

Current evidence for histone deacetylase inhibitors in pancreatic cancer

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Abstract

Pancreatic cancer is one of the most aggressive human cancers, with more than 200 000 deaths worldwide every year. Despite recent efforts, conventional treatment approaches, such as surgery and classic chemotherapy, have only slightly improved patient outcomes. More effective and well-tolerated therapies are required to reverse the current poor prognosis of this type of neoplasm. Among new agents, histone deacetylase inhibitors (HDACIs) are now being tested. HDACIs have multiple biological effects related to acetylation of histones and many non-histone proteins that are involved in regulation of gene expression, apoptosis, cell cycle progression and angiogenesis. HDACIs induce cell cycle arrest and can activate the extrinsic and intrinsic pathways of apoptosis in different cancer cell lines. In the present review, the main mechanisms by which HDACIs act in pancreatic cancer cells *in vitro*, as well as their antiproliferative effects in animal models are presented. HDACIs constitute a promising treatment for pancreatic cancer with encouraging anti-tumor ef-

fects, at well-tolerated doses.

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Key words: Pancreatic cancer; Histone deacetylases; Histone deacetylase inhibitors; Experimental studies

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INTRODUCTION

Pancreatic cancer is one of the most lethal human cancers and continues to be a major unsolved health problem at the beginning of the 21st century. Worldwide, over 200 000 people die annually of pancreatic cancer, with the highest incidence and mortality rates found in developed countries. Pancreatic cancer is the 4th and 6th leading cause of cancer death in United States and Europe, respectively. Pancreatic cancer incidence and mortality rates are almost equal because of the high fatality rate. A lack of illness indicators and screening tests mean that pancreatic cancer is usually diagnosed at the late stages of the natural history of the disease^[1,2].

In Western communities, the 1- and 5-year survival rates for pancreatic cancer are less than 25% and 5%, respectively, and the mortality rates are essentially identical. Although survival rates are highest (16.6%) when the tumor is localized at diagnosis, less than 10% of tumors are detected at that time. On the other hand, the survival rates have been only slightly improved over the past decade because of a lack of significant medical advances in early detection and the poor outcome of treatment approaches. Pancreatic cancer is rare in the first three decades of life. The majority of pancreatic cancers occur

in the exocrine pancreas and the vast majority (> 90%) have ductal differentiation. Men consistently have higher incidence and mortality rates than women, worldwide^[3].

Treatment of advanced pancreatic cancer

Traditionally, 5-fluorouracil (5-FU)-based chemotherapy and/or radiotherapy have been used in the treatment of locally advanced pancreatic cancer; however, the value of radiotherapy remains unclear^[4]. Today, *gemcitabine*-based therapy is the acceptable treatment approach for both unresectable locally advanced and metastatic pancreatic cancer. Several phase III trials were undertaken with gemcitabine in combination with a range of chemotherapy agents. However, combining gemcitabine with 5-FU^[5], as well as *irinotecan*, *oxaliplatin*, *pemetrexed*, *exatecan* and *cisplatin*^[6-10], all failed to show superiority over gemcitabine monotherapy. In a recent phase III trial, the combination of *capecitabine* with gemcitabine significantly improved the objective response rate and progression-free survival, but did not show superiority in overall survival in patients with advanced pancreatic cancer^[11]. Additionally, in a randomized phase II trial of *folfirinox* (5-FU/leucovorin, irinotecan, and oxaliplatin) versus gemcitabine, the median overall survival was 11.1 mo in the folfirinox group compared with 6.8 mo in the gemcitabine group, indicating that folfirinox is an option for the treatment of patients with metastatic pancreatic cancer^[12].

Targeted therapies have also been investigated for advanced pancreatic cancer. Erlotinib is a small-molecule tyrosine kinase inhibitor of the human epidermal growth factor receptor (EGFR). A multicenter, randomized, double-blind, placebo-controlled phase III clinical trial of erlotinib in combination with gemcitabine, in patients with locally advanced or metastatic pancreatic adenocarcinoma met its primary endpoint, with the combination regimen being the first gemcitabine combination to demonstrate a statistically significant survival advantage over gemcitabine monotherapy and the regimen was consequently approved for metastatic disease^[13].

Many molecular-targeted agents that interact with crucial pathways for cell survival in pancreatic cancer are currently being explored. These include agents that target *poly ADP-ribose polymerase*, histone deacetylase (*HDAC*), *Src/Abl kinases*, and mammalian targets of rapamycin^[14].

Histone acetyltransferases and deacetylases

The principal structure of eukaryotic chromatin is the nucleosome. Each nucleosome consists of approximately 146 bp of DNA wrapped around a core of eight basic proteins called histones, two each of H2A, H2B, H3 and H4. Nucleosomal structure not only facilitates packing DNA into a relatively small nucleus, but also exerts important regulatory functions. The N- and C- terminal tails of the nucleosomal core undergo post-translational modifications, participating in chromatin assembly regulation and/or DNA accessibility. Nucleosomes containing highly charged hypoacetylated histones bind tightly to the phosphate backbone of DNA, inhibiting transcrip-

tion, preventing transcription factors, regulatory complexes and RNA polymerase to access the DNA. Acetylation neutralizes the charge of the histones generating a more open DNA conformation. Transcription factors may then access the DNA, promoting the expression of the corresponding genes. Therefore, histone acetylation is generally associated with transcriptional activation. Histone acetylation is carried out by a group of proteins called histone acetyl transferases (HATs), and the acetyl groups can be removed by HDACs. These molecules play a pivotal role in cellular functions, such as chromosome remodelling, gene transcription and cell proliferation^[15,16].

