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Overcoming nucleoside analog chemoresistance of pancreatic cancer: A therapeutic challenge

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Abstract

Clinical refractoriness to nucleoside analogs (e.g., gemcitabine, capecitabine) is a major scientific problem and is one of the main reasons underlying the extremely poor prognostic state of pancreatic cancer. The drugs' effects are suboptimal partly due to cellular mechanisms limiting their transport, activation, and overall efficacy. Nonetheless, novel therapeutic approaches are presently under study to circumvent nucleoside analog resistance in pancreatic cancer. With these new approaches come additional challenges to be addressed. This review describes the determinants of chemoresistance in the gemcitabine cytotoxicity pathways, provides an overview of investigational approaches for overcoming chemoresistance, and discusses new challenges presented. Understanding the future directions of the field may assist in the successful development of novel treatment strategies for enhancing chemotherapeutic efficacy in pancreatic cancer.

Keywords

Pancreatic cancer; Gemcitabine; Chemoresistance; Therapeutic; Nucleoside analog

1. Introduction

Pancreatic cancer continues to be a devastating malignancy with a 5-year patient survival rate remaining less than 6% [1]. Since the approval of the chemotherapeutic agent gemcitabine (2',2'-difluorodeoxycytidine, dFdC) in 1996 [2,3], the advancement in the treatment of pancreatic cancer has progressed slowly. Therefore, gemcitabine monotherapy currently remains the standard of care, although it only extends survival by a mere 5 weeks [4]. In addition, the innate and acquired chemoresistance of patients to the drug has emerged as a major concern as observed by a very low patient response rate [5]. The chemoresistance of gemcitabine in pancreatic cancer can be attributed to several anatomical (e.g., dense desmoplastic stroma), pathophysiological (e.g., amplification of growth, cell survival, and anti-apoptotic pathways), and pharmacological (e.g., requirement of phosphorylation for drug activation) barriers. Innumerable clinical trials have ensued over the past decade investigating the efficacy of gemcitabine in chemotherapeutic combinations [6]. Unfortunately, this strategy has proven largely unsuccessful, leading to further evaluation of the molecular aspects of the disease biology as well as approaches for overcoming chemoresistance. As the field shifts towards targeted therapy, several new approaches have recently developed. This review highlights the cellular and molecular mechanisms that contribute directly to gemcitabine chemoresistance as well as the contemporary approaches

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under investigation for overcoming chemoresistance and the therapeutic challenges they pose. Understanding the future directions of the pancreatic cancer treatment may lead to the successful development of new therapeutic strategies for enhancing chemotherapeutic efficacy.

2. Determinants of chemoresistance in the gemcitabine pathway

The dismal circumstance of pancreatic cancer is largely due to late diagnoses (i.e., locally-advanced or metastatic disease) at which chemoresistance in patients is a critical issue. The driving force for metastasis has been a long-standing question in the field, with precise answers not yet available. However, with the advent of tumor genomic sequencing projects, genetic mutations in both the primary carcinoma and subsequent metastatic tumors have been compared and contrasted. These studies have concluded that although genomic instability leading to genetic mutations contributes to metastasis, non-genetic factors are involved as well [7,8]. Particularly, there is evolutionary selection that drives clones with particular genetic mutations or epigenetic changes to form metastatic tumors.

Among the factors for the evolutionary drive includes cellular signaling that favor expansion of cellular populations with specific mutations. Several cell signaling pathways vital for both intrinsic and acquired chemoresistance in pancreatic cancer have been identified [4,9]. Although not necessarily directly related to the gemcitabine cytotoxicity mechanisms, these pathways involved in both the tumor and stromal microenvironment play critical roles in determining drug resistance. Specifically, signaling pathways involved in growth, proliferation, differentiation, apoptosis, invasion, and angiogenesis, such as the notable Akt, EGFR, SHH, Notch, MAPK, and NF κ B pathways, appear to either directly or indirectly influence chemosensitivity. Furthermore, with respect to differentiation, epithelial-to-mesenchymal transition (EMT) and cancer stem cells are two emerging aspects of interest that drive chemoresistance and clonal selection in pancreatic cancer.

Recently, Yachida et al. showed that the progression of pancreatic cancer takes place over approximately 17 years: over a decade is needed to progress from the initiating mutation to a primary carcinoma, about five years is needed for the tumor to obtain metastatic ability, and patients die approximately two years after [6]. Although this confirms that current intervention is indeed at the terminal phase of pancreatic cancer, it is apparent that a large window of opportunity exists for early detection and diagnosis of the disease. However, since early diagnostic methods are not yet available, it is also important to focus on treatment aspects at the end stage of pancreatic cancer, that is, when diagnosis occurs. Undoubtedly, there is a large spectrum of genetic and molecular alterations that contributes to chemoresistance in pancreatic cancer, many of which have been extensively reviewed elsewhere [4,9-11]. Therefore, this review will focus primarily on chemoresistance due to alterations in the gemcitabine pathway.

2.1. Transporters and metabolic enzymes

Due to its hydrophilicity ($\log P$ of -1.33) [12], gemcitabine relies on numerous transporters to pass through the cellular lipid bilayer and exert its cytotoxicity. Earlier studies by Mackey et al. identified key transporters involved in the uptake of gemcitabine and demonstrated the need for their activity in order to confer gemcitabine sensitivity [13,14]. While they found that the human equilibrative transporters 1 and 2 (hENT1 and hENT2) were able to mediate gemcitabine transport (K_m of 160 and 740, respectively), the human concentrative nucleoside transporter 1 (hCNT1) had the greatest intrinsic transport activity ($V_{max}:K_m$ of 0.24) [14]. Since then, both hENT1 and hCNT1 have been highly implicated in gemcitabine chemoresistance as well as patient outcome. Although the clinical correlation between hENT1 expression, both transcriptionally and immunohistochemically, and disease-free as

