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Anti-Tumour Treatment

Epigenetic targeting in pancreatic cancer

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ABSTRACT

The prognosis of pancreatic cancer patients is very poor, with a 5-year survival of less than 6%. Therefore, there is an urgent need for new therapeutic options in pancreatic cancer. In the past years it became evident that deregulation of epigenetic mechanisms plays an important role in pancreatic carcinogenesis. This review focuses on the exploitation of drugs that alter histone modifications, DNA methylation and microRNA expression as options for the treatment of pancreatic cancer.

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Introduction

Pancreatic cancer is one of the most aggressive of all human malignancies. It is the fourth leading cause of cancer-related death among men and women in the USA and Western Europe with a 5-year survival about 6% [1,2]. Early detection is difficult because of the absence of disease-specific symptoms and a reliable biomarker. Therefore, the majority of patients present with locally advanced or metastatic disease [3]. For years, the standard treatment for patients with advanced pancreatic cancer has been the nucleoside analogue gemcitabine, which showed improved alleviation of disease-related symptoms over 5-fluorouracil (5FU) treatment [4]. Subsequently, the epidermal growth factor receptor (EGFR) inhibitor erlotinib was found to have a small, but significant survival benefit in combination with gemcitabine over gemcitabine alone, albeit with more serious adverse effects of treatment [5,6]. Furthermore, the FOLFIRINOX regimen (oxaliplatin, irinotecan, fluorouracil, and leucovorin) also showed a significant survival benefit over gemcitabine [7]. However, the gained survival advantage remains limited, at the cost of more toxicity. Therefore, there is an urgent need for new therapeutic options in pancreatic cancer.

This review will focus on the use of drugs that target epigenetic regulation for the treatment of pancreatic cancer.

Epigenetics

In 2008, the core signaling pathways affected in pancreatic cancer were revealed [8]. The best characterized genes in pancreatic cancer are the oncogene *KRAS*, and the tumor-suppressor genes *TP53*, *SMAD4* and *CDKN2A* [8,9]. The identification of these pathways and genetic alterations provides potential molecular targets for drug development. But despite of encouraging results in preclinical studies, many drugs did not show a survival benefit over gemcitabine [10–12]. Recently, other molecular events, such as epigenetic alterations, are identified to contribute to the development of pancreatic cancer. Epigenetics is defined as the study of heritable changes in gene expression caused by mechanisms other than changes in the underlying DNA sequence. The best known

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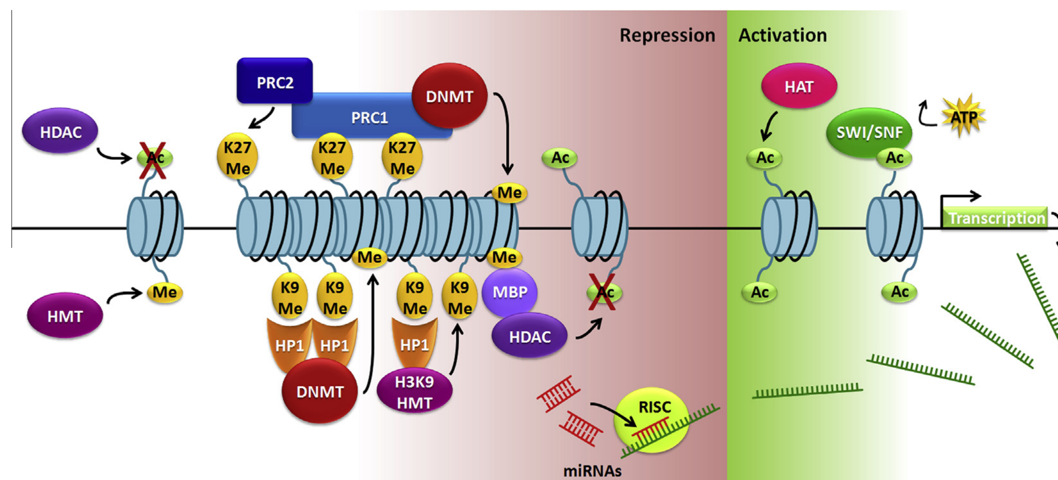

Fig. 1. Schematic overview of epigenetic mechanisms. When nucleosomes (blue cylinders) are densely compacted (left), the chromatin is in a repressed state. This repressive signal is induced and maintained by several proteins like HDACs. Activation is mediated by HATs and chromatin remodelling complexes, which decondense chromatin (right). DNA methylation is regulated by DNMTs. In addition, miRNAs can silence gene expression by associating with mRNA of transcribed genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

epigenetic mechanisms are histone modifications, DNA methylation and microRNAs (miRNAs) (Fig. 1). These epigenetic mechanisms might serve as suitable targets for therapeutic interventions as they play a pivotal role in the regulation of cellular processes.