Eighteen different human HDAC isoforms have been described and are classified into four classes. Class I HDACs (HDACs 1, 2, 3 and 8) are associated with RPD3 deacetylase, and are primarily located in the nucleus. Class II HDACs are divided into two subclasses, class II a (HDACs 4, 5, 7 and 9) and class II b (HDACs 6 and 10) and are homologous to the yeast Hda1 deacetylase. Class III HDACs consists of seven HDACs (SIRT1 to SIRT7) that share homologies with the yeast silent information regulator 2 (Sir2) family. The class IV family of HDACs has only one member, HDAC11. Classes I, II and IV require Zn²⁺ for activity, while class III has a unique catalytic mechanism that requires the co-factor NAD⁺. To achieve the acetylation of histones, an acetyl group of acetyl coenzyme A is linked to the ε-amino group of lysine by HATs, which can be removed by HDACs. When HDACs remove the acetyl group from a histone lysine, a positive charge on the lysine residue condensing the structure of nucleosomes is restored. The active site of HDACs consists of a cylindrical pocket in which the lysine residue fits when deacetylation takes place. Zn²⁺ is located near the bottom of the cylindrical pocket^[17].

In addition to deacetylating histones, HDACs have also been reported to interact with non-histone proteins. Such protein targets of HDACs include transcription factors and regulators, signal transduction mediators, DNA repair enzymes, nuclear import regulators, chaperone proteins, structural proteins, inflammation mediators and viral proteins. These HDAC substrates are involved in numerous important cell pathways, including control of gene expression, regulation of cell proliferation, differentiation, migration and death. Altered expression of HDACs has been reported in several types of human neoplasms^[18,19].

HDAC inhibitors

HDAC inhibitors (HDACIs) have three common structural characteristics: a Zn binding moiety, an opposite capping group, and a straight chain alkyl, vinyl or aryl linker connecting the two. The majority of HDACIs are designed to interfere with the catalytic domain of HDACs, blocking substrate recognition and inducing gene expression. Aberrant expression of different HDAC isoforms has been associated with different malignancies; thus, HDACIs represent a potent and specific strategy for cancer treatment. Most of the described HDACIs only

affect the Zn-dependent classes I and II HDACs. The HDACs described so far vary in structure and origin, being divided into different classes based on their chemical properties. The *hydroxamic acids* include trichostatin A (TSA), SAHA (vorinostat), LBH589 (panobinostat) and PXD101 (belinostat). The *short-chain fatty acids* comprise another class, including sodium butyrate (NaBu), 4-phenylbutyrate and valproic acid. A third class includes the *cyclic tetrapeptides*, such as FK228/depsipeptide (romidepsin). A fourth class of HDACs is the *benzamides*, including MS-275 (entinostat), CI-994 and MGCD0103^[20,21].

The mechanisms of action of HDACs are complex and not completely understood. HDACs have multiple biological effects related to acetylation of histones and many non-histone proteins, such as those involved in regulation of gene expression, apoptosis, cell cycle progression, DNA repair, cell migration and angiogenesis. HDACs induce cell cycle growth arrest in both normal and transformed cells, and can activate the extrinsic and intrinsic pathways of apoptosis. Both *in vitro* and *in vivo* data and ongoing clinical trials have indicated that HDACs could be used against different solid tumors and hematological malignancies; thus, comprising one of the most promising classes of new anticancer agents^[22,23]. In the present review, the latest knowledge on the effect of HDACs on pancreatic cancer is discussed.

EXPERIMENTAL *IN VITRO* STUDIES

The data available so far regarding the different classes of HDACs used in pancreatic cancer cell lines are presented in the following section. Additionally, the targets modulated by different HDACI compounds are listed in Table 1.

Hydroxamic acids

Suberoylanilide hydroxamic acid (SAHA, *N*-hydroxy-*N*²-phenyl-octanediamide, vorinostat) is a synthetic hydroxamic acid that is structurally related to the natural product, trichostatin A {TSA, 7-[4-(dimethylamino)phenyl]-*N*-hydroxyl-4,6-dimethyl-7-oxo-(2*E*,4*E*,6*R*)-2,4-heptadienamide}, which is produced by selected strains of *Streptomyces platensis*, *Streptomyces hygroscopicus* Y-50 or *Streptomyces sioyaensis*. Hydroxamic acids have a high affinity to biometals, including Fe³⁺, Ni²⁺ and Zn²⁺. The synthesis of SAHA and its potency to induce differentiation of murine erythroleukemia (MEL) cells was first reported in 1996. SAHA and TSA contain a hydroxamic acid-based metal-binding domain that coordinates the catalytic Zn²⁺ in the HDAC active site, a 5 (TSA) or 6 (SAHA)-membered carbon-based linker that mimics the C α functional group of lysine, and a hydrophobic motif that interacts with the periphery of the HDAC binding pocket^[24].

TSA: TSA strongly inhibited the cellular growth of nine pancreatic adenocarcinoma cell lines (MiaPaCa-2, PANC1, PSN1, PT45P1, CFPAC1, HPAF-II, T3M4, PaCa44 and PC), although a marked difference in sen-

sitivity to the drug was noted. TSA-induced cell cycle arrest was associated with a block in the G2 phase and apoptotic death. TSA treatment in T3M4 and PaCa44 cell lines resulted in p21WAF1/CIP1 induction, an increase in caspase-3 activity and the downregulation of p27 and cyclin A2 mRNA expression^[25].

Global gene expression profiles were also examined in several pancreatic cancer cell lines (CFPAC1, HPAF, MiaPaCa-2, Panc1, PC, PSN1, PT-45P1 and PaCa44) post-TSA treatment. Three point four percents of genes involved in a wide variety of cellular processes, such as cell proliferation, signaling, regulation of transcription, and apoptosis, were altered after TSA treatment. The cyclin-dependent kinase (cdk) inhibitors p21, p19 and p57 were all upregulated, while cyclin A and cdk10 were downregulated. Additionally, BIM, a proapoptotic BCL-2 family member, was significantly induced, while the expression of the antiapoptotic genes *BCL-XL* and *BCL-W* was repressed by TSA treatment^[26].