well as overall survival of pancreatic cancer patients has been well-established [15-19], the correlation between hENT1 expression and innate and acquired chemoresistance in cultured pancreatic cancer cells remains somewhat incongruous and context-dependent [13,20-23]. This may be due to the bidirectional nature of the transporter resulting in drug efflux at higher concentrations, the relative role of the transporter among the presence of other nucleoside transporters, and the expression characteristics of the transporter in normal versus tumor cells [21] including variations in cell cycle characteristics [24]. In pancreatic cells, mislocalization of hENT2, rather than changes in expression levels, proposes an alternative mechanism for gemcitabine chemoresistance [25]. Similarly, the loss of hCNT1 cell surface expression and activity frequently observed in pancreatic cancer cells was found to correlate directly with high gemcitabine chemoresistance [21,24]. Structure-activity characterization of hCNT3 revealed electrostatic interaction by the 3'-hydroxyl position to play a major role in the transport of substrates, including gemcitabine [26]. While the exact role of hCNT3 in gemcitabine chemoresistance has yet to be evaluated, patients with elevated immunocytochemical expression of the transporter were observed to experience a lower risk of disease recurrence and longer overall survival [18].

In addition to influx transporters, a few efflux transporters have also been implicated in gemcitabine resistance of pancreatic cancer including the ATP-binding cassette (ABC) family of multidrug resistance-associated proteins (MRPs). Increased expression of MRP2 mRNA and protein in pancreatic cancer tissues has been associated with both intrinsic and acquired resistance to a regimen of gemcitabine plus cisplatin [27]. Likewise, MRP7, expressed in both normal pancreas [28] and most established pancreatic carcinoma cell lines [29], has been demonstrated to be able to transport gemcitabine, although direct correlations with cytotoxicity have not yet been demonstrated [30]. This may be in part due to the ability of MRPs to efflux the active metabolite of gemcitabine, allowing for reduction of drug concentrations inside the cell [31]. Although the overall significance of transporters in determining gemcitabine chemosensitivity has been well demonstrated, the precise mechanisms and alterations that occur in pancreatic tumors resulting in chemoresistance remain unclear.

Once inside the cell, gemcitabine is phosphorylated by deoxycytidine kinase (dCK) before further phosphorylation into its active diphosphate (dFdC-DP) and triphosphate (dFdC-TP) forms. Activated gemcitabine, in the form of dFdC-TP, induces masked chain termination during which the nucleotide is incorporated into DNA, followed by another deoxynucleotide, prohibiting further DNA polymerase action [32]. Since involved in the rate-limiting step for gemcitabine cellular activation, a deficiency in dCK expression and activity has been shown to be associated with gemcitabine resistance in pancreatic cancer. Loss of dCK mRNA in highly-resistant pancreatic cancer cells has been observed [20,33,34], and a clear correlation between dCK activity and gemcitabine sensitivity in murine and human pancreatic tumor xenografts has been demonstrated [35]. Overexpression of dCK was found to significantly enhance gemcitabine sensitivity in two of three pancreatic cancer cell lines [36]. Furthermore, simultaneous expression of dCK and p8 in a gemcitabine-resistant pancreatic cancer cell line, PANC-1, significantly decreased the cytotoxic IC₅₀ of gemcitabine and enhanced apoptosis and caspase-3 activity; tumor growth inhibition was also noticeably improved in nude mice [37]. Clinically, low immunohistochemical expression of dCK was correlated with both decreased overall survival as well as older age of patients, suggesting a role of age-related methylation in patients [38]. Low expression of the RNA-binding protein HuR has also been shown to correlate with a 7-fold increase in the risk of mortality for pancreatic cancer patients due to its ability to regulate dCK protein levels and confer gemcitabine chemoresistance [39,40].

Gemcitabine is rendered inactive by cytidine deaminase (CDA), which removes the NH₂ group from the pyrimidine [41], allowing the uracil metabolite to be exported from the cell. Interestingly, gemcitabine-induced inhibition of cytidine deaminase activity leads to a decrease in dFdC-TP catabolism, hence propelling the self-potential of gemcitabine activity [42]. Although the focus of most studies involving CDA and gemcitabine in pancreatic cancer has been on generalized adverse effects (i.e., high-grade neutropenia) due to genetic polymorphisms rather than cancer cell cytotoxicity [43-47], a few studies have identified a dramatic increase in chemosensitivity, up to 54-fold, of cell lines with the inhibition of CDA [36,48]. Although further studies are necessary, alterations in CDA expression levels in tumors could be a promising mechanism for improving gemcitabine sensitivity.

2.2. Other molecular targets

Additional candidates of interest for the manipulation of gemcitabine cytotoxicity include effectors of DNA synthesis and repair. In particular, ribonucleotide reductase subunits 1 and 2 (RRM1 and RRM2) are inhibited by dFdC-DP and dFdC-TP and hindered from repairing flawed DNA. In addition to impeding DNA repair, inhibition of RR, the rate-limiting enzyme in deoxyribonucleoside triphosphate (dNTP) synthesis, reduces the endogenous dNTP pool, lessening competition and indirectly facilitating dFdC-TP incorporation into DNA [49]. The transcriptional upregulation of the larger subunit, RRM1, has been consistently observed as pancreatic cancer cell lines acquire gemcitabine resistance [20,50]. Consistently, low expression of RRM1 in tumors is correlated with enhanced response to gemcitabine specifically in recurrent cases [50,51]. The subunit appears to have no correlation with disease-free or progression-free survival, and its correlation with overall survival is variable [51,52]. Therefore, the expression of RRM1 seems to be more relevant to acquired rather than innate gemcitabine resistance in both pancreatic cancer cell lines and patients.