Histone modifications

The DNA of eukaryotic cells is packaged into the nucleus in the form of chromatin, which is made up of nucleosomes. A nucleosome consists of an octameric histone core with DNA tightly wrapped around it. In general, this core consists of dimers of histone proteins H2A, H2B, H3, and H4. These histones all have a flexible N-terminus protruding from the core, called histone tail, which can be post-translationally modified, leading to changes in chromatin compaction [13]. Two major histone modifications are acetylation and methylation.

Acetylation is mediated by histone acetyltransferases (HATs) and can occur on lysine residues in histone tails (Fig. 1). Lysine acetylation promotes transcriptional activation; it neutralizes the positive charge of lysine, thereby loosening the interaction with negatively charged DNA and enabling transcription. Furthermore, it recruits ATP-dependent chromatin remodeling complexes such as the SWI/SNF complex, which facilitate the binding of transcriptional proteins by decondensing the DNA [13]. Acetylation of histone tails is a reversible process. Histone deacetylases (HDACs) remove acetyl groups from the histone tails. This restores the positive charge on the lysine residue and reestablishes the electrostatic interaction with DNA, thereby repressing transcription [14].

Histone methylation is mediated by histone methyltransferases (HMTs) and can occur on lysine and arginine residues (Fig. 1). Methylation of lysine (K) 9 and 27 in the histone tail of H3 induces the formation of heterochromatin [13,15,16]. This H3K9 methylation recruits heterochromatin protein 1 (HP1), which can then oligomerize with other HP1 proteins to form heterochromatin. Furthermore, HP1 can recruit DNA methyltransferases (DNMTs) and HMTs to propagate the repressive mark along the DNA [15]. Transcriptional silencing induced by H3K27 methylation is mediated by polycomb repressor complexes PRC1 and PRC2. PRC2 functions as a HMT that adds methyl groups to H3K27. H3K27 methylation by PRC2 forms a binding site for PRC1, which condenses the DNA into heterochromatin [16]. Furthermore, polycomb complexes can associate with DNMTs [17].

DNA methylation

DNA methylation is the covalent addition of a methyl group to a cytosine in mainly CpG dinucleotides and is mediated by DNMTs (Fig. 1). It is a key element in the formation and maintenance of condensed chromatin. DNA methylation induces transcriptional silencing by interfering with the binding of transcriptional proteins and it can recruit methyl-CpG binding proteins (MBPs), which in turn recruit HDACs leading to transcriptional repression [15,18].

MicroRNAs

Although miRNAs are under epigenetic control themselves, they too are heritable regulators of gene expression without changing the underlying DNA sequence [19]. Unlike histone modifications and DNA methylation, miRNAs are post-transcriptional regulators of gene expression. They are small non-coding RNAs that can base-pair with complementary protein coding mRNAs, thereby inhibiting translation or inducing degradation of these mRNAs (Fig. 1) [20]. There are more than 1800 human miRNAs that can have multiple targets [21]. They are predicted to regulate approximately 30% of the protein coding genome, including important cellular processes such as cell growth, differentiation and apoptosis [20,22].

Therapeutic options of epigenetic targeting in pancreatic cancer

Several studies have investigated the role of epigenetic mechanisms in pancreatic cancer and the results reflect their importance in pancreatic cancer development and progression.

HDACs have been shown to regulate important cellular processes such as proliferation, apoptosis, differentiation, migration and angiogenesis in cancer [23]. In pancreatic cancer, several HDACs are overexpressed and our understanding of the downstream effects is increasing [24]. Low expression of tumor-suppressor gene *TGFBR2* is observed in 50% of pancreatic cancers, but genetic alterations of *TGFBR2* are rare [25–27]. It has been shown that HDAC1 and HDAC2/SIN3a are recruited to the *TGFBR2* promoter, leading to its repression [28–30]. Also, Von Burstin et al. observed recruitment of SNAIL together with HDAC1 and HDAC2 to the promoter of *CDH1* in a highly metastatic pancreatic cancer cell line [31]. *CDH1* encodes for the epithelial marker E-cadherin. Loss of E-cadherin is a key element in epithelial-mesenchymal

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transition (EMT), leading to cancer invasion and metastasis [32]. Overexpression of HDAC2 leads to apoptotic resistance of cancer cells via silencing of the death-inducing *NOXA* gene and attenuates TRAIL-induced apoptosis of pancreatic cancer cells [33,34]. In addition, HDAC3 has been shown to activate SKP2 which recognizes phosphorylated $p27^{Kip1}$, targeting the cell cycle regulator for ubiquitination and subsequent degradation by the proteasome [24].