Different pancreatic cancer cell lines co-express high-level TNF-related apoptosis-inducing ligand receptor (TRAIL-R), Fas and TNF-R1 but are strongly resistant to apoptosis triggered by the death receptors. The drug combinations geldanamycin/PS-341, TSA/PS-341 and TSA/geldanamycin with low-dose TRAIL were tested and all were found to be effective in initiating apoptosis in four pancreatic cancer cell lines (AsPC-1, BxPC-3, MiaPaCa-2 and Panc-1) compared with single drug-based treatments. This killing effect was enhanced when Bcl-XL was depleted. When Bcl-XL-depleted cells and control counterparts were exposed to TSA/PS-341, TRAIL induced cell death in Bcl-XL knockdown cells. However, under the same experimental conditions fewer control cells were killed, indicating that Bcl-XL depletion significantly increased TSA/PS-341 killing effects on pancreatic cancer cells in the presence of TRAIL^[27].

TSA and SAHA induced apoptosis in pancreatic cancer cell lines IMIM-PC-1, IMIM-PC-2 and RWP-1, independently of their intrinsic resistance to conventional antineoplastic agents. Caspase-3 activity was slightly increased in IMIM-PC-1 and RWP-1 cells, but significantly increased in IMIM-PC-2 cells after TSA treatment. On the other hand, caspase-8 and -9 activities were not altered. In addition, PARP-1 was only partially cleaved after TSA treatment. An inhibitor of the human serine protease Omi/HtrA2, called ucf-101, was able to block the cell death induced by TSA in the three cell lines through a caspase-independent mechanism. In the same experimental setting, Bax protein levels were dramatically increased, but those of Bcl-2 and p21 were not significantly modified^[28].

TSA and SK-7041, a novel hybrid synthetic HDACI, both induced apoptosis and G2-M cell cycle arrest in the pancreatic cancer cell lines Panc-1 and ASPC-1. They caused increased H4 histone acetylation, and also suppressed the expression of the antiapoptotic proteins Mcl-1 and Bcl-XL, but did not affect either Bcl-2 or the proapoptotic Bax and Bak proteins. TSA and SK-7041 also enhanced the expression of p21 and of cyclin D2

Table 1 Histone deacetylase inhibitors and targets modulated in different pancreatic cancer cell lines

HDACI compound		Targets modulated		Ref.
		Upregulated	Downregulated	
Hydroxamic acids	TSA	p21, p19, p57, Bim, Bax, caspase-3, -7, cyclin D2, TGF- β , OC18, MGMT, TFAR19, maspin, CDKN1C, MUC2, MUC5B, miR-107	p27, cyclin A2, -B1, cdk6, cdk10, Bcl-XL, Mcl-1, PARP-1, NF- κ B, K-Ras, MEK1/2, phosph. MEK, ERK1/2, NPM, TCTP	[25-30,37-52]
	SAHA	p21, p27, p57, Bax, RARa, E-cadherin, C/EBPa, caspase-3, -7, HHIP, RELN, DAB1	Bcl-2, cyclin D1, -B1, c-myc, Ptc-1, survivin, EGFR, NF- κ B, RelA/p65	[53-60,62,63,66]
Cyclic peptides	FK228	p21, p16, caspase-3, DR5, GATA4	Survivin	[68-70]
Short chain fatty acids	VPA	NEP/CD10, RELN, DAB1		[62,73]
	4-PB	p21, p16, miR-127, caspase-8, Bid, JNK	Bcl-6, PARP	[78-80]
	NaBu	ALP, K23, NEP/CD10, caspase-3, -7, -9, GrT-IVa, 5-hydroxytryptamine, CEA, DU-PAN-2, CA19-9, IRT, Lcu7, synaptophysin, p21, p27, Bax	Bcl-XL, TGF- α , PKC, b4 and b7 integrin, EGFR, ezrin, cyclin D1, mut-p53, Bcl-2, survivin	[73,82-99]
Benzamides	MS-275	p21, p27, gelsolin, caspase-3	pRb, Bcl-2, cyclin D1	[101,102]

HDACI: Histone deacetylase inhibitor; TSA: Trichostatin A; SAHA: Suberoylanilide hydroxamic acid; VPA: Valproic acid; NF- κ B: Nuclear factor kappa B; TGF: Transforming growth factor; EGFR: Epidermal growth factor receptor; CEA: Carcinoembryonic antigen.

and reduced that of cyclin B1^[29].

TSA and the selective 26S proteasome inhibitor PS-341, synergistically induced apoptosis in eight pancreatic adenocarcinoma cell lines (AsPC-1, BxPC-3, CFPAC-1, Capan-2, Mia PaCa-2, Panc-1, SU86, and SW1990). Combining TSA with PS-341 induced apoptosis by increasing caspase-3 and -7 activities and enhanced PARP cleavage. Their combination also effectively blocked nuclear factor kappa B (NF- κ B) signaling pathway and downregulated the NF- κ B dependent anti-apoptotic factor Bcl-XL. Moreover, they inactivated the Ras-MAP kinase pathway by depleting several key components of MAP kinase cascades, including K-Ras, MEK1/2, phosphorylated MEK and ERK1/2^[30].

TSA strongly inhibited proliferation of pancreatic endocrine carcinoma cell lines (CM, metastatic insulinoma; BON, metastatic carcinoid; and QGP-1, somatostatinoma) by causing cell cycle G2/M arrest and apoptosis. TSA-induced apoptosis of CM cells was shown to be a retarded event with respect to that observed in BON and QGP-1 cells. Such effect was ascribed to modifications in the expression of proteins related to cell proliferation, gene expression, signal transduction, cytoskeleton organization, chromatin organization, as also RNA splicing and protein folding^[31]. Another study examined the effect of TSA or 5-Aza-C, a DNA methyltransferase inhibitor, treatment on the proliferation of the pancreatic endocrine cancer cell lines QGP-1, CM and BON. TSA treatment resulted in cell cycle arrest at G1 (QGP-1) or G2 (BON and CM) phase, whereas 5-Aza-C blocked the cell cycle in G2 phase only in BON cells. The combined treatment did not significantly increase the cytostatic effect obtained with TSA alone, suggesting that the synergistic cell growth inhibition by the two drugs may be not caused by cell cycle arrest^[32].