For RRM2, some studies have shown increased transcript and protein expressions in pancreatic cancer cell lines [20,53], while others have additionally identified the significance of the alteration in determining gemcitabine sensitivity in pancreatic cancer. Specifically, RNA interference of RRM2 attenuated chemoresistance and invasiveness in cells [33,54], while it suppressed tumor growth, enhanced apoptosis, and inhibited metastasis in xenograft models [55]. Moreover, Ohhashi et al. found a reduction in cellular proliferation with the inhibition of RRM1 and RRM2 even in the absence of gemcitabine treatment [33]. Clinically, low RRM2 mRNA expression levels correlated with significantly enhanced disease-free, median, and overall survival as well as overall response rate in gemcitabine-treated patients [56,57]. Reduction of the dNTP pool by gemcitabine inhibition of RRM2 is clear in its effects on nuclear DNA (i.e., facilitating the incorporation of dFdC-TP into replicating DNA). However, this mechanism may also hold true for mitochondrial DNA (mtDNA) although evidence has not yet been provided. Nevertheless, gemcitabine has been shown to directly affect mtDNA by inhibiting its γ -polymerase [58].

3. Approaches to enhancing gemcitabine delivery

3.1. Prodrugs by chemical modification

An apparent approach for enhancing gemcitabine delivery to cells is to bypass its dependence on transporters for entering the cell. This can be achieved by chemically modifying the drug and creating various prodrugs, such as acyl derivatives, with increased lipophilicity. For example, lipophilic prodrugs of troxacitabine, a nucleoside analog drug with an unnatural L-configuration, were created with the addition of linear aliphatic chains to the amino group; sensitivity of pancreatic cancer cells to the modified drugs was greater

than 100-fold compared with troxacitabine [59]. Likewise, lipophilic prodrugs of gemcitabine were synthesized by linking the 4-amino group with acyl derivatives (i.e., valeroyl, heptanoyl, lauroyl, and stearyl) [60,61]. In addition, by masking the N-terminus, this modification also protects gemcitabine from rapid deamination and improves its half-life. In particular, 4-(N)-stearyl-gemcitabine was found to decrease the cytotoxic IC₅₀ of a cervix, breast, colorectal, and nasopharyngeal cell line by 1.5-5-fold [62,63]. Furthermore, a novel gemcitabine-cardiolipin conjugate was shown to induce cytotoxicity in several gemcitabine-resistant cell lines independent of nucleoside transporter activity. *In vivo*, the conjugate demonstrated less adverse effects compared with gemcitabine as measured by body weight and white blood cell count, while inhibiting tumor growth by 20% greater than gemcitabine. A high dose of the treatment increased the median survival of tumor-bearing mice by 73%, while the same dose and schedule of gemcitabine led to toxic death of all mice [64]. Another lipophilic prodrug of gemcitabine, CP-4126, was created by esterifying an elaidic fatty acid at the 5' position. The compound was found to be as effective as gemcitabine in chemoresistant cell lines, but both were ineffective in cells devoid of dCK activity. However, CP-4126 maintained its efficacy in nucleoside transporter-inhibited cells, while the IC₅₀ for gemcitabine increased 200-fold. In various xenograft models, both gemcitabine and CP-4126 were equally effective, but the prodrug was able to be administered orally [65]. The potential of this approach has extended to a phase I study with a novel oral gemcitabine prodrug, LY2334737, evaluated in patients with advanced or metastatic solid tumors. This prodrug protects the amine group of gemcitabine with a covalent bond to valproic acid, preventing extensive pre-systemic deamination. As the first human clinical trial for LY2334737, the study demonstrated safe administration of the drug, despite the caveat of blood-brain barrier effects, with minor adverse effects and observations of antitumor activity [66].

An alternative approach for enhancing efficacy includes the creation of a phosphoramidate prodrug. By creating a variant of the monophosphate form (i.e., dFdC-MP), the rate-limiting phosphorylation step is bypassed. Wu et al. demonstrated that a phosphoramidate prodrug of gemcitabine was approximately 4-fold more effective than gemcitabine in dCK-deficient variants of cancerous cell lines. Furthermore, inhibition of transporter activity did not diminish the prodrug's activity in the dCK variants, although the same did not hold true for the parental cell lines [67]. Similarly, another phosphoramidate prodrug, GemMP [10], was found to reduce thyroid cancer cell proliferation by arresting cells in S phase at concentrations 5-10-fold lower than gemcitabine [68]. In addition to increasing lipophilicity, reducing deamination, and bypassing the rate-limiting phosphorylation step, chemical modifications of gemcitabine can also be conducted in order to enhance its interaction with delivery vesicles such as liposomes [60,61], which will be further discussed below.

3.2. Nanoparticle drug delivery

Nanoparticle drug delivery has been shown to be a promising approach for overcoming several anatomical, pathophysiological, and pharmacological barriers [69] and attenuating chemoresistance in pancreatic cancer. By encapsulating or adsorbing gemcitabine in nanoparticles, reduced pre-systemic metabolism, lower dosages, and sustained release are possible. For example, encapsulation of gemcitabine into chitosan and albumin nanoparticles produced sustained release profiles as well as improved antitumor activity *in vitro* compared with the administration of free drug [70]. Additional therapeutic advantages of nanoparticle drug delivery include altered pharmacokinetic parameters (e.g., decreased drug clearance), increased drug concentration in tumor tissues via the enhanced permeability and retention (EPR) effect (i.e., nanoparticles tend to greater accumulate in tumor rather than normal tissues) [71], increased local plasma T3 levels, and the potential for targeted delivery.

Furthermore, combination therapies can also be utilized with nanotechnology. One study reported that a combination of gemcitabine and curcumin in nanoparticles enhanced the inhibition of tumor growth, abolished systemic metastases, and reduced the activation of nuclear factor κ B in a pancreatic cancer xenograft model as compared with either agent alone [72]. Similarly, gemcitabine and paclitaxel preconjugated with a hydrolysable linker and subsequently loaded into a drug carrier were found to significantly improve the chemotherapeutic activity as compared with their free form [73].