HMTs are also involved in pancreatic cancer. EZH2 is a HMT of the PRC2 complex [16]. Nuclear accumulation of EZH2 is a hallmark of poorly differentiated pancreatic cancer. Genetic depletion of EZH2 results in re-expression of cell cycle regulator $p27^{Kip1}$ and decreased pancreatic cancer cell proliferation. In addition, it sensitized pancreatic cancer cells to doxorubicin and gemcitabine [35]. Another HMT implicated in pancreatic cancer is SUV39H1. Baumgart et al. have shown that SUV39H1 is recruited by NFAT to the $p15^{INK4b}$ promoter in pancreatic cancer leading to H3K9 trimethylation and subsequent binding by HP1 resulting in a repressed chromatin state and gene silencing [36].

Many promoters of tumor-suppressor genes are hypermethylated in pancreatic cancer [37,38]. One typical example is the classic tumor-suppressor gene *CDKN2A*, which encodes for cell cycle regulator p16 and undergoes methylation-induced silencing in almost all those pancreatic cancers (around 15–20% of cases) that do not have bi-allelic genetic inactivation of *CDKN2A* [39].

An increasing amount of miRNAs has also been associated with pancreatic cancer [40]. For example, miR-21 is upregulated in pancreatic cancer [41,42]. Overexpression of miR-21 precursor in pancreatic cancer cells resulted in increased proliferation, invasion and chemoresistance compared with control cells [41]. MiR-21 targets many apoptosis related genes such as *PTEN* and *PDCD4*, leading to inhibition of apoptosis and consequently increased tumorigenicity [43]. Furthermore, miR-21 positively correlated with the mRNA expression of invasion-related genes, matrix metalloproteinase-2 and −9, and vascular endothelial growth factor [41].

These observations emphasize the importance of epigenetics in pancreatic carcinogenesis. Moreover, they led to the investigation of histone deacetylase inhibitors (HDACIs), histone methyltransferase inhibitors (HMTIs), DNA demethylating strategies and miRNA expression modulators as therapeutic options in pancreatic cancer (Table 1). Although other epigenetic proteins, such as HATs, are also implicated in cancer, there are currently no studies investigating drugs targeting these proteins in the context of pancreatic cancer.

Histone deacetylase inhibitors

HDACs can be divided into four classes. The class I, II and IV HDACs rely on zinc-dependent catalysis for their activity. Class III HDACs are called SIRT 1 to 7 and use NAD^+ as a co-factor for deacetylation activity [24,44,45]. Class I HDACs 1, 2, 3 and class II HDAC 7 have been reported to be overexpressed in pancreatic cancer [24,46–50]. Four different types of HDACIs are used in pancreatic cancer studies: hydroxamic acids, short-chain fatty acids, cyclic peptides and benzamides (Table 1) [45].

Hydroxamic acids

Hydroxamic acids are metal chelators, which can scavenge the zinc ions necessary for HDAC activity. Furthermore, they can bind to the active site of HDACs [51]. The hydroxamic acid HDACIs most studied in pancreatic cancer are trichostatin A, vorinostat, panobinostat and belinostat (Table 1) [52].

Trichostatin A (TSA) is a natural HDACI that inhibits class I and class II HDACs [53]. Its effect has been studied *in vitro* in many pancreatic cancer cell lines and also *in vivo* using xenografts in nude mice [54–57]. Although the results of these studies indicate that TSA is a very potent anti-tumor agent, it has not been tested in clinical trials [58].