TSA and gemcitabine synergistically inhibited the proliferation of several human pancreatic adenocarcinoma cell lines (T3M4, PANC1, PC, CFPAC1, YAPC, DANG and Panc-89). In the cell lines tested, TSA enhanced apoptosis, but not the cell cycle arrest induced by

gemcitabine^[33,34]. TSA significantly inhibited the viability of BxPC-3 cells in a time- and dose-dependent manner by inhibition of cell proliferation and induction of apoptosis. Cell cycle analysis showed an increase of cells in the G0/G1 phase post-TSA treatment, indicating cell cycle arrest. Additionally, TSA induced the apoptosis of BxPC-3 cells and led to alterations in the expression levels of miRNAs. Although some variation at the gene transcription level was observed among Panc-1, BxPC-3, SOJ-6 and MiaPaCa-2 cell lines, the amount of HDAC proteins produced seemed to be comparable^[35]. The effects of known inhibitors of class III HDACs, such as Nicotinamide and Sirtinol, on the growth of pancreatic cancer cells, in addition to those of TSA, were also examined. Treatment of pancreatic cells with different drugs concentrations resulted in a dose-dependent inhibition of cell growth, with TSA being the most effective compound. Sirtinol induced G1 arrest in SOJ-6; however, TSA induced G1 arrest in BxPC-3 cells. Treatment of cells with HDACIs resulted in elevated cell numbers in the sub-G1 peak region, suggesting induction of cell DNA degradation by Sirtinol and TSA. Sirtinol and TSA treatment also involved the mitochondrial pathway of apoptosis induction^[36].

Transforming growth factor beta (TGF- β) plays a significant role in the growth inhibition of most normal epithelial, and some cancer, cells. TGF- β mediates its biological effects through cell surface receptors known as type I (RI) and II (RII) receptors. TGF- β resistance caused by loss of receptors expression has been linked to tumor formation and progression. The TGF- β RII promoter contains two consensus Sp1 sites. The Sp gene family consists of four members, whose protein products are referred to as Sp1-Sp4. Sp1, Sp2 and Sp4 are activators of gene transcription, whereas Sp3 can be an activator or a repressor. In this aspect, TSA treatment of MiaPaCa-2 cell line induced accumulation of acetylated histones in chromatin associated with the TGF- β RII gene. MiaPaCa-2 pancreatic cancer cells acquired resistance to growth inhibition by TGF- β associated with

reduced transcription of TGF- β RII. Accumulation of TGF- β RII with highly acetylated histones H3 and H4 was noted in TSA-treated compared to untreated MiaPaCa-2 cells^[37]. Furthermore, TSA activated TGF- β RII promoter activity in a panel of five pancreatic cancer cell lines (BxPC-3, PANC-1, CFPAC-1, MiaPaCa-2 and UK Pan-1), by mechanisms involving induction of Sp1 acetylation and changes in a multiprotein complex containing p300, PCAF, Sp1 and NF-Y^[38,39].

Treating the pancreatic carcinoma cell line MIA PaCa-2 with TSA, increased the O(6)-Methylguanine-DNA methyltransferase (MGMT) mRNA and protein levels by 2-3-fold, caused by increased histone acetylation in the endogenous MGMT promoter region, which was also associated with CBP/p300. MGMT is a suicide enzyme that repairs pre-mutagenic, pre-carcinogenic and pre-toxic DNA damage O(6)-methylguanine. MGMT also likely protects against therapy-related tumor formation caused by highly mutagenic drugs. The MGMT expression level provides important information on cancer susceptibility and the success of therapy^[40]. TSA treatment also resulted in a marked (5-fold) induction of 2.3% of genes in AsPC1, 1.9% in Hs766T, 1.1% in MiaPaCa2, and 2.5% in Panc1. A large panel of novel targets for silencing by histone deacetylation were identified, including several known tumor suppressor or cell cycle-regulatory genes, such as *ING1*, *p57KIP2*, *CHE31*, *CHFR* and *GADD45B*. One of the novel findings of this study was that TSA alone induced the expression of four of the 11 genes whose CpG islands were identified as aberrantly methylated in pancreatic cancer^[41]. Among the proteins with altered expression post-TSA treatment in Paca44 and T3M4 pancreatic cancer cell lines, of particular interest are the two downregulated proteins nucleophosmin and translationally controlled tumor protein, which are involved in oncogenesis and tumor reversion, respectively. Additionally, several other proteins were found to be upregulated, including programmed cell death protein 5 (TFAR19), which is involved in the regulation of cell apoptosis, and stathmin (OC18), which promotes microtubule depolymerization during interphase and late mitosis. TSA could inhibit cell proliferation of the pancreatic adenocarcinoma cell line Paca44 by cell growth arrest at the G2 phase and apoptosis^[42,43].