As previously noted, decreasing the hydrophilicity of gemcitabine aids in the incorporation of the drug into liposomal formulations. In particular, stearyl gemcitabine nanoparticles better managed tumor growth as compared with free gemcitabine, and results were further enhanced by PEGylation which increases drug half-life [74]. Such nanoparticles were also shown to overcome gemcitabine resistance related to the overexpression of RRM1 in both cell culture and mice. Furthermore, the enhanced cytotoxicity of these nanoparticles was additionally observed in dCK-deficient tumor cells with the induction of apoptosis through caspase activation [75]. Similarly, conjugation of gemcitabine with squalene, a precursor to cholesterol synthesis, resulted in amphiphilic molecules that were shown to have enhanced anticancer activity due to protection of the drug from rapid deamination [76]. Such nanoparticles were demonstrated to possess greater ability to induce S phase arrest and apoptosis of cancer cells as compared with free gemcitabine [77]. Furthermore, it has been reported that albumin enhanced the passive diffusion of squalenoyl gemcitabine nanoparticles rather than relying on membrane transporters [78]. This was confirmed by higher activity of the nanoparticles than free gemcitabine in transporter-deficient, gemcitabine-resistant tumor cells [79]. Furthermore, the penetration of the gemcitabine-squalene molecules was increased due to the presence of cholesterol in the monolayer, which may contribute to the increased anticancer activity since cancer cell membranes are rich in cholesterol [80]. Squalenoyl-based gemcitabine nanoparticles were found to overcome gemcitabine resistance with increased cytotoxicity in gemcitabine-resistant cell lines deficient in both dCK and hENT1 [77].

Nanoparticles can also be modified for targeted delivery of gemcitabine to pancreatic cancer cells. The addition of monoclonal antibodies has been utilized as a targeting moiety for pancreatic tumors. A recent study demonstrated that Herceptin (HER2)-conjugated chitosan nanoparticles loaded with gemcitabine led to an increase in antiproliferative activity *in vitro* along with enhanced S-phase arrest as compared with free gemcitabine. In addition, the targeted nanoparticles were efficiently taken up by the cells and prolonged intracellular retention was obtained. Sustained *in vitro* release of the drug from the nanoparticulate system indicated proper diffusion of the drug from the polymeric matrix [80]. Likewise, gold nanoparticles with anti-epidermal growth receptor antibodies demonstrated that a lower dose of gemcitabine was able to inhibit the proliferation of pancreatic cancer cells as well as orthotopic tumor growth [81].

An additional method for targeting includes the use of magnets which are directed to the desired site upon application of an external magnetic field. The drug can then be released with the help of various triggering factors like ultrasound or changes in physiological conditions (e.g., pH, temperature) or by simple diffusion [82,83]. Such systems prepared by the inclusion of magnetic nanocrystals into squalenoyl gemcitabine bioconjugates have been reported. Upon injection into a mouse tumor model, magnetically guided nanoparticles showed enhanced anticancer activity [84].

Nanogel formulations with tumor-specific molecules were also efficient in overcoming resistance by enhancing tumor growth inhibition with stable release of the drug over several days in nucleoside transporter-deficient and dCK-deficient lymphogenic cancer cells [85].

Such nanogel formulations containing the active form of the nucleoside analog (i.e., dFdC-TP) demonstrated greater cytotoxicity and reduced resistance as compared with nucleoside analog prodrugs. The drug delivery systems aid in the protection of the active drugs and enhance intracellular retention [86,87].

3.3. Dosage and schedule modifications

The mechanism of action of gemcitabine is atypical since the drug depends on the cell cycle and only a few key proteins for efficacy. The importance of these key players for gemcitabine is unique and evident by the significant influence on active metabolite concentration and drug efficacy with the modification of only a single target. Therefore, the efficacy of gemcitabine is dependent on a balance of both dosage and schedule.

Gemcitabine pharmacokinetics can be described by a linear, 2-compartment model with a half-life of 42–94 min (short infusion of <70 min) depending on age and gender. While the rate of clearance varies with age and gender, 92–98% of a typical gemcitabine dose (i.e., 1000 mg/m²/30 min infusion) is excreted, predominantly in the urine. The volume of distribution of gemcitabine is highly affected by infusion duration and gender, while effects of plasma protein binding are negligible [2].

Currently, the standard regimen consists of 1000 mg/m² gemcitabine given weekly as a 30 min infusion for 3 weeks followed by one week of rest. While that dosage is believed to saturate dCK activity, pharmacokinetic studies have determined that a 1000 mg/m²/h infusion rate was optimum for intracellular phosphorylation of gemcitabine [88]. Nonetheless, several studies have shown 1500 mg/m² as the maximum tolerated dose (MTD), with high-grade neutropenia, granulocytopenia, and thrombocytopenia being dose-limiting factors [89,90]. Trials comparing a fixed dose rate (FDR) of gemcitabine (1500 mg/m² at a rate of 10 mg/m²/min weekly for 3 weeks out of a 4-week cycle) with standard gemcitabine treatment indicated increased efficacy of the FDR but with much greater hematological toxicities (i.e., neutropenia, anemia, and thrombocytopenia) in patients [91–93]. Furthermore, although one study established the MTD of gemcitabine at 6500 mg/m² with hematopoietic progenitor support, its efficacy was not superior than that reported with lower-dosage FDR schedules [94]. A high dose of gemcitabine was also investigated with 2200 mg/m² administered as an infusion for 30 min on days 1 and 15 for 6 months. The regimen was deemed safe, tolerable, and effective for palliative treatment of advanced pancreatic cancer patients [95]. Collectively, these data indicate that altering the exposure level of gemcitabine in patients is vital for obtaining specific desired effects.

When comparing schedules, 1000 mg/m² gemcitabine as a 30 min infusion given weekly for either 3 consecutive weeks every 4 weeks or 2 consecutive weeks every 3 weeks indicated comparable efficacy although the 3-week schedule produced lower toxicity in patients [96]. Another schedule of 1000 mg/m² gemcitabine given for 7 consecutive weeks followed by a week of rest and then weekly for 3 weeks out of 4 identified increased toxicities compared with the conventional regimen [97]. Metronomic dosing, or continuous and timed administration of low dosage, of gemcitabine (1 mg/kg/d for a month) was found to exert equal cytotoxicity in orthotopic models of human pancreatic carcinoma in nude mice compared with the conventional schedule of 100 mg/kg/d 0, 3, 6, and 9 post-implantation. However, an anti-angiogenic effect was additionally observed with the metronomic regimen [98].