Vorinostat is a synthetic compound, which like TSA, is a class I/II HDACI [59]. Vorinostat is approved by the U.S. Food and Drug Administration for treatment of relapsed or refractory cutaneous T cell lymphoma (CTCL) [60]. In 2007, Arnold et al. were the first to describe the effect of vorinostat in pancreatic cancer cell lines. They showed that vorinostat induced G_1 cell cycle arrest via upregulation of p21 in BxPC-3 cells and COLO-357 cells, but not in gemcitabine resistant PANC-1 cells. Furthermore, vorinostat had an additive effect on growth inhibition with gemcitabine in BxPC-3 and COLO-357 cells, and sensitized PANC-1 cells to gemcitabine [61]. In contrast, Kumagai et al. (2007) showed that vorinostat treatment did result in growth inhibition of PANC-1 cells and induced upregulation of p21 in these cells and that vorinostat induced G_2/M instead of G_1 cell cycle arrest [62]. More recent studies investigated the effect of vorinostat in combination therapy. *In vitro* it is found that the combination of gemcitabine, the proteasome inhibitor bortezomib and vorinostat resulted in the greatest inhibitory effect on cell growth. Experiments *in vivo* in nude mice, however, showed no significant benefit of the triple-combination over gemcitabine with bortezomib [63]. Another study by Millward et al. (2012), currently in a phase I clinical trial, reported marked synergy of the proteasome inhibitor marizomib and vorinostat *in vitro* in tumor cell lines derived from patients with non-small cell lung cancer, melanoma and pancreatic carcinoma. However, in the clinical trial so far, they detected no tumor response [64]. Although little encouraging results were found in pre-clinical and clinical trials, the anti-cancer effect of vorinostat observed *in vitro* and in other cancers triggers researchers to set up new clinical trials with vorinostat in pancreatic cancer. In an ongoing phase I/II clinical trial, the combination of vorinostat with radiation therapy and 5FU is tested in patients with locally advanced pancreatic cancer [65]. Furthermore, a phase I trial is recruiting participants to evaluate the combination of vorinostat, capecitabine, a prodrug of 5FU, and radiation therapy in patients with non-metastatic pancreatic cancer [66].

Panobinostat inhibits all zinc dependent HDAC classes and is therefore called a pan-HDAC inhibitor [67]. It was tested in the context of pancreatic cancer for the first time in 2008 by Haefner et al. They showed that panobinostat induced G_2/M cell cycle arrest, upregulation of p21 and apoptosis *in vitro*. *In vivo*, it significantly reduced tumor mass in nude mice and potentiated the efficacy of gemcitabine, but apoptosis was only slightly increased and there was no significant reduction of cell proliferation [68]. Panobinostat was recently tested in a phase II clinical trial in combination with bortezomib in patients with pancreatic cancer progressing on gemcitabine therapy. Unfortunately, the study was terminated after inclusion of seven patients due to a complete lack of treatment responses and early grade 4 thrombocytopenia and diarrhea [69]. However, a recent study with panobinostat in combination with PI3K and mTOR inhibitor BEZ235 showed growth inhibition both *in vitro* and *in vivo* using xenografts in nude mice [70].

Belinostat is a relatively new pan-HDAC inhibitor [67]. In 2010, it was tested in a phase I clinical trial in combination with carboplatin, and/or paclitaxel in patients with solid tumors, including three cases of pancreatic cancer. Interestingly, one of the pancreatic cancer patients had a partial response to belinostat with carboplatin [71]. Like panobinostat, belinostat has also been tested in a pre-clinical study in combination with bortezomib. The results showed synergistic antiproliferative and pro-apoptotic effects of the two drugs in pancreatic cancer cell lines [72]. More recently, two studies showed that belinostat induces growth inhibition, *in vitro* and *in vivo* in immunodeficient mice, alone or synergistically with gemcitabine [73,74].

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Table 1
Epigenetic targeting strategies in pancreatic cancer.