Maspin is a unique member of the serpin family of protease inhibitors with tumor suppressive activity in different cancer types. Interestingly, the maspin gene is located on chromosome 18q21.3 and its promoter region contains the binding sites of several transcription factors that positively regulate its expression, including Ets, AP1, HER and p53. When PANC-1 cells were exposed to 5-Aza-C, maspin mRNA expression was restored in a dose dependent manner. TSA also led to re-expression of maspin^[44]. Five pancreatic cancer cell lines (AsPC1, CFPAC1, Hs766T, MiaPaCa-2, and Panc1) were screened for genes that displayed expression patterns associated with hypomethylation. This analysis identified 1485 transcripts that were likely to be variably expressed in pan-

creatic cancer cell lines. Among the 1485 transcripts identified, 392 were found to be elevated by 3-fold or greater in any of the pancreatic cancer cell lines after combined treatment with 5-Aza-C and TSA. This list included several genes that have been reported to be overexpressed in pancreatic cancer, such as those encoding cysteine-rich protein 1 (CRIP1), decay accelerating factor for complement (CD55), maspin/SERPINB5, S100 calcium-binding protein P (S100P), and tissue-type plasminogen activator (PLAT). Treatment of MiaPaCa-2 cells with 5-Aza-C restored the expression of maspin mRNA, whereas treatment with TSA alone did not, but combined treatment with 5-Aza-C and TSA strongly induced the maspin expression in a synergistic manner^[45,46]. CDKN1C is a potent inhibitor of several G1 cyclin complexes, and is a negative regulator of cell proliferation. Treatment of the pancreatic cancer cell lines AsPC1 and BxPC3, where the CDKN1C gene was silenced, with 5-Aza-C or TSA, or their combination, resulted in restoration of CDKN1C expression, more potently with TSA and the combined treatment^[47].

MUC2, *MUC5AC*, *MUC5B* and *MUC6* mucin genes encode large secreted O-glycoproteins that participate in mucus formation and play an important role as a physiological barrier against various attacks on the underlying epithelia. Among the four 11p15 mucin genes, *MUC2* and *MUC5B* were highly susceptible to DNA methylation and histone modifications, whereas *MUC5AC* was rarely influenced by epigenetic regulation and *MUC6* was not. In this context, pancreatic cell lines CAPAN-1 and PANC-1 were treated with 5-Aza-C or TSA. In PANC-1 cells TSA treatment induced *MUC2* expression, while in CAPAN-1 cells 5-Aza-C treatment induced an increase of *MUC5AC* mRNA and *MUC5B* expression. Histone deacetylation was also involved in *MUC5B* repression, as TSA treatment induced its expression^[48]. Treatment of the human pancreatic cancer cell lines PANC1 (*MUC2*-negative) and BxPC3 (*MUC2*-positive) with both 5-Aza-C and TSA, resulted in a definite increase of the expression level of *MUC2* mRNA^[49]. In pancreatic cancer, *MUC4* overexpression is associated with a bad prognosis and has become an important molecular target. In this aspect, the pancreatic cancer cell lines PANC-1, CAPAN-1 and CAPAN-2 were tested. In *MUC4*-nonexpressing pancreatic PANC-1 cells, treatment with 5-Aza-C and TSA induced *MUC4* expression at the mRNA level, and at protein level in a small number of cells. In *MUC4*-high expressing pancreatic CAPAN-1, 5-Aza-C treatment did not affect *MUC4* mRNA, whereas TSA treatment induced a strong inhibition of *MUC4* mRNA levels, correlated to a strong decrease of the apomucin level in the cells^[50].

Two pancreatic cancer cell lines, MiaPACA-2 and PANC-1, were treated with 5-Aza-C or TSA, and their combination. Fourteen miRNAs were upregulated by ≥ 2 -fold in each of the cell lines following exposure to both agents. Enforced expression of miR-107 in MiaPACA-2 and PANC-1 cells downregulated *in vitro* growth that was associated with repression of the putative miR-107 target,

adk6, thereby providing a functional basis for the epigenetic inactivation of this miRNA in pancreatic cancer^[51].

Evaluating the *in vitro* growth inhibition of several pancreatic cancer cell lines established from primary tumors, as well as that of others established from metastatic tumors, to gemcitabine and 5-FU, newer generation cytotoxic agents (oxaliplatin, irinotecan), targeted therapy (gefitinib) and TSA, demonstrated that the combination of TSA and irinotecan increased growth inhibition on the highest percentage of cell lines (80%). TSA proved to be the best partner for all drugs, with the exception of 5-FU. Notably, PSN1, the most sensitive cell line to single-drug treatments, became the most resistant cell line to all combined treatments. In addition, all pairwise combinations were less effective in PaCa3, which contains a functional p53 gene, supporting the hypothesis that the p53 gene status may be not relevant for cell sensitization by TSA^[52].

SAHA: Both SAHA and its novel compounds 17a and 9, inhibited PANC-1 and PT-45 cells' proliferation in a dose dependent manner, with the novel compounds having a more potent antiproliferative activity. Although p21 gene expression of PANC-1 cells was significantly increased after treatment with SAHA and 17a, none of the HDACIs tested affected the expression of the p27 gene^[53].

In another study, SAHA inhibited the growth of BxPC-3 and COLO-357 cell lines, by 42% and 50%, respectively, but not that of PANC-1 cells. SAHA induced a G1 cell cycle arrest and upregulation of p21 in BxPC-3 and COLO-357. On the other hand, PANC-1 cells remained unaffected. According to this study, p21 upregulation was necessary for SAHA-induced cell cycle arrest in COLO-357, but not in BxPC-3 cells. PANC-1 cells, which were resistant to gemcitabine alone, exhibited a marked increase in sensitivity when treated with both gemcitabine and SAHA^[54].

Additive and time-dependent reduction of cell proliferation and induction of apoptosis by SAHA and the novel DNA methyltransferase inhibitor Zebularine was reported in pancreatic cancer cell lines YAP C, DAN G and Panc-89. In fact, the apoptosis induction was associated with downregulation of Bcl-2 and upregulation of Bax^[55]. SAHA inhibited the cell growth in six pancreatic cancer cell lines in a dose-dependent manner. G2/M cell cycle arrest was also induced by SAHA in most cell lines. Remarkably, SAHA and 5-Aza-C treated cells, presented higher levels of acetylated-H3 than those noted in cells treated with SAHA alone. Treatment with SAHA markedly enhanced histone H3 acetylation in the promoter region of the p21 gene. In PANC-1 cells, p21 protein expression increased to the same levels after exposure to either 5-Aza-C, SAHA or both, while p27, Bax and Bcl-2 levels remained unaltered. Levels of p57, E-cadherin and RAR α increased in the presence of SAHA, either alone or with 5-Aza-C. Additionally, SAHA decreased expression of cyclin D1, B1 and c-myc independently of the β -catenin pathway and increased C/EBP α ^[56].

