Trametinib (MEKi) | NCT00138085 |
| | II | Completed | AdvST | NCT00920257 |
| Rapalog | Sirolimus (rapamycin) | mTORC1 (FKBP12) | I | Completed | Pancreatic cancer | Sunitinib (RTKi) | NCT00583063 | 150 |
| | I | Completed | Pancreatic cancer | Sorafenib (RTKi) | NCT00499486 |
| | II | Completed | Pancreatic cancer | NCT00276744 |
| | II/II | Ongoing | PDAC | Metformin | NCT02048384 |
| | I | Completed | Pancreatic cancer | Vismodegib (SMOi) | NCT01537107 |
| | Temsirolimus (CCI-779, Torisel) | I | Completed | Pancreatic cancer | Lenalidomide | NCT01183663 |
| | I | Terminated | PDAC | Gemcitabine | NCT00593008 |
| | II/II | Ongoing | Pancreatic cancer | Nivolumab (PD-1i) | NCT02423954 |
| | Everolimus (RAD001) | I | Completed | Pancreatic cancer | Sorafenib (RTKi) | NCT00981162 |
| | I | Completed | Pancreatic cancer | Trametinib (MEKi) | NCT00955773 |
| | I/II | Completed | PDAC | Gemcitabine | NCT00560963 |
| | I/II | Completed | Pancreatic cancer | Cetuximab (EGFRi) and capetitabine | NCT01077986 |
| | II | Terminated | Pancreatic cancer | Erlotinib (EGFRi) | NCT00640978 |
| | IX | Completed | Pancreatic cancer | NCT00409292 |
| | II | Terminated | Pancreatic cancer | NCT00640978 |
| | I/II | Recruiting | PDAC | Ribociclib (CDKi) | NCT02985125 |
| | Ridadirolimus | I | Completed | AdvST (12% pancreatic cancer) | Bevacizumab (VEGFRi) | NCT00781846 |

### PI3K inhibitors

| PI3K isoform p110α | Alpelisib (BYL719) | I | Ongoing | Pancreatic cancer | Gemcitabine and abraxane | NCT02155088 |
|---------------------|---------------------|-------|----------|---------------------|--------------------------|--------------|
| Pan-PI3K | Buparlisib (BKM120) | I | Completed | Pancreatic cancer | mFOFOX6 | NCT01571024 |
| | I | Completed | Pancreatic cancer | LDE225 (SMOi) | NCT01576666 |
| | I | Completed | Pancreatic cancer | Trametinib (MEKi) | NCT01155453 |
| | I | Ongoing | AdvST | MEK163 (MEKi) | NCT01363232 |
| | PX-866 | I | Completed | AdvST (5% PDAC) | Docetaxel | NCT01204099 |
| | ZSTK474 | I | Completed | AdvST | NCT01280487 |
| | Copanlisib (BAY 80–6946) | I | Completed | AdvST (18% pancreatic cancer) | NCT00962611 |

Continued
Concordantly, evidence for the validity of downstream pathway targeting is highlighted in a genetically engineered mouse model of mutant KrasG12D-driven PDAC, which was applied in concert with a sleeping beauty transposon library, both conditionally expressed (ie, LSL-KrasG12D and LSL-SB11) under pancreas-specific Pdx1-Cre.88 89 These approaches identified several genes within the MAPK and PI3K pathways as cooperating mutations for KrasG12D-driven PDAC. Similarly, recent assessment of kinases with the highest levels of absolute and differential expression in a panel of pancreatic cancer cell lines demonstrated significantly reduced cell number after knockdown of EGFR, Akt2, PLK2 or MET.90 This review will focus on PI3K pathway targeting in PDAC (see also table 2, PI3K pathway inhibitors under clinical investigation in PDAC).

**PI3K pathway inhibitors in the clinic**

After the discovery and isolation of rapamycin on the island of Kapa Nui from Streptomyces hygroscopicus, over 30 years of research continues to find new therapeutic applications for this compound.91 For example, the mTOR inhibitor (mTORi) rapamycin was recently assessed in PDAC driven by activated PI3K/AKT pathway components, allowing the negation of this feedback loop with promising therapeutic potential (table 2).84 98 Importantly, new combination therapies with rapalogs should consider the combined toxicity with other targeted compounds. For example, combination of everolimus with the RTKi cetuximab was found to be too toxic for patients with PDAC in a phase I/II clinical trial, while the single agents show minimal toxicity.94 99 100 With this in mind, trials are still ongoing in PDAC using rapalogs under clinical investigation in PDAC.