Group	Drug	Important targets	Clinical trial	Results	Ref.
HDAC inhibitors					
Hydroxamic acids	Trichostatin A	HDAC class I and II			
	Vorinostat	HDAC class I and II	NCT00948688	In combination with marizomib; no tumour response detected.	[64]
			NCT00948688	Ongoing: in combination with 5-FU and radiation therapy.	[65]
			NCT00983268	Ongoing: in combination with capecitabine and radiation therapy.	[66]
	Panobinostat	HDAC pan-inhibitor		In combination with bortezomib. Terminated because of lack of response and early toxicity.	[69]
	Belinostat	HDAC pan-inhibitor			
Short-chain fatty acids	Valproic acid	HDAC class I and II		In combination with epirubicin. Partial response of one pancreatic cancer patient.	[77]
			NCT01333631	Ongoing: in combination with gemcitabine and radiation therapy.	[81]
	Butyrate	HDAC class I and II			
Cyclic peptides	Romidepsin	HDAC class I and II, G9a and SUV39H1	NCT00379639	In combination with gemcitabine. Adverse events but also partial response and stable disease in patients with solid tumour including pancreatic cancer.	[90,91]
Benzamides	Entinostat	HDAC class I		The only patient with pancreatic cancer was removed due to disease progression.	[93]
				In combination with 13-cis retinoic acid. No tumour response, the only patient with pancreatic cancer showed stable disease.	[94]
HMT inhibitors	BRD4770	G9a			
	Romidepsin	HDAC class I and II, G9a and SUV39H1		See romidepsin cyclic peptides.	
	Curcumin/CDF	EZH2/miR-101, miR-200, miR-21, miR-22 and miR-199a⁎		Alone and in combination with gemcitabine. Some tumour responses to curcumin.	[99–101]
			NCT00094445	Ongoing: single agent study.	[102]
DNA methylation	5-aza-2′-deoxycytidine/ 5-azacytidine	Methylated CpG islands	NCT01167816	Ongoing: in combination with gemcitabine.	[108]
			NCT01845805	Ongoing: single agent study.	[109]
	Curcumin/EF31/ UBS109	DNMT1		See curcumin/CDF HMT inhibitors.	
Modulators of miRNA expression	Retinoic acid receptor antagonists	miR-10a			
	Indole-3-carbinol	miR-21			
	Lentiviral vector	miR-21			
	Curcumin/CDF	EZH2/miR-101, miR-200, miR-21, miR-22 and miR-199a⁎		See curcumin/CDF HMT inhibitors.	
	Garcinol	miR-21			
	3,3′-diindolylmethane (DIM) and isoflavone	miR-200, miR-146a and let-7			
	5-aza-2′-deoxycytidine/ Vorinostat	miR-34a		See vorinostat hydrozamic acids and 5-aza-2′-deoxycytidine / 5-azacytidine DNA methylation.	
	Trichostatin A	miR-200c and miR-21			
	Metformin	miR-101, miR-200, let-7 and miR-26a	NCT01210911	Ongoing: in combination with erlotinib and gemcitabine.	[129]
			NCT01167738	Ongoing: in combination with capecitabine, cisplatin, epirubicin and gemcitabine.	[130]
			NCT01666730	Ongoing: in combination with oxaliplatin, leucovorin calcium, fluorouracil.	[131]
			NCT01488552	Ongoing: in combination with gemcitabine, nab-paclitaxel, FOLFORINOX.	[132]
	Glargine	miR-95			
	Nanovectors	miR-34a, miR143/145 and let-7			

Short-chain fatty acids

Short-chain fatty acids (SCFAs) are less potent HDACIs than hydroxamic acids, presumably because they are not able to access the zinc ion in the HDAC active-site pocket [75]. In pancreatic cancer, the best studied and most promising SCFAs are valproic acid and butyrate.

Valproic acid (VPA) is a class I/IIa HDACI, which was first discovered as an antiepileptic drug. Later, the HDAC inhibitory effects of VPA became apparent [76]. In a phase I clinical trial of VPA and the anthracycline drug epirubicin in solid tumors, one pancreatic cancer patient showed partial response to this drug combination [77]. *In vitro*, VPA has shown to potently down-regulate the proliferation and adhesion capacity of pancreatic cancer cells [78]. In 2011, two separate studies by Iwahashi et al. in pancreatic cancer cell lines showed that VPA alone did not induce significant growth inhibition, but potentiated growth inhibition by 5FU and the combination of gemcitabine and

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pegylated interferon-α2b [79,80]. Recently, a phase II study has been set up to evaluate the toxicity and efficacy of VPA in combination with gemcitabine and radiotherapy [81].

Butyrate is a class I/II HDACI [82]. It has been shown to induce apoptosis and inhibits invasion in pancreatic cancer cell lines and is suggested to potentiate chemotherapy [83,84]. However, it is also reported to have poor pharmacological properties such as a short half-life and first-pass hepatic clearance [85]. Prodrugs of butyrate with better pharmacological properties could provide an alternative therapeutic option as is demonstrated by the growth inhibition of pancreatic cancer cells by the butyrate prodrug tributyrin [86]. (Pre-)clinical trials are needed to assess the therapeutic value of butyrate derivatives.