Infusion duration has also been identified as a key factor that influences gemcitabine efficacy. One study demonstrated that the chemotherapeutic retained its antitumor activity at doses as low as 300 mg/m² when infusion time was prolonged [99]. Other trials increased the infusion rate of 100 mg/m² to 24 h weekly for 3 weeks in a 28-day cycle and found an

improvement in the quality of life of patients but also only marginal antitumor activity [100]. Furthermore, an investigational method of hypoxic abdominal stop-flow perfusion demonstrated increased efficacy of gemcitabine in patients compared with the standard treatment at doses up to 1125 mg/m². However, this complicated modality remains in its infancy, and further studies are needed for its complete assessment [101].

4. Approaches to directly modifying determinants of gemcitabine cytotoxicity

4.1. Gene therapy

Even though gene therapy has not yet been well-developed for the application of cancer (as compared with other disorders such as cystic fibrosis), the approach has become appealing for altering specific targets with its potential for producing a bystander effect within heterogeneous tumors. A fusion gene of dCK and uridine monophosphate kinase (UMK) (dCK::UMK) was found to sensitize pancreatic cancer cells to gemcitabine by markedly decreasing viability. Tumor volume was also reduced as an antitumoral bystander effect was observed due to apoptosis of untransduced cells. Additionally, the use of a synthetic carrier (i.e., polyethyleneimine (PEI)) further induced tumor regression [102]. A combination of dCK::UMK with siRNA against RRM2 and thymidylate synthase (TS), whose inhibition activates hENT1, and gemcitabine promoted chemosensitivity even more with a 40-fold decrease in cytotoxic IC₅₀ in PANC-1 cells. Tumor volume was reduced dramatically and mouse survival prolonged significantly due to an increase in apoptosis and decrease in cellular proliferation [103]. Recently, it has also been found that the overexpression of dCK and knockdown of p8 with recombinant adenoviral vectors also significantly decreased gemcitabine resistance in PANC-1 cells and inhibited tumor growth with enhanced apoptosis and caspase-3 activity [37].

The herpes simplex virus 1 thymidine kinase (HSV-TK)/ganciclovir (GCV) strategy has thus far been successful up to the clinical level, and the same technique may be adapted to target increased RR in cancer cells. RR has been a target of interest with gene therapy since its overexpression is an attribute of mitotic cancerous cells. Therefore, vectors lacking the RR gene are attractive options due to their ability to complement with overexpressed mammalian RR and selectively target rapidly dividing cells. For example, a herpes simplex virus (HSV) with an RRM1 deletion mutant (i.e., ICP6Δ) was found to enhance the expression of adeno-associated viruses along with their kinase genes both in pancreatic cancer cells and xenografts, demonstrating potential for the combination therapy [104]. Similarly, an HSV vector lacking the RR gene (i.e., hrR3) was found to extend survival in 70% of mice receiving both the vector and ganciclovir, 40% of mice receiving the vector alone, and 0% of untreated mice [105].

Overexpression of hENT1 has also been conducted with a recombinant adenovirus (i.e., Ad-hENT1) in human pancreatic cancer cells. Although expression of the transporter is known to vary with cell cycle and cell type, treatment with the vector improved response to gemcitabine [106]. Other targets of gene therapy in pancreatic cancer include p53 and p16 [107] and Bax [108], although they are not directly related to gemcitabine metabolism.

4.2. Epigenetic approaches

The field of epigenetics has been rapidly emerging since the identification of its influence on the initiation, progression, and resistance of various cancers. The ability to reverse aberrant epigenetic alterations renders this targeted approach highly attractive. Among the various mechanisms, DNA methylation has been the most widely studied. Abnormal promoter hypermethylation can silence tumor suppressor genes and have been implicated in the

clinicopathological features and promotion of tumorigenic properties of pancreatic cancer. One study identified that low expression of dCK in pancreatic cancer patients correlated with overall survival as well as age, suggesting a role of age-related methylation of the dCK gene [38]. It was also found that the CDA gene was methylated in an entire cohort of colorectal cancer patient samples, although no correlation was observed between methylation status and clinicopathological parameters [109]. By utilizing microarrays, countless other aberrantly methylated genes have been identified in pancreatic cancer, particularly those related to growth, differentiation, angiogenesis, and apoptotic signaling [110-113]. However, further information about the methylation status of the targets of the gemcitabine pathway is limited.

FDA-approved inhibitors of DNAmethylation are currently available with several more under investigation. For example, the nucleoside analogs 5-azacytidine (5-aza-C) and 5-aza-2'-deoxycytidine (5-aza-dC) have been approved for the treatment of high risk myelodysplastic syndromes [114] and are currently under study for acute and chronic myeloid leukemias and ovarian cancer [115]. Both compounds have been found to be substrates for hCNT1 [116] and rely on dCK for their phosphorylation [117]. Combination of 5-aza-dC with gemcitabine was shown to inhibit pancreatic cancer cell growth to a greater extent than with gemcitabine alone [118]. The DNA methylation inhibitor cladribine, also a nucleoside analog, has been studied in conjunction with rituximab for the treatment of mantle cell lymphoma; high levels of activity with minimal toxicity were found [119].