### Table 2 Continued

| Target | Inhibitor | Phase | Status | Patients | Combination | NIH number | Reference(s) |
|--------|-----------|-------|--------|----------|-------------|------------|--------------|
| Dual PI3K pathway inhibitors | mTORC1/2 | Vistusertib (AZD2014) | I | Completed | AdvST | NCT01026402 | 98 |
| | II | Recruiting | AdvST (RICTOR amplified) | NCT03166904 |
| | II | Recruiting | AdvST: combination with Selumitinib (MEKI) | NCT02583542 |
| | II | Recruiting | AdvST (TSC1/2 loss or mutation) | NCT03166176 |
| | II | Recruiting | AdvST: combination with Olaparib (PARPi) | NCT02576444 |
| | p70-S6K and Akt | LY2780301 | I | Complete | AdvST (2% pancreatic cancer) | NCT0115751 | 274 |
| | | Dactolisib (NVP-BEZ2235) | I | Completed | AdvST | NCT01337765 |
| | | NVP-BGT226 | I | Completed | AdvST (2% pancreatic cancer) | NCT00600275 | 275 |
| | | Voxtalisib (SAR245409, XL265) | I | Completed | AdvST (4% pancreatic cancer) | NCT00485719 | 276 |
| | | SF1126 (LY294002 prodrug) | I | Completed | AdvST (5% pancreatic cancer) | NCT00907205 | 277 |
| | | Gedatolisib (PF-05212384, PKI-587) | I | Terminated | AdvST (5% pancreatic cancer) | NCT01347866 | 279 |
| | | | I | Completed | AdvST (4% PDAC) | NCT00940498 |
| | | | I | Recruiting | AdvST | NCT03065062 |

AdvST, advanced solid tumours (including pancreatic cancer); CDKi, cyclin-dependent kinase inhibitor; EGFR, epidermal growth factor receptor; EGFRi, EGFR inhibitor; MEKi, MAPK/ERK kinase inhibitor; mFOLFOX6, modified FOLFOX (ie, 5-fluorouracil and oxaliplatin); MST, metastatic solid tumours (including pancreatic cancer); mTOR, mechanistic target of rapamycin; NIH, National Institutes of Health; PARP, poly (ADP-ribose) polymerase; PARPi, PARP inhibitors; PDAC, pancreatic ductal adenocarcinoma; PD-1, programmed death-1; PD-1i, PD-1 inhibitor; PI3K, phosphoinositide 3-kinase; RTKi, receptor tyrosine kinase inhibitor; SMOi, smoothened inhibitor; VEGFRI, vascular endothelial growth factor receptor inhibitor.

**Recent advances in basic science**

Recent advances in basic science have demonstrated a clear benefit for rapalogs as single agents (table 2);93 however, no significant improvements have been identified for rapalogs as single agents in PDAC.94 95 This has been attributed to an upstream feedback loop where inhibition of mTORC1 alone relieves the inhibitory phosphorylation of insulin receptor substrate 1 (IRS1) by p70-S6K and mTORC1, leading to an upregulation of Akt phosphorylation (figure 3).96 97 Hence, while trials of rapalogs may benefit from stratification for patients with high PI3K pathway activity, newer agents that target both mTORC1 and mTORC2, or other pathway components, allow the negation of this feedback loop with promising therapeutic potential (table 2).84 98