Treatment of PANC1, MiaPaca2 and ASPC-1 cells

with concentrations of SAHA and sorafenib that are sustainable in patient serum resulted in a greater than additive increase in tumor cell killing, as assessed by short-term death assays, and a synergistic increase in killing assessed by colony formation assays. Suppression of caspase 8 function, as well as expression of dominant negative caspase 9 or Bcl-XL also blunted sorafenib-SAHA lethality. Sorafenib-SAHA, but not treatment with the individual drugs, activated CD95 and caused formation of a death-inducing signal complex containing caspase 8, FADD, ATG5 and Grp78/BiP. Additionally, a clinically relevant and sustainable concentration of sodium valproate, another HDACI, enhanced sorafenib lethality in a synergistic fashion in pancreatic tumor cells derived from either humans or rodents. Finally, it was speculated that small-molecule antagonists of Bcl-2 family proteins (HA14-1, GX15-070) enhanced sorafenib-HDACI lethality *via* autophagy and partial activation of the intrinsic apoptosis pathway, independently of death receptor functionality^[57-59].

A combination of SAHA and the Smoothed antagonist, SANT-1, was evaluated for their ability to suppress growth of the gemcitabine-resistant pancreatic adenocarcinoma cell lines Panc-1 and BxPC-3. The combination of SAHA and SANT-1 supra-additively suppressed cellular proliferation and colony formation *via* induction of apoptotic cell death, cell cycle arrest in G0/G1 phase and ductal epithelial differentiation. Cell death was associated with nuclear localization of survivin, increased Bax expression and activation of caspases-3 and -7. Consistent with the cell cycle arrest and cytodifferentiation, the CdkIs p21 and p27 were upregulated and cyclin D1 was downregulated. The potentiated anti-proliferative effect by the combination of SAHA and SANT-1 was attributed to cooperative suppression of the Hedgehog pathway activity, as shown by the upregulation of hedgehog interacting protein by SAHA, and enhanced repression of Ptc-1 mRNA expression^[60].

SAHA induced apoptosis in IMIM-PC-1, IMIM-PC-2 and RWP-1 cell lines with a serine protease-dependent and caspase-independent mechanism. SAHA induced a decrease in the number of cells in S phase in all three cell lines, while an increase in the sub-G1 peak was noted, suggesting that the three cell lines underwent apoptosis^[28].

SAHA and the proteasome inhibitor bortezomib (PS-341) were tested in a panel of pancreatic cancer cell lines. Both SAHA and TSA blocked bortezomib-induced aggresome formation in pancreatic cells, which is a cytoprotective mechanism, and dramatically sensitized aggresome-positive cells to bortezomib-induced apoptosis^[61].

RELN, a key regulator of neuronal migration, is frequently silenced in pancreatic cancers. RELN is a secreted extracellular protein that plays an essential role in brain development and function. Underexpression of RELN and its components (ApoER2, VLDLR and DAB1) is related to cell motility, invasiveness, and colony-forming ability in cancer. Treatment of pancreatic adenocarcinoma cell lines Panc1 and AsPC1 with SAHA and valproic acid, restored

the expression of RELN and DAB1 in a dose-dependent manner, and also inhibited cell migration^[62]. SAHA inhibited proliferation of MiaPaCa-2 and AsPC-1 PDAC cells in a dose-dependent manner and further enhanced the radiation-induced apoptosis (additive but not synergistic). Radiosensitization of these cells was ascribed to inhibition of DNA repair and suppression of radiation-induced EGFR and NF- κ B prosurvival signaling pathways^[63]. In another study, using 3-D agarose colonies of MiaPaCa cells, synergy occurred when SAHA was combined with carboplatin at short exposure times, providing evidence that SAHA may allow a reduction in the standard dose of carboplatin, with improvement in the overall therapeutic index^[64]. Thirty genes were identified as constitutively silenced in PANC-1 cells, and were upregulated post-SAHA and/or 5-Aza-C treatment. Interestingly, among them, 10 genes were known cancer antigens, suggesting that many of these antigens may be silenced by acetylation and/or methylation in PANC-1 cells^[65]. The Rel/NF- κ B family consists of various members of transcription factors, such as RelA/p65, which are responsible for the regulation of cytokines, their receptors and cell adhesion molecules. Overexpression or dysregulation of certain regulatory proteins of the NF- κ B pathway, have been associated with poor prognosis in different cancer types. Treatment of the pancreatic cancer cell line PANC-1 with SAHA resulted in a time dependent reduction of RelA/p65 activity of up to 50%, while valproic acid (VPA) treatment decreased RelA/p65 activity by approximately 25%, affecting also its subcellular localization. Neither SAHA nor VPA affected the protein levels of I κ B α , but inhibited its phosphorylation. According to this study, strong antineoplastic effects of SAHA could be partly based on an alteration of the NF- κ B signaling pathway^[66].

Cyclic peptides

FK228 (FR901228, depsipeptide, romidepsin): Depsipeptide (1S,4S,7Z,10S,16E,21R)-7-ethylidene -4,21-bis (1-methylethyl)-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo [8.7.6] tricos-16-ene-3,6,9,19,22-pentone, is a bicyclic peptide isolated from *Chromobacterium Violaceum* and has demonstrated potent *in vitro* cytotoxic activity against human tumor cell lines and *in vivo* efficacy against human tumor xenografts. Upon entering cells, FK228 is reduced to an active compound, capable of preferentially interacting with the zinc in the active site of the HDAC class I enzymes; however, it is still generally classified as a broad-spectrum inhibitor as it also inhibits class II enzymes. It was approved by the United States Food and Drug Administration for the treatment of cutaneous T-cell lymphoma^[67].