In addition to DNA, epigenetic modifications, namely methylation and acetylation, can also occur at the histone level. Unlike DNA methylation, histone methylation is a mechanism that has yet to be thoroughly studied in the context of cancer. Histone acetylation, on the other hand, is a mechanism well-studied in the development and progression of cancer since aberrant activity promotes the expression of various oncogenes. This process is reversibly catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Currently, FDA-approved HDAC inhibitors include vorinostat and romidepsin for the treatment of T cell lymphoma, although many others remain under study in clinical trials [115]. Romidepsin has also been demonstrated to have antiproliferative activity in five human pancreatic cancer cell lines (IC₅₀: 1–500 nM) via cell cycle arrest at both G1 and G2/M phases followed by apoptosis [120]. Additionally, the HDAC inhibitor MS-27-275 was shown to decrease cells in S phase cells while increasing G1-phase cells. Antitumor efficacy was observed in both Capan-1 cells and mouse xenograft [121]. Trichostatin A [122], another HDAC inhibitor, has been widely studied and shown to induce antitumor effects in pancreatic cancer cell lines at submicromolar concentrations [123,124]. Furthermore, a novel compound derived from both MS-27-275 and TSA was shown to cause cell cycle arrest and subsequent apoptosis in pancreatic cancer cell lines [125]. Several studies have shown the increased efficacy of combining an HDAC inhibitor with gemcitabine in pancreatic cancer cell lines including the synergistic apoptotic effects of TSA with gemcitabine in pancreatic ductal carcinoma cells [126-130]. With 50% reduction of pancreatic cancer xenografts and no observed toxicity, Donadelli et al. further identified that TSA did not affect the gene expressions of targets in the gemcitabine pathway [127]. Nonetheless, a phase II trial of CI994 (n-acetyl dinoline), an HDAC inhibitor, with gemcitabine demonstrated no additional advantage in survival rate compared with gemcitabine alone [131], warranting further studies for the better understanding of this therapeutic approach.

The rapidly expanding field of microRNAs (miRNAs), short (~22 nt) RNA sequences that post-transcriptionally regulate gene expression by binding to complementary mRNA, is also appealing for its potential to influence cancer epigenetic therapy. Their ability to regulate expression of canonical oncogenes and tumor suppressor genes as well as the recent

demonstration of their direct involvement in oncogenic processes emphasizes their potential to intervene in dysfunctional cancer pathways. There is also increasing evidence demonstrating that miRNAs are critical regulators of drug resistance in pancreatic cancer. For example, re-expression of the miR-200 family or downregulation of miR-21 in gemcitabine-resistant cells led to sensitization of the cells to gemcitabine [4]. Since miRNA expression patterns are unique to each tumor type, aberrant expression profiles (e.g., the downregulation of the *let-7* family) have been shown to contribute to cancer pathogenesis and tumor development and progression [4,132]. In addition to being epigenetic targets, miRNA activity can also be modulated by inhibition with antisense oligonucleotides or overexpression with synthetic miRNAs, as reviewed recently [4,132]. The development of curcumin as a potential therapeutic for pancreatic cancer patients has been under investigation due to its interactions with DNA methyltransferase, HDACs, and HATs and ability to alter miRNA expression, including upregulation of miR-200 and downregulation of miR-21 [133]. In this regard, several recent reviews identify key growth, proliferation, and differentiation (e.g., PI3K/Akt, MAPK, K-ras, STAT-3, Notch-1, Notch-2), invasion (e.g., ABCG2, cadherin, ZEB1, vimentin), and apoptosis (e.g., CDK6, p53, caspase 3) proteins altered by miRNAs [4,132,134]. Therefore, targeting miRNAs offers great promise for tackling pancreatic cancer chemoresistance and providing diagnostic and therapeutic values.

Studies from our laboratory show miRNAs influencing the expression of direct targets of the gemcitabine pathway. We have identified that miR-214, miR-339-3p, and miR-650 overexpression significantly reduced CNT1, but not ENT1, protein levels. Furthermore, several miRNAs were found to markedly reduce both CNT1 transport activity as well as gemcitabine cytotoxicity in a pancreatic cancer cell line [24]. Likewise, our recent study showed that the differential processing of the *let-7* family of miRNAs altered gemcitabine chemosensitivity in pancreatic cancer cells through its activity on RR (unpublished data). Both studies identify the potential for modulating miRNA expression to enhance the efficacy of gemcitabine, although the ability of pancreatic cancer cells to process miRNA precursors should be carefully considered while choosing precursor miRNA-based therapies. In addition to overcoming chemoresistance, epigenetic and miRNA alterations also have the potential to act as prognostic and predictive biomarkers for the guidance of therapy.

4.3. Molecular therapeutic agents

Several small and large molecule inhibitors demonstrate potential as therapeutic agents. For example, proteasomal inhibitors, such as bortezomib, have been shown to increase cytotoxicity in combination with gemcitabine [24,135]. Similarly, tetrahydropyridine (ThU), a competitive inhibitor of CDA, has been shown to sensitize pancreatic cancer cells to gemcitabine up to 54-fold [36]. Although pharmacokinetic studies have been performed on the compound, its therapeutic value as well as safety and toxicity profiles remain under study in humans [136]. RNA interference has also been commonly used for the study of specific targets in pancreatic cancer chemosensitivity. While Ohhashi et al. noticed no change in gemcitabine sensitivity or cellular proliferation with hENT1-targeted siRNA [33], others found that knockout of TS with siRNA decreased chemoresistance in pancreatic cancer cell lines [137]. Furthermore, shRNA against hENT1 combined with 5-fluorouracil (5-FU) treatment attenuated cell viability and gemcitabine cytotoxic IC₅₀ values. Given the dual treatment, cells were arrested in G0/G1 phase, while mouse xenografts had diminished tumor volumes [26]. Furthermore, modulation of transporter activity and, consequently, gemcitabine uptake was recently conducted with siRNA against hCNT1 [24]. Similarly, silencing of dCK with siRNA in gemcitabine-resistant cells further reduced gemcitabine sensitivity without affecting cellular proliferation [33]. Treatment of gemcitabine-resistant PANC-1 cells with hydroxyurea led to a 4-fold increase in chemosensitivity [138]. RRM1-

and RRM2-targeted siRNA in pancreatic carcinoma cell lines increased gemcitabine sensitivity, apoptosis, and caspase-3 activity while lessened cell proliferation and invasiveness [33,54,55,139]. In an orthotopic xenograft model, synergism was observed between RRM2 silencing and gemcitabine, leading to reduced tumor growth, enhanced tumor apoptosis, and inhibition of metastasis [55].