Next-generation dual PI3K pathway inhibitors are being developed that take advantage of the homology of the kinase domains from class I, II and III PI3Ks and those of phosphoinositide 3-kinase-related kinases, such as mTOR, ATM and DNA-PK (figure 3, table 2).101 However, these dual inhibitors have been linked with drug-related dosage-dependent toxicities, such as hyperglycaemia, nausea, vomiting and diarrhoea, consistent with PI3K isoform targeting, and reinforce the need for preclinical assessment of the additive or synergistic toxicities when developing novel combination therapies.81 Moving forward, improvements in solubility are driving greater oral bioavailability,
where lower drug dosages can show equivalent drug efficacy and gastrointestinal toxicities are readily reduced. One exciting example of a dual PI3K pathway inhibitor is AZD2014, which was developed by iterative structure–activity relationship medicinal chemistry approaches to have high aqueous solubility and a potent inhibitory effect against both mTORC1 and mTORC2. Recent preclinical work by our group has demonstrated potent antiproliferative and anti-invasive effects in the KPC (LSL-KrasG12D, LSL-Trp53R172H and Pdx1-Cre) GEM PDAC model and after a promising phase I clinical trial in advanced solid tumours (AdvSTs), AZD2014 has progressed to phase II biomarker-driven clinical trials either alone or in combination with a MAPK/ERK kinase inhibitor (MEKi) (table 2). The additional anti-invasive role for AZD2014 is consistent with increasing evidence for the emerging antimetastatic and anti-invasive effect of PI3K pathway targeting (figure 1). Indeed, the opposing roles for the different Akt isoforms in cell motility have identified an invasion and metastasis promoting role for Akt2 but an inhibitory role for Akt1. The dual role of Akt1 in either promoting tumour growth or metastasis was recently shown to be regulated by the inositol polyphosphate 5-phosphatase (PIPP), where PIPP ablation resulted in reduced metastasis but increased tumour growth. Similarly, mTORC1 and mTORC2 have been shown to regulate migration and invasion through Rac1 and RhoA. Furthermore, mTOR inhibition dramatically reduced metastasis in prostate cancer, highlighting the broader potential of PI3K pathway therapeutics as antimetastatic agents.

Intriguingly, the mTOR inhibitor, everolimus, resulted in a partial response in a patient with pancreatic cancer that was induced by Peutz-Jeghers syndrome (PJS). PJS is caused by a tumour-suppressor gene mutation in the serine threonine kinase 11 gene (STK11, also known as LKB1), which results in ~11%–36% of patients with PJS developing pancreatic cancer. This loss of STK11 leads to a loss of suppression of mTOR signalling and raises the tantalising possibility that mTOR inhibition could have monotherapy efficacy in PDAC in selected cases with a similar genetic background.

**Figure 3** Simplified schematic of the PI3K pathway, which highlights the common targets for small molecule inhibitors. Briefly, signalling from growth factors activates RTKs and recruits PI3K and other scaffold proteins to the cell membrane, where PIP, is converted to PIP. This recruits phosphoinositide-dependent kinase-1 (PDK1) and Akt to the membrane and leads to downstream signalling through the kinase activities of Akt. (1) Single-strand break repair is regulated primarily by PARP and inhibition of PARP can lead to genomic instability. (2) Double-stranded break repair is primarily regulated by a complex with BRCA2, which is lost in familial pancreatic cancer and some PDAC cases and can lead to genomic instability. Genomically unstable tumours require the PI3K pathway to maintain survival pathways and PI3K pathway inhibition may be an emerging option for patients with BRCA2 mutations or in combination with PARP inhibitors. More exhaustive pathway maps can be found in refs 61 80. P13K, phosphoinositide 3-kinase; RTKs, receptor tyrosine kinases.

**EMERGING OPPORTUNITIES FOR COMBINATION THERAPIES**

Opportunities for patients with RTK amplification or mutation

The RTK family comprises several subfamilies that are not limited to ErbB, fibroblast growth factor receptors (FGFRs), insulin and insulin-like growth factor receptors, platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor (VEGF) receptor (VEGFR) and Axl and the Ephrin receptors. Inhibition of these receptors using RTK inhibitors (RTKi) generally takes one of three forms: antibody or recombinant protein inhibition of the extracellular ligand binding domain, inhibition of the ligand itself, or targeting of the cytoplasmic tyrosine kinase domain.

In recent work, 19 PDAC cell lines from the American Type Culture Collection and 17 patient-derived cell lines (PDCls) from the Australian Pancreatic Cancer Genome Initiative collection, sequenced as part of the International Cancer Genome Consortium (ICGC), were used to assess global phosphotyrosine (pTyr) profiles in PDAC by mass spectrometry. Recent advances in basic science

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Recent advances in basic science

to the overlapping use of both genomic and mass spectrometry approaches to assess aberrant pathway expression, mutation status and, importantly, activation state and provides clear motivation for the incorporation of both techniques into the drug discovery pipeline (figure 2).