Cyclic peptides

Romidepsin is a pentapeptide that can interact with the zinc ion in the active site of the HDACs and it is classified as a class I/II HDACI. In 2009, romidepsin was approved by the U.S. FDA for treatment of patients with CTCL [87]. Romidepsin induces G_1 or G_2/M cell cycle arrest and subsequent apoptosis in refractory human pancreatic cancer cells [88]. Furthermore, romidepsin also induces growth inhibition *in vivo* in a human pancreatic cancer xenograft [89]. Recently, a phase I study by Jones et al. (2012) tested romidepsin in combination with gemcitabine in solid tumors, including pancreatic cancer. Although additive hematological toxicity of the combination was observed, the disease stabilization in 14 patients and partial response in two patients warrant further research [90,91].

Benzamides

Entinostat is a synthetic benzamide derivative that inhibits class I HDACs [67]. Its anti-tumor activities were first studied in 1999 by Saito et al. They reported strong anti-tumor efficacy against human cancers in nude mice [92]. In 2008, a phase I study of entinostat included one metastatic pancreatic cancer patient, who had disease progression [93]. A recent phase I study of entinostat in combination with 13-*cis* retinoic acid in solid tumors, again included one pancreatic cancer patient who reached prolonged stable disease [94].

Inhibiting histone methyltransferases

The use of HMTIs in pancreatic cancer is rare. Recently, Yuan et al. (2012) described the discovery of a novel HMTI called BRD4770. This HMTI inhibits HMT G9a, which mediates H3K9 methylation. They showed that BRD4770 induced cellular senescence in PANC-1 cells [95]. Furthermore, they found that gossypol, a natural product from cottonseeds and a putative BH3 mimetic, acts in a synergistic manner with BRD770, inducing autophagy-related cell death in PANC-1 cells [96]. The therapeutic value of these new drugs *in vivo* is unknown.

With hindsight, HMT inhibition was observed for agents targeting other cellular processes. For example, Wu et al. (2008) have shown that the HDACI romidepsin suppresses expression of HMTs G9a and SUV39H1 [97]. Bao et al. (2012) found that difluorinated-curcumin (CDF), an analogue of curcumin, increases expression of a panel of tumor-suppressor miRNAs, including miR-101, *in vitro*. Re-expression of miR-101 mediated by CDF reduced HMT EZH2 levels and inhibited cell growth. Furthermore, reduced EZH2 levels and tumor growth inhibition was also observed *in vivo* in an orthotopic xenograft model of human pancreatic cancer [98]. As curcumin as well as CDF inhibit NF-κB, a transcription factor that is often constitutively active in pancreatic cancer, it should be taken into account that the anti-tumor effects observed of curcumin or CDF might not be through EZH2 suppression. Phase II trials of curcumin alone and in combination with gemcitabine showed that curcumin was well tolerated and might have some biological activity in patients with pancreatic cancer [99–101]. Furthermore, there is another ongoing phase II clinical trial of curcumin in patients with advanced pancreatic adenocarcinoma [102].

DNA demethylation

As mentioned before, it has been well established that many genes are hypermethylated in pancreatic cancer [37,38]. DNA demethylating agents such as the cytidine analog 5-azacytidine and other DNMT1 inhibitors have been used to reduce methylation in pancreatic cancer.

Intracellularly, 5-azacytidine is converted to the activated 5-aza-deoxycytidine-triphosphate, and subsequently incorporated into DNA. This results in an irreversible covalent complex with DNMT1, triggers proteasome-mediated DNMT1 degradation, and leads to reduced DNA methylation levels [103]. In 2005, Missiaglia et al. have shown that 5-aza-deoxycytidine (5-aza-dC) treatment results in global DNA demethylation, growth inhibition and apoptosis of pancreatic cancer cell lines. Furthermore, they showed that pretreatment with 5-aza-dC increased the sensitivity of pancreatic cancer cells to other chemotherapy agents, including gemcitabine [104]. However, in 2003, Sato et al. showed that 5-aza-dC treatment induced aberrant expression of matrix metalloproteinases (MMPs) in pancreatic cancer cell lines, promoting tumor invasion and metastasis [105]. In 2009, Kumari et al. showed that treatment of pancreatic cancer cell lines with 5-azacytidine resulted in the reduction of telomerase activity. Telomerase activity, exerted by TERT, is a key component of cellular immortality and in this way may play a role in tumorigenesis. The mechanism behind the 5-azacytidine induced loss of telomerase activity is far from clear. Although, counterintuitive it may be that demethylation of the CpG island at the TERT promoter directly leads to reduced expression [106].