5. Novel therapeutic combinations with gemcitabine

In addition to radiosensitization, potentiating chemosensitization of gemcitabine by other pharmaceutical agents has been a longstanding area of interest. Although combination therapies with gemcitabine have been studied extensively both *in vitro* and in clinical trials, only a few have consistently resulted with significant improvements in patient survival. Since several mechanisms of drug resistance exist, combination therapy can simultaneously undertake multiple mechanisms and pathways, allowing the drugs to act in concert. This rationale is well-illustrated by the current approach towards breast cancer treatment. Nonetheless, combinations of gemcitabine with other chemotherapeutics such as other nucleoside analogs (i.e., 5-FU, capecitabine), platins (i.e., cisplatin, oxaliplatin), and taxoids (i.e., docetaxel [122,140], paclitaxel [141]) have shown variable results with no significant enhancement of patient survival [6].

With the interests geared towards targeted therapy, numerous novel therapeutics have been studied with gemcitabine: antibodies (i.e., bevacizumab, cetuximab), growth factor inhibitors (i.e., erlotinib, aflibercept, axitinib [142], saracatinib [143]), topoisomerase I inhibitors (i.e., irinotecan, exatecan, rubitecan), MMP inhibitors (i.e., marimastat, BAY 12-9566), tyrosine kinase inhibitors (i.e., masitinib, sorafenib, sunitinib [144], ARQ 197 [145]), and other inhibitors (i.e., pemetrexed, tipifarnib, celecoxib, imitinib [146], PX-12 (a novel inhibitor of thioredoxin) [141] and enzastaurin [147]) [6]. Among the sizeable list, several agents show promise. However, erlotinib has been the only agent thus far to have consistently demonstrated equal or significantly enhanced median overall survival compared with gemcitabine (1-year survival rates of 23% and 17%, respectively) [6]. Therefore, the EGFR inhibitor is currently FDA-approved for use in combination with gemcitabine for the treatment of pancreatic cancer. Other agents of interest include curcumin (a natural product) [6], leucovorin (an adjuvant agent) [6], tegafur (a prodrug of 5-FU) [148,149], and MGCD0103 and valproic acid (HDAC inhibitors) [150]. In particular, notable combinations under study include GTX (gemcitabine, docetaxel, and capecitabine) [151,152], GemOx (gemcitabine, oxaliplatin) [153], GemOxCet (gemcitabine, oxaliplatin, cetuximab), and Gemoxel (gemcitabine, oxaliplatin, capecitabine) [154]. Nonetheless, novel combinations not including gemcitabine such as Xeliri (capecitabine, irinotecan) [155], Folfiri (leucovorin, 5-FU, irinotecan) [155], and Folfirinox (leucovorin, 5-FU, irinotecan, oxaliplatin) [156] have also shown promise in combating pancreatic cancer, although more studies are needed to determine toxicity and efficacy profiles.

An alternative approach studied is the use of agents for modulating the tumor microenvironment. Several clinical trials have been conducted with prophylactic anticoagulants (i.e., nadroparin, enoxaparin, dalteparin) with gemcitabine, although minimal significant results for the advancement of pancreatic cancer therapy were obtained [6]. Similarly, the use of pomalidomide, an investigational immunomodulating drug that both inhibits angiogenesis as well as exerts antitumoral effects, was found to be feasible and safe in most patients [157]. When co-administered with gemcitabine, a drug depleting tumor-associated stromal tissue via inhibition of the Hedgehog cellular signaling pathway, IPI-926, was found to facilitate gemcitabine intratumoral delivery and extend survival of a spontaneous mouse pancreatic tumor model [158]. Furthermore, a novel ceramide analog, AL6, has been shown to increase the dCK/CDA gene expression ratio, leading to increased

apoptosis in two pancreatic ductal adenocarcinoma cell lines [159]. A study of gemcitabine in combination with imexon, a pro-oxidant small molecule, was found to significantly increase chemotherapeutic efficacy in PANC-1 xenograft tumors. This is thought to be due to the inhibition of RR by imexon, with inhibition effects even greater with the addition of gemcitabine [160]. Overall, combinations of a targeting agent with gemcitabine have shown to be well-tolerated with promise for enhanced efficacy. Further studies as well as meta-analysis of data from clinical trials are warranted for consistent efficacy results.

6. Therapeutic challenges in overcoming chemoresistance

Despite the abundance of common and novel chemotherapies, gemcitabine remains the superior drug of choice for the treatment of pancreatic cancer due to its ability to extend survival, although only by a mere few weeks, and improve the quality of life with better tolerance. As one of the many enduring nucleoside analog drugs, gemcitabine has been predicted to remain as the drug of choice for pancreatic cancer for the near future even though its effects are truly suboptimal. In spite of considerable efforts to improve the drug of choice, several other approaches (e.g., targeting stromal pathways in tumors) for overcoming chemoresistance in pancreatic cancer have been underway, bringing with them the new challenges they pose.

It is inarguable the abundance of consistent genetic and molecular alterations observed in panIN, primary, and metastatic pancreatic neoplasms. Numerous large-scale genomic sequencings and proteomic screenings have been conducted with novel discoveries identified from the experiments. However, the direct applicability of these potential biomarkers in patient populations remains unknown. For example, perhaps the most recognized alteration noted in pancreatic cancer is the mutation of Kras. Although targeting this player has thus far been unsuccessful due to the redundancy of cellular signaling pathways and perhaps also the lessened dependency of cells on this pathway as the disease progresses, a novel small molecule inhibitor of the Ras oncoprotein may develop with promise [161]. While the utilization and targeting of such genetic and molecular modifications remain a challenge in overcoming chemoresistance, the identification of precise biomarkers is vital since they may be used for targeting tumor cells and for guiding therapy. Identifying cell surface markers could allow for the targeting of tumor cells alone, leaving surrounding healthy cells in the heterogeneous tumor microenvironment to thrive. Also, utilizing these markers could stratify patient groups for individualized and tailored treatments. In addition to earlier diagnosis, biomarkers also have the potential for therapeutic monitoring of patients, especially since pancreatic cancer is known to undergo genetic mutations at a high frequency and rapid changes in disease biology. Currently, challenges arise in both identifying appropriate biomarkers for use in patient populations as well as in the practicality of screening a patient in the narrow time period between initial diagnosis and start of treatment. Clinical trial inconsistencies pose another challenge as the patient populations are often low in number and heterogeneous (e.g., with some patients already under treatment and some chemo-naïve). Well-annotated, patient-derived tumor samples remain a poorly available resource for further research.