The ErbB family
RTKs are transmembrane receptors that communicate signals from ligands outside of the cell by activating their cytoplasmic tyrosine kinase domains, which facilitate downstream signalling within the cell, typically through activation of the MAPK and PI3K pathways. The ErbB family contains four RTKs structurally related to the epidermal growth factor receptor (EGFR; human epidermal growth factor receptor (HER) 1 and ErbB-1). EGFR expression is observed in normal pancreatic ducts but has been shown to increase from the early stages of PanIN development through to PDAC.\textsuperscript{118–120} Targeting of the EGFR receptor with the small molecule inhibitor erlotinib in combination with gemcitabine resulted in a statistically significant, but clinically modest, improvement in overall survival compared with gemcitabine monotherapy in patients with metastatic disease and has also been evaluated in the adjuvant setting.\textsuperscript{121, 122} These studies subsequently motivated the assessment of predictive markers that would stratify patients for this treatment.\textsuperscript{123–126} These studies found conflicting evidence for KRAS mutational status as a predictive or prognostic marker for erlotinib response but suggested that mutations or amplification of EGFR may be sufficient to stratify patients for therapy. Interestingly, expression of ErbB-3 (HER3) has been associated with sensitivity to erlotinib treatment in pancreatic cancer cell lines and therefore may prove an effective biomarker for adjuvant erlotinib for patients with PDAC.\textsuperscript{127–129} ErbB-3 requires heterodimerisation for downstream signalling through the PI3K pathway and expression in PDAC is a poor prognostic factor for survival.\textsuperscript{127–129} Another emerging personalised approach to PDAC therapy comes from the success of targeting ErbB-2 (neu and HER2) amplified tumours with a humanised monoclonal antibody.\textsuperscript{130} ErbB-2 amplification in PDAC has a relatively low prevalence of 2%;\textsuperscript{130} 131 132 however, clinical trials with trastuzumab (Herceptin) in combination with chemotherapy have shown beneficial responses in metastatic PDAC patients with ErbB-2 amplification.\textsuperscript{133, 134} and studies are still ongoing in metastatic or recurrent PDAC.\textsuperscript{135} ErbB-4 (HER4) is the last member of the ErbB family but is only weakly expressed in PDAC.\textsuperscript{135, 136} However, given the established importance of the other ErbB family members in PDAC progression, they may also prove effective biomarkers for inhibition of the PI3K pathway, which is less sensitive to changes in receptor dimerisation.

FGFR, PDGFR and VEGFR stromal targeted therapies and biomarkers
The FGFR, PDGFR and VEGFR families share sufficient structural homology that targeting of these receptors often has overlapping responses. In PDAC, overactivation of FGFR signalling has been associated with 2% of patients, and targeting of this receptor in PDAC using dovitinib has recently completed a phase 1 clinical trial in combination with chemotherapy, after a promising preclinical study, where dovitinib was found to exert its effect through decreased Akt activity (NCT01497392).\textsuperscript{130, 137} Furthermore, FGFR and PDGFR upregulation in a proof-of-principle study using Kras-deficient PDAC was recently linked with increased sensitivity to PI3K pathway targeting highlighting the essential supportive role this pathway plays in PDAC progression and the potential of RTKs as biomarkers for patient stratification.\textsuperscript{138} Interestingly, inhibition of FGFR alone or in combination with PDGFR inhibition was not sufficient to decrease cancer cell proliferation to the same degree as PI3K pathway inhibitors, indicating that multiple RTK pathways feed into PI3K activation in PDAC and that PI3K inhibition may provide an opportunity for targeting of multiple de-regulated RTK pathways simultaneously (figure 3).\textsuperscript{138}

Due to the highly desmoplastic reaction characteristic of PDAC, it is important to consider the stromal responses to therapies and even look for new targets within this compartment. Moreover, the effect of FGFR targeting in stromal pancreatic stellate cells has also demonstrated a beneficial outcome by reducing cancer cell invasion and hence better containing the tumour.\textsuperscript{139} This suggests that PI3K pathway inhibition may also have an antistromal effect that reduces the protumorigenic role of the activated cancer-associated fibroblasts and stellate cells, but as yet, this effect has not been assessed. Interestingly, overexpression of FGFR has also been used in a less conventional approach, where targeting this cell-surface receptor with an antibody-conjugated adenovirus specifically delivered a viral gene.\textsuperscript{140} This viral gene then predisposed these cells to antiviral therapy by ganciclovir. While this work has not progressed beyond preclinical models, other alternative therapies, such as antibody-conjugated nanoparticles, toxins, viruses or CAR-Ts,\textsuperscript{141–144} highlight the variety of emerging therapies that could potentially combat this primarily treatment-refractory disease.