FK228 markedly inhibited the proliferation of five pancreatic cancer cell lines, with the greatest effect on MIAPaCa-2 cells. FK228 treatment induced cell cycle arrest at the G1 or G2/M phase and subsequent apoptosis. The induced hyperacetylation of histone H3 was accompanied by p21 overexpression and caspase-3 activation, leading to cleavage of p21, and by dramatic downregulation of survivin^[68]. Treatment with FK228, bortezomib

or both, sensitized two out of three pancreatic cancer cell lines tested, to NK cells. Tumors sensitized to NK cell cytotoxicity showed a significant increase in surface expression of DR5, which induces the TRAIL pathway of apoptosis. Expressions of MHC class I, MIC-A/B, DR4 and Fas did not alter in different cell lines^[69]. Interestingly, in another study, depsipeptide could induce demethylation of both the p16 and GATA4 promoters in PANC1 cells, among other cancer cell lines, assayed with bisulfite sequencing (region D of the p16 promoter and region B of the GATA4 promoter). Additionally, depsipeptide could induce a significant inhibition of cell proliferation in PANC1 cells. In this study, a novel mechanism of HDAC-mediated DNA demethylation *via* suppression of histone methyltransferases was suggested^[70].

Short chain fatty acids

VPA: VPA is now an established antiepileptic drug through its effect on the function of the neurotransmitter GABA. The finding that VPA was an effective inhibitor of HDACs came from the observations that VPA was able to relieve transcriptional repression of a peroxisomal proliferation and activation of a glucocorticoid receptor (GR)-peroxisome-proliferation-associated receptor (PPAR) ϵ hybrid receptor and a RAR-dependent reporter gene expression system, suggesting that it acts on a common factor in gene regulation, such as corepressor-associated HDACs, rather than on individual transcription factors or receptors. Consistent with this finding, it was shown that VPA causes hyperacetylation of the N-terminal tails of histones H3 and H4 *in vitro* and *in vivo* and was found to inhibit HDAC enzymatic activity at a concentration of 0.5 mmol/L^[71]. VPA has shown potent antitumor effects in a variety of *in vitro* and *in vivo* systems, by modulating multiple pathways, including cell cycle arrest, apoptosis, angiogenesis, metastasis, differentiation and senescence. Most of preclinical and clinical data on the anticancer effects of VPA has been generated for malignant hematological diseases^[72].

Neutral endopeptidase (NEP/CD10) is a cell surface Zn metalloprotease that inactivates multiple physiologically active peptides. Loss of, or decrease in, NEP/CD10 expression has been reported in many types of malignancy. VPA treatment resulted in increase of NEP/CD10 protein expression accompanied with a significantly reduced growth in PATU-8988T and HUP-T4 cells. The VPA effect on the proliferation in HUP-T4, as well as in HUP-T3, cells was much lower than in the high NEP/CD10-expressing PATU-8988T cells^[73]. VPA treatment of DanG cells resulted in a significant reduction of cell proliferation, an effect that was depended on the drug exposure time. VPA evoked a significant blockade of both DanG tumor cell adhesion to HUVECs, particularly when the compound was applied for 5 days. Additionally, VPA treatment modified the integrin surface profile on pancreatic cancer DanG cells^[74]. MiaPaCa2 and Panc1 cell lines were also treated with the topoisomerase II inhibitor etoposide and VPA. VPA-mediated depletion of HDAC2

was observed in MiaPaCa2 and Panc1 cells. The apoptotic fraction of VPA/etoposide co-treated MiaPaCa2 and Panc1 cells was significantly increased compared to etoposide-treated pancreatic cancer cells. Sensitization by VPA to etoposide (DNA-damage induced apoptosis) was noted in both cell lines. This effect was not observed when VPA was used in combination with other drugs, such as gemcitabine, 5-FU or oxaliplatin. Such data suggested that HDAC2 inhibition might result in specific sensitization towards DNA damage-induced apoptosis^[75]. Treatment of Panc1 cells with VPA and SAHA, at different concentrations, induced the mRNA expression of RELN and DAB1 in a dose-dependent manner. VPA was also shown to prevent the epigenetic downregulation of RELN, leading to the inhibition of migration of Panc-1 cells^[62].

4-phenylbutyrate: 4-phenylbutyrate (4-PB) is a short-chain fatty acid that reversibly inhibits class I and II HDACs. It is considered as an HDAC inhibitor of the first generation, as the HDAC inhibitory effect is not specific. Working concentrations are rather high, in the millimolar range, and the effects are pleiotropic. 4-PB exerts multiple effects in the cell, including the modulation of protein isoprenylation, which regulates the ras proto-oncoprotein, and activation of the nuclear steroid PPAR^[76]. 4-PB exerts a potent anti-tumor effect *in vitro* and causes growth inhibition and differentiation in various human cancer cell lines^[77].