In addition to identifying genetic and molecular alterations, it is crucial to understand when the changes occur during the progression of the disease. Pancreatic cancer, like any other solid tumor, consists of multiple defined stages, yet all patients are treated with the same drug (mainly due to diagnoses occurring at advance-stage disease). With better comprehension of the changes that occur throughout the cancer, including variations between the primary and secondary tumors and the primary and recurrent tumors, tailored drug therapies can be developed. Furthermore, management of genetic and molecular changes and disease monitoring are vital with disease progression. Identification of genetic

and molecular signatures that can segregate pancreatic tumors into categories, such as those from Collison et al. (i.e., classical, quasimesenchymal, and exocrine-like) [162], could lead to separate and appropriate treatment and management strategies. Therefore, it is imperative to understand how the chemotherapeutic drugs exert their efficacy as this information may be particularly useful when considering the heterogeneity of pancreatic tumors.

Pancreatic tumors are well-known for their dense desmoplastic stroma and sparse, collapsed vasculature, all characteristics impeding effective chemotherapy. Although the tumors consist of both stromal and epithelial tissues, most earlier studies have focused mainly on epithelial components. However, currently, an increased number of studies are investigating the stroma of pancreatic tumors, including methods to target eminent stromal pathways such as Hedgehog, TGF- β , and Wnt/PI3K as well as deplete stromal tissues in order to stimulate angiogenesis and increase drug delivery. The latter approach is currently under investigation in numerous Phase I-III clinical trials. Enhanced knowledge of the heterogeneity of pancreatic tumors may lead to improved methods for overcoming chemoresistance due to tumor diversity. Furthermore, accurate methods for diagnosis as well as disease and therapeutic monitoring can be developed.

Significant advances have been made in the study of pancreatic cancer, including the development of *in vitro* cell lines and *in vivo* mouse models. Although a plethora of pancreatic cancer cell lines exist for study, their ability to mimic the wide range of pancreatic tumor characteristics is lacking. For example, Collison et al. identified cell lines that represented the classical and quasimesenchymal subtypes of pancreatic cancer but not the exocrine-like subtype [162]. Although the use of cell lines for cancer study remains valid, it is important to note the potential flaws of the model systems. Furthermore, although novel mouse models such as the orthotopic and K-ras^{LSL-G12D/+}; *Trp53*^{LSL-R172H/+}; *Cre* (KPC) have been developed, there remains the issue of interspecies differences in the development of chemoresistance. It is not yet fully determined how well these murine models represent human pancreatic tumors in both development and treatment outcomes. For example, the orthotopic model does not generate enough stroma to mimic human tumors, and the KPC model does not contain the entire spectrum of genetic mutations observed in human tumors. Although developmental issues are evident, the relative significance of these differences on influencing therapeutic outcome remains unknown. Ultimately, although great strides have been made with the development and use of novel mouse models, there remains a lack of model that has the ability to completely recapitulate human disease.

In addition to identifying new and far more improved drugs, an integrated approach is needed to overcome chemoresistance. For example, the rational combinations of the reviewed therapies (Fig. 1), such as epigenetic agents, gene therapy, antibodies, and signaling inhibitors, along with the use of chemotherapeutics still need further explication. The direct applicability of these therapies in combination (e.g., precise biomarker combinations to target cancer cells of all stages) in guiding treatment remains unknown. Furthermore, disconnect between benchtop, clinical, and regulatory science propels an additional, complex challenge in overcoming pancreatic cancer. Currently, numerous clinical trials remain at early investigational stages and are difficult to escalate to the level of approved patient-use. Although divisions between specialized fields still exist, the process of streamlining translational research is gaining momentum, and its persistence may ultimately lead to overcoming the therapeutic challenges of pancreatic cancer.

7. Conclusions

As the current standard of care, gemcitabine is predicted to remain as the drug of choice for the treatment of pancreatic cancer for the near future. Determinants of chemoresistance in the gemcitabine pathway are well-known, yet many challenges remain in pursuing the targets of interest to improve efficacy. Furthermore, other than the gemcitabine pathway, numerous other cellular and tumoral determinants pose as obstacles in overcoming chemoresistance. Nonetheless, novel therapeutic approaches are currently underway to circumvent limitations in gemcitabine transport, phosphorylation, and overall efficacy. Although therapeutic challenges are clear and present, countless studies, both at the benchtop and clinical levels, continue to provide clues for maximizing chemotherapeutic efficacy in patients.

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Fig. 1.

Novel therapeutic approaches for overcoming limitations in the gemcitabine pathway. Gemcitabine enters the cell via nucleoside transporters (i.e., CNT1, CNT3, ENT1, ENT2). The drug is either phosphorylated into its active form (i.e., dFdC-phosphate) by dCK or deaminated into dFdU by CDA and eliminated from the cell. Activated gemcitabine can then terminate the cell by directly targeting DNA or inhibiting RRM1 and RRM2 to deplete the dNTP pool necessary for DNA replication. Descriptions in boxes (red) indicate known methods for targeting a particular determinant (green) of gemcitabine chemosensitivity in pancreatic cancer. Abbreviations: CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; dCK, deoxycytidine kinase; CDA, cytidine deaminase; RR, ribonucleotide reductase; dFdC, 2',2'-difluorodeoxycytidine; dFdU, 2',2'-difluorodeoxyuridine; CDP, cytidine diphosphate; dCDP, deoxycytidine diphosphate; dCTP, deoxycytidine triphosphate.