PDGFR is less commonly mutated in PDAC, but upregulation of PDGFR signalling has been implicated as a mechanism for metastatic progression in p53-mutated tumours.\textsuperscript{42, 145, 146} Interestingly, one patient with PDAC who responded well to AZD2014 therapy in a phase I trial was found to have a PDGFRA mutation, and this may present a novel biomarker for therapies aimed at PI3K pathway inhibition.\textsuperscript{98} An important function of PDGFR signalling is an overlapping role with VEGFR signalling for angiogenesis, which has been extensively assessed as a target in PDAC.\textsuperscript{147} After promising clinical trials led to approval of the mTORC1 inhibitor rapamycin and the broad-spectrum RTKi Sunitinib in pNETs, which are typically highly vascularised, several clinical trials began looking at the effectiveness of these inhibitors in PDAC (table 2).\textsuperscript{93, 148} However, the antiangiogenic effects in PDAC provided minimal clinical benefit and future clinical trials are looking at the application of RTKis as part of combination therapies (table 2).\textsuperscript{149–152} One interesting target that has emerged from VEGFR targeting strategies is the discovery that placental growth factor, a VEGF homologue, is specifically upregulated in tumour vasculature and provides a target for disease-specific angiogenesis, without affecting normal healthy vessels.\textsuperscript{153} However, the effectiveness of this strategy remains controversial and has yet to progress to the clinic.\textsuperscript{154}

Ephrin receptors as predictive biomarkers or novel targets
The largest known RTK family is that of the Ephrin receptors, of which both the EphrinA and EphrinB subfamilies are associated with poorer survival in patients with PDAC and are predictive of tumour proliferative and growth capacity.\textsuperscript{155, 156} Indeed, increased activity of EphrinA2 has been associated with Kras-driven PDAC progression and knockdown in a mouse model of PDAC decreased metastasis.\textsuperscript{153, 157} Furthermore, axon guidance GPs in which EphrinA5 and EphrinA7 play a role have been implicated in PDAC development, providing further motivation for application of the EphrinA/EphrinB receptors as predictive biomarkers for aggressive disease.\textsuperscript{38} Their continued association with PDAC has led to several approaches to therapeutically

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target these receptors. For example, a recent toxin-conjugated monoclonal antibody against the EphrinA2 receptor MEDI-547 completed phase I clinical trials in treatment-refractory solid tumours. Similarly, the broad-spectrum small molecule tyrosine kinase inhibitor dasatinib has an established inhibitory effect on the intracellular kinase domains of Ephrin receptors and provides a parallel approach for targeting of other RTKs. After promising preclinical studies, dasatinib has progressed to clinical trials for metastatic PDAC in combination with FOLFOX (NCT01652976) or gemcitabine/erlotinib (NCT01660971) chemotherapy. However, dasatinib in combination with gemcitabine did not improve overall compared with gemcitabine and placebo in locally advanced, non-metastatic PDAC. Another common approach to target upregulated Ephrin signalling is to inhibit the downstream pathways, such as the MAPK or PI3K pathways. Importantly, as PDAC therapy necessarily turns towards predictive biomarkers to guide personalised therapies, upregulation of Ephrin family members may predict response to RTK, MAPK or PI3K pathway inhibition in PDAC.

Canonical and non-canonical inhibition of aberrant transforming growth factor β (TGFβ) signalling

The membrane-bound TGFβ receptor is mutated at a relatively low frequency in PDAC. However, disruptions in other pathway components occur in ~47% of patients, including mutations in SMAD4, SMAD3, TGFBR1, TGFBR2, ACVR1B and ACVR2A. There is a complex relationship between TGFβ signalling and either tumour suppression or metastatic spread. Indeed, loss of SMAD4 is indicative of a poorer prognosis, while TGFβ pathway activation is associated with an epithelial-to-mesenchymal transition, one of the driving factors for metastatic dissemination. This has made TGFβ signalling the focus of recent clinical trials combining TGFβ receptor inhibition with gemcitabine (NCT02154646 and NCT01373164) or immunotherapy (NCT02734160). However, these trials are not biomarker driven and hence are not stratified for SMAD4 mutational status, which is associated with failure of adjuvant chemotherapies in PDAC. The role of SMAD4 in TGFβ signalling is primarily tumour suppressive, and this function may limit application of TGFβ receptor inhibitors, where they would best be applied to patients with SMAD4 deletion. A key non-canonical mediator of TGFβ signalling is the PI3K pathway, which was shown to be inhibited by TGFβ receptor inhibitors and activated by endogenous TGFβ. Hence, an alternative route, independent of the tumour-suppressive functions of TGFβ signalling, may be through inhibition of these non-canonical signalling pathways.