Recently, Shakya et al. (2013) tested 5-aza-dC in an aggressive stroma-rich mouse model of pancreatic carcinoma. They showed that 5-aza-dC was able to slow disease progression and even transient tumor growth inhibition [107]. Currently, a phase I trial is recruiting patients to determine the maximum tolerated dose of 5-azacytidine and gemcitabine in subjects with previously untreated and unresectable pancreatic cancer [108]. Furthermore, a phase II trial is set up to assess the effect of 5-azacytidine on progression free survival in patients with resected pancreatic cancer [109].

Another recent study by Nagaraju et al. (2013) investigated the effect of curcumin and two curcumin analogues EF31 and UBS109 on DNMT1 expression in pancreatic cancer. Treatment of pancreatic cancer cell lines with curcumin, EF31 and UBS109 resulted in growth inhibition, downregulation of DNMT1, decreased DNA methylation and re-expression of silenced genes such as *CDKN2A*. Furthermore, they show that EF31 and UBS109 reduce DNMT1 expression and DNA methylation, and re-express p16. In addition, these agents increased the sensitivity of pancreatic cancer cells to the combination of 5FU and oxaliplatin. In all these experiments, UBS109 proved to be the most potent drug [110].

MicroRNA expression modulators

In pancreatic cancer, both overexpression and downregulation of miRNAs have been associated with poor overall survival and prognosis [111]. Many studies have evaluated the effect of inhibiting overexpressed miRNAs and re-expressing silenced miRNAs [40] and have thereby attempted to identify new therapeutic applications. The miRNAs involved in pancreatic cancer have been

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summarized extensively by others [111–114]. Here, we focus on modulators of miRNA expression tested in the context of pancreatic cancer.

In 2009, Weiss et al. showed that miR-10a is overexpressed in metastatic pancreatic cancer. Furthermore, they show that miR-10a is a retinoid acid target and that retinoic acid receptor antagonists effectively repress miR-10a expression and completely block metastasis in zebrafish xenotransplants [115]. Currently, no clinical trials are investigating these agents in pancreatic cancer [58].

Also miR-21 is upregulated in pancreatic cancer. It is associated with apoptosis inhibition resulting in chemoresistance [40]. *In vitro* studies have shown that miR-21 inhibition sensitizes cells to gemcitabine and 5FU. Recently, Paik et al. (2013) have shown that indole-3-carbinol (I3C) suppressed miR-21 expression and sensitized PANC-1 cells to gemcitabine [116]. Furthermore, Sicard et al. (2013) reported miR-21 downregulation using lentiviral vectors *in vitro* and *in vivo*. They show that targeting miR-21 strongly inhibits pancreatic cancer tumor growth [117].

In 2008 already, Sun et al. demonstrated that curcumin upregulated miR-22 and downregulated miR-199a* in pancreatic cancer cell lines. Furthermore, upregulation of miR-22 suppressed the ESR1 and SP1 protein expression, suggesting a new mechanism by which curcumin mediates its effects on cell growth and apoptosis [118]. As mentioned before, curcumin analogue CDF is able to re-express miR-101 leading to tumor growth inhibition *in vivo* in immunodeficient mice via EZH2 downregulation [98]. Furthermore, CDF has been shown to induce re-expression of miR-200, which is also downregulated in pancreatic cancer [40]. Re-expression of miR-200 has been shown to result in upregulation of apoptosis regulator *PTEN*, and downregulation of matrix metalloproteinase-1, which is often activated and expressed in tumor cells with significant invasive properties, and is associated with poor prognosis of patients [119]. In addition, CDF was found to downregulate miR-21 expression and sensitize cells to gemcitabine [120,121].

Garcinol, a natural agent associated with anti-cancer activity, has been shown to alter the expression of several miRNAs, including downregulation of miR-21. It can synergize with gemcitabine to inhibit cell proliferation and induce apoptosis in pancreatic cancer cells [122]. Furthermore, garcinol has been shown to synergize with curcumin to inhibit growth and induce apoptosis in pancreatic cancer cells [123]. Other natural agents like 3,3′-diindolylmethane (DIM) and isoflavone have been shown to upregulate the expression of miR-200 and let-7 in gemcitabine resistant cells, thereby reducing the EMT characteristics of these cells [124]. Furthermore, pancreatic cancer cells treated with DIM or isoflavone showed increased miR-146a expression and caused a downregulation of epidermal growth factor receptor, metastasis-associated protein 2, interleukin-1 receptor associated kinase 1, and NF-κB with subsequent inhibition of pancreatic cancer cell invasion [125].