Targeting DNA repair defective tumours

Aberration in DNA repair pathways, such as mutations in BRCA1, BRCA2, PALB2 or ATM, are commonly associated with increased risk of familial pancreatic cancer, but also occur in the later stages of PanIN development and PDAC. Patients with mutations in this pathway in other cancers have shown beneficial responses to PARPi, and recent clinical trials in PDAC have been performed to assess the beneficial role of second-line olaparib monotherapy in BRCA1/2-deficient patients, following failure on gemcitabine. PARPi work on the basis of synthetic lethality whereby tumours with defects in double-stranded DNA repair pathways become dependent on PARP to repair the resultant collapsed replication forks and maintain chromosomal stability and cell cycle progression. Another option for patients with mutations in DNA repair pathways is by causing further DNA damage in these defective cells by either platinum-based therapies or mitomycin C. Furthermore, the PI3K pathway has a well-established role in DNA damage repair, and promising combination therapies in endometrial and breast cancers have motivated clinical trials in PDAC to assess the effect of PARPi in combination with PI3K pathway inhibitors (table 2, figure 3). The clear responses seen in patients presenting with these DNA repair defects provides a promising personalised approach to therapy when standard of care is found to be ineffective.

Sensitisation of cell cycle defective tumours to cyclin-dependent kinase (CDK) inhibitors as combination therapy

Mutations in CDKN2A, CCND1 and/or CDK4/6 commonly occur in PDAC, and recent work has demonstrated that the reliance of some tumours on this pathway may sensitise them to CDK inhibitors (CDKi). In recent work, the sensitivity of a panel of PDCCLs was assessed for their response to a CDKi, which identified PDCCLs with high expression of retinoblastoma protein and low expression of p16INK4A were significantly correlated with improved response to CDKi, in combination with gemcitabine. This is in line with work in breast and ovarian cancers, and melanoma, where this same expression pattern is common. Furthermore, it has been shown that in PDAC and other cancers, combinations of CDKi with PI3K pathway inhibition in subsets of patients can have an even greater response, thus stratification in this setting may warrant further investigation (table 2).

Histone deacetylases (HDACs) and mutant p53 inhibitors

Loss or mutation of the tumour suppressor p53 occurs in ~75% of patients with PDAC, where gain-of-function mutations occur at a higher prevalence and are thought to provide a growth advantage, as well as driving metastatic progression. The primary role of p53 is to bind DNA as a transcriptional activator or repressor, mediating transcriptional networks responsible for cell death and replicative senescence in response to genotoxic or oncogenic stress. HDACs work by regulating gene expression at an epigenetic level and have been associated with upregulation of mutant p53 in several cancers, including PDAC. Furthermore, several HDACs are overexpressed in PDAC, prompting assessment of the clinical benefit of their inhibition. Recently, a phase I clinical trial of vorinostat with chemoradiation in PDAC showed promising overall survival benefits. In parallel, emerging studies in other AdvSTs demonstrated promising synergistic benefits when combining vorinostat with the broad-spectrum RTKi sorafenib and subsequently led to a new phase I trial of vorinostat and sorafenib with chemoradiation in PDAC (NCT02349867).

One of the key mediators of p53 protein stability is mouse double minute 2 (MDM2), which is responsible for ubiquitination and subsequent degradation of p53 by the proteasome. Mutation of CDKN2A occurs in 35% of PDAC tumours leading to loss of expression of the tumour suppressors p16INK4A and p14ARF (p19ARF in murine tumours). ARF inhibits MDM2, and hence loss of this tumour suppressor leads to increased levels of MDM2 and a decrease in p53 pathway activity. Another key mediator of MDM2 activation is Akt, which activates MDM2 in parallel with other survival pathways (figure 3). Furthermore, the tumour suppressor PTEN has been shown to bypass MDM2 and stabilise p53 protein levels, leading to downstream
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Development of small molecule inhibitors for oncogenic KRAS and MAPK signalling

Mutations in KRAS occur in ~93% of PDAC cases, and this has prompted several efforts to target both mutant KRAS and the resultant aberrant downstream signalling.26 38 The predominant KRAS mutations in PDAC are KRASG12D and KRASG12V, where KRASG12D accounts for 83% of KRAS mutations in PDAC and has been shown to classify into more aggressive molecular subtypes.41 217 This aggressive classification may be due to the downstream signalling cascades that have been linked to specific KRAS mutants, where KRASG12D predominantly activates the MAPK and PI3K pathways, whereas KRASG12V predominantly activates Ral signalling.218 Several attempts have been made to inhibit oncogenic Ras isoforms by either competitive inhibition of GTP binding or by preventing membrane translocation but have so far failed to successfully inhibit Ras at a low enough dose for clinical efficacy.219 Similarly, the farnesyl transferase inhibitor, tipifarnib, did not prolong overall survival compared with gemcitabine alone in advanced PDAC.219 Interestingly, RNA interference approaches have been efficaciously applied to PDAC tumours that were metabolically reprogrammed by mutant Ras, where inhibition of the mutant isoform was sufficient to delay tumour growth.220–222 With this in mind and by successfully delivering small inhibitory RNAs (siRNAs), one group was able to demonstrate the in vivo application of a miniature biodegradable polymeric matrix for delivery of a KRASG12D-targeted siRNA.223 Knockdown of oncogenic KRASG12D at the transcript level effectively inhibited downstream pathways and reduced in vivo tumour burden. Another recent approach to deliver siRNAs to PDAC tumours is using fibroblast-derived exosomes, termed iExosomes, which maintain CD47 expression and hence show increased bioavailability and tumour uptake.224

As an alternative approach to inhibition of oncogenic KRAS, innumerable inhibitors have been developed to target the key signalling cascades immediately downstream, namely the MAPK and PI3K pathways. Several clinical trials have been performed using MEKi in combination with gemcitabine,223–225 but these have so far failed to demonstrate significant improvements in survival, compared with gemcitabine alone. Inhibition of the MAPK pathway is regularly associated with an increase in PI3K pathway activity.225 226 Hence, new treatment strategies have emerged that aim at inhibiting both of these key effector pathways (table 2). While these follow from promising preclinical studies, where the combined efficacy of dual MAPK and PI3K pathway inhibition provides significant tumour growth inhibition, the combined toxicity of this approach can present a strong limiting factor.226 227 Notably, the sequential effect of targeting these pathways may increase tumour susceptibility to inhibition while potentially minimising toxicity.228

MICROENVIRONMENTAL INFLUENCES ON DRUG RESPONSE

PDAC is characterised by a highly desmoplastic reaction, commonly associated with high levels of stromal infiltration, ECM deposition and tumour hypoxia.47 229 Targeting of the ECM or associated stroma has shown some efficacy in PDAC, by improving drug delivery and sensitising tumours to chemotherapies,230–232 although the viability of targeting the stroma in PDAC remains controversial. Complete stromal ablation in PDAC was shown to enhance cancer aggressiveness,233 234 which calls for more subtle and targeted approaches to normalising instead of completely ablating the tumour-associated stroma.235

This effect is partly thought to occur due to a normalisation of the tumour vascular network and manipulation of the ECM/stroma, improving drug efficacy in the tumour.231 233 236 Another common feature resultant from enhanced desmoplasia is the development of a hypoxic environment (figure 4). Hypoxia is strongly associated with increased radioresistance, chemoresistance and metastasis237–239 and PDAC is among those cancers with a propensity for high levels of tumour hypoxia, which is predictive of poorer patient prognosis.229 240