In 2011, Nalls et al. demonstrated that treatment of pancreatic cancer stem cells with 5-Aza-dC or HDACI vorinostat inhibits cell proliferation, induces apoptosis, and reduces invasive potential. They demonstrate that this is due to restored expression of miR-34a, which targets among others the Notch signaling component RBPJ and EMT inducer ZEB1 [126]. Furthermore, HDACI TSA inhibits cell proliferation in pancreatic cancer cells through upregulation of miR-200c and downregulation of miR-21, which results in cell cycle arrest and increased apoptosis [127]. Recently, Bao et al. (2012) showed that metformin, a diabetes mellitus drug associated with a reduced risk of pancreatic cancer, caused re-expression of miR-101, miR-200, let-7 and miR-26a. Treatment of pancreatic cancer cell lines with metformin significantly decreased cell survival, clonogenicity, sphere-forming capacity, and increased disintegration of spheres in both gemcitabine-sensitive and gemcitabine-resistant pancreatic cancer. Furthermore, it has been shown to inhibit tumor growth in PANC-1 xenografts [128]. Currently, four phase II clinical trials are recruiting patients to investigate the effect of metformin in combination with other drugs such as gemcitabine and capecitabine [129–132]. At the same time, Li et al. (2012) demonstrated that high doses of glargine, an insulin analog used in diabetes mellitus, altered miRNA expression. Most obvious was the raise of miR-95, which can cause increased pancreatic cancer cell proliferation, invasion and migration and inhibits its apoptosis [133].

Upcoming strategies to directly modify miRNA levels are replacement and inhibition therapies. Inhibition of overexpressed miRNAs can be done with antisense oligonucleotides, as studied by Park et al. (2009). They showed that antisense inhibition of miR-21 and miR-221 resulted in cell cycle arrest and/or induction of apoptosis *in vitro*, and sensitizes pancreatic cancer cells to the effects of gemcitabine [134]. Lentiviral vectors and non-viral lipid based nanoparticles can be used for restitution of miRNAs. Pramanik et al. (2011) demonstrated that systemic intravenous delivery with miR-34a or miR-143/145 nanovectors inhibited growth of subcutaneous MiaPaCa-2 xenografts [135]. Restoring let-7 levels in pancreatic cancer cells through plasmid-based synthetic miRNAs or lentiviral induction inhibited cell proliferation, *KRAS* expression, and mitogen-activated protein kinase activation. Unfortunately, inhibition of tumor growth after intratumoral gene transfer failed [136].

Conclusion

This review shows that a rapidly increasing number of studies is focused at drugs that target epigenetic mechanisms. Although promising results have been obtained in many *in vitro* and *in vivo* studies, clinical trials with pancreatic cancer patients predominantly show disappointing outcomes [63,64,69,93,94]. This might be attributed to the lack of specificity of epigenetic drugs.

The targets and effects of epigenetic drugs are not fully elucidated yet. These drugs might have numerous unknown adverse effects. Although epigenetic drugs are regarded as targeted therapies, they often affect many different pathways and tissues by exerting their effect throughout the whole genome in both tumor and normal cells. In addition, it is complicated to determine whether reported responses to epigenetic drugs are strictly mediated via epigenetic mechanisms [137]. For example, HDACIs are not only involved in deacetylation of histone tails. A large number of other non-histone proteins is also acetylated and can thus be affected by HDACIs [23]. 5-aza-dC exerts its demethylating activity throughout the whole genome. Thus, this drug leads to global hypomethylation, re-expression of hypermethylated tumor-suppressor genes, but possibly also activation of oncogenes [105]. Furthermore, new miRNAs and new targets of known miRNAs are continuously discovered. Moreover, miRNA targeting not only affects pathways involved in carcinogenesis but also normal pathways leading to unwanted side effects.

The currently unknown effects of drugs targeting epigenetics might play a role in the adverse effects and poor performance in clinical trials. Careful review of research in other solid tumors and, further research is needed to establish all targets and downstream effects of epigenetic targeting. This will provide a basis for understanding the cause of adverse effects, tumor specificity and drug resistance, and can ultimately lead to optimal drug regimens in pancreatic cancer, patient selection and safety.

Conflict of interest statement

The authors report no sources of funding and declare no conflicts of interest.

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