Reduced oxygen consumption and increased glycolysis were recently identified by mitochondrial genome sequencing in PDAC PDCLs.241 This is a key aspect of the Warburg effect, which predicts tumours to rely more heavily on glycolysis for their metabolism.242 While this presents an advantage for tumours that experience reduced vascularity and oxygen levels, it also presents an opportunity to potentially starve the tumour in these hypoxic regions.243 One of the first steps for tumours to switch to glycolytic metabolism is an increase in lactate dehydrogenase (LDH) activity, which converts pyruvate into lactate.244 Inhibition of LDH has recently been shown to synergise with gemcitabine in vitro and may provide a novel strategy for PDAC.245 Another important aspect of PDAC metabolism is the metabolic reprogramming resultant from KRAS mutation, which upregulates glucose uptake and biomass synthesis.246 Furthermore, the upregulation of MUC1 during PDAC progression, along with HIF1α in hypoxic tumour regions, has been shown to cooperate by upregulating anabolic metabolism through the pentose phosphate pathway, resulting in gemcitabine resistance.247 The glycolytic switch in hypoxia can lead to a decrease in pH, and hence pH-regulating proteins are also an important downstream target of the cellular hypoxic response.248 Inhibitors of these pH-regulatory components are currently being assessed, after promising preclinical work, for their role in limiting tumour growth.249 250 The PI3K pathway also plays an important role in glucose uptake, amino acid metabolism and response to cellular stress.58 60 61 In hypoxia, Akt activity is upregulated, along with glucose transporters, to facilitate the switch to anaerobic metabolism.105 238 239 244 Moreover, treatment of PDAC with PI3K pathway inhibitors is less effective in hypoxia, highlighting an important microenvironmental consideration for future stratified clinical trials.105 251

With this in mind, we recently demonstrated that a hypoxia-activated prodrug (HAP) could alleviate hypoxia-induced resistance to a PI3K pathway inhibitor in a combination...
Moving forward, the design and synthesis of HAPs with defined molecular targets are emerging for specific applications. For example, hypoxia-activated chk1 inhibitors were recently developed as proof-of-principle molecules for targeting the hypoxic compartment of tumours, where chk1 is an important component of the DNA damage response and cell cycle progression. From these studies, it is clear that the emerging application of microenvironmental-targeted agents in combination therapies can improve patient outcomes, and as newer generation inhibitors are developed, we are likely to see a wider application of these agents entering the clinic.

CONCLUSIONS
Given the lagging improvements in therapy, there is a dire need to find new biomarkers and targets to move pancreatic cancer towards personalised medicine approaches (figure 2). To guide clinical success, emerging combinations would benefit from a preclinical platform of evidence in at least one in vivo model, as well as optimisation of solubility for reduced toxicity and, importantly, identification of at least one suitable biomarker for therapies with reduced off-target effects.

Box 2  What may improve clinical trials?

► Patient subtyping from tumour biopsies by genomic and/or mass spectrometry approaches.
► Biomarker identification prior to progression to phase II/III studies to ensure appropriate patient stratification for maximal benefit (circulating cell-free DNA/genomic approaches/HC).
► Incorporation of non-invasive imaging for hypoxic tumour burden, such as electron paramagnetic resonance imaging, MRI or positron emission tomography with $^{18}$F-fluorodeoxyglucose.
► Testing of promising lead compounds against stratified patient-derived xenograft/Avatar cohorts prior to phase I clinical trials.
► Development of new produgs to use in combination therapies with reduced off-target effects.
► Raising the bar when defining preclinical ‘success’.

Figure 4 Schematic of the formation of a hypoxic environment and the potential targeting of this microenvironment with HAPs. RBCs transport oxygen through the blood vasculature, and hypoxia forms when this diffusion-limited process delivers insufficient oxygen to cells distant to the vasculature (blue cells). The extreme case of anoxia (grey cells) regularly results in necrotic cell death. HAPs take advantage of the hypoxic environment of tumours to deliver cytotoxic compounds to these tumour regions, where the prodru is either enzymatically cleaved by the cells metabolic machinery or undergoes a conformational change in response to the low oxygen partial pressure. HAPs, hypoxia-activated produgs; RBCs, red blood cells.
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patient stratification at the level of clinical trials (figure 2). The emerging efficacy of PI3K pathway inhibitors for PDAC and the convergence of several aberrantly expressed signalling cascades highlights a clear progression towards their application for this disease. For example, patients with aberrant DNA damage repair pathways have responded well to PI3K pathway inhibition as part of combination therapies, and trials are already underway in PDAC. Furthermore, given the complex demarcation of the ERβ family of RTKs and the association of EPhrin receptors with more aggressive PDAC subtypes, RTKs may provide biomarkers for patients that would respond efficaciously to PI3K pathway inhibition. Moving forward, one of the key goals of the ICGC2 is to link bioinformatics approaches, such as molecular subtyping of patients, to clinical data, and we expect this to drive an increase in biomarker-driven clinical trials (proposed in box 2). This is a necessary step to decrease the attrition of lead compounds in the pharmaceutical industry and to ensure that next-generation inhibitors progress to patients that are appropriately subtyped for maximum benefit.

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