A set of normal fibroblast and cancer cell lines, among them the pancreatic cancer CFPAC-1, were treated with 5-Aza-C and 4-PB. Upregulation of the cell cycle inhibitors p16 and p21 was induced post-5-Aza-C and 4-PB treatment. Interestingly, both normal and cancer cells presented very similar induction levels after combination treatment^[78]. Expression profiling of different cancer cell lines revealed upregulation of several miRNAs by simultaneous treatment with 5-Aza-C and 4-PB. One of these, miR-127, is embedded in a CpG island and is highly induced from its own promoter after treatment. miR-127 is usually expressed as part of an miRNA cluster in normal but not in cancer cells, suggesting that it is subject to epigenetic silencing. Additionally, the proto-oncogene *BCL6*, a potential target of miR-127, was translationally downregulated after treatment of several lines with the combination of drugs^[79]. 4-PB inhibited HDAC activity by 60%-70% in the cancer cell lines T3M-4 and BxPc3. Treatment with 4-PB inhibited growth and induced apoptosis of Panc1, T3M-4, COLO 357 and BxPc3 pancreatic adenocarcinoma cell lines in a dose- and time-dependent manner. Concentration-dependent cell cycle arrest was noted in T3M-4 and COLO 357 cells, which was not verified in Panc 1 and BxPc3 cells. In addition, 4-PB increased gap junction communication between T3M-4 cells, allowing exchange of apoptotic signals between neighboring cells. Furthermore, 4-PB inhibited cellular export mechanisms. 4-PB increased gemcitabine-mediated apoptosis of two resistant cell lines T3M-4 and BxPc3. Activation of caspase-8 was enhanced, as well as that of Bid and PARP-cleavage.

No differences in the expression levels of caspase -2 and -3 and IAPs were noted. Finally, no influence of MEK or p38 on gemcitabine-mediated cell death in these cells was found. In contrast, inhibition of JNK completely abolished the sensitizing effect of 4-PB^[80].

Sodium butyrate (NaBu): Sodium butyrate has multiple effects on cultured mammalian cells, including inhibition of proliferation, induction of differentiation and induction or repression of gene expression. NaBu inhibits most HDACs, except class III HDAC and class II HDAC6 and -10. Promoters of butyrate-responsive genes have butyrate response elements, and the action of butyrate is often mediated through Sp1/Sp3 binding sites^[81].

NaBu induced a dramatic decrease in cell proliferation and an increase in ALP activity in PANC-1 cells. NaBu also induced a number of morphologic alterations in these cells, including increase in the cytoplasmic secretory elements and enhancement of differentiation^[82]. Both NaBu and the natural butyrate prodrug tributyrin, inhibited growth and induced apoptosis in MiaPaca-2 and Capan-1 cells and stimulated differentiation in Capan-1 cells, as indicated by alterations of ALP levels^[83]. NaBu also induced differentiation and apoptosis in the human pancreatic cancer cell line AsPC-1, as well as increased K23 mRNA levels^[84]. NaBu treatment resulted in a significant reduced cell growth of PATU-8988T cells and an increase of NEP/CD10 protein levels^[73]. Additionally, NaBu treatment induced cell growth inhibition and apoptosis in four lines (ASPC-1, PANC-1, PT45 and PACA44) with different susceptibility. Bcl-xL expression was strongly down-regulated by NaBu in a time-dependent manner, whereas Bax expression was not affected. NaBu enhanced the intrinsic pathway of apoptosis, including mitochondrial membrane depolarization, cytochrome c translocation to the cytosol, caspase-3 and -9 activation, although it had no effect on caspase-8. NaBu also enhanced the extrinsic pathway of apoptosis, sensitizing pancreatic cancer cell lines to Fas-mediated signals^[85]. Moreover, NaBu inhibited the ability of several pancreatic cell lines to form colonies in soft agar. Cellular ALP levels were markedly increased post-treatment^[86,87]. Treatment of pancreatic cancer cell line CAPAN-1 with 1 mmol/L NaBu reduced the rate of cellular growth and inhibited colony forming ability in soft agar, but did not suppress cell growth. These effects were completely reversible on removal of NaBu and were therefore not causing a terminal differentiation step or a loss of cell viability. Significant changes in proteins and glycoproteins of CAPAN-1 occurred with NaBu treatment^[88]. Treatment with NaBu strongly inhibited growth of pancreatic carcinoid BON cell line. It was found that NaBu increased levels of 5-hydroxytryptamine in the cells, as a differential effect^[89]. TGF- α but not TGF- β mRNA levels were decreased after NaBu treatment in CAPAN-1 cells, while the membrane-bound protein kinase C activity was also reduced^[90].

Oligosaccharide antigens are commonly used as tumor markers. Such antigens control tumor cell adhesion,

motility and invasiveness, being synthesized by a series of glycosyltransferases. In MiaPaCa-2, PSN-1 and PK59 cell lines, the expression of GnT-IVa (N-acetylglucosaminyltransferase-IVa) was increased after NaBu treatment^[91]. Carcinoembryonic antigen (CEA) expression of human pancreatic adenocarcinoma cells and differentiation features were studied and compared in the well differentiated and CEA-producing CAPAN-1 and the poorly differentiated PANC-1 cell line post-NaBu treatment. NaBu reduced colony formation in both cell lines by approximately 50%. Significant ultrastructural alterations were noted only in the PANC-1 cells, including increased intercellular desmosomes, tonofilaments and lipid droplets. NaBu increased CEA expression in CAPAN-1 cells, but had no effect on CEA expression in PANC-1 cells. Thus, CEA expression and state of differentiation were independently affected^[92]. NaBu inhibited the growth of pancreatic cancer cell lines PC-1 and PC-1.0 (hamster) and HPAF, CD11, CD18 and PANC-1 (human), and induced cell enlargement, an increase in secretory material, microfilaments and pseudopodia. NaBu increased the expression of blood group A, DU-PAN-2 and CA 19-9 tumor associated antigens^[93]. Treatment with NaBu, slightly increased immunoreactive trypsin 1 (IRT) levels in both human pancreatic adenocarcinoma cell lines CF-PAC-1 (established from a patient with cystic fibrosis) and CAPAN-1, while growth inhibition was significant. Consequently, IRT levels or differentiation state did not correlate with cellular growth^[94].

NaBu treatment inhibited the cell growth of four pancreatic cancer lines (PT45, PaTu-II, Panc-1 and A818-1) more potently than all-trans retinoic acid (ATRA). Additionally, neuroendocrine markers synaptophysin and Lcu7 in Panc-1 cells were highly expressed^[95]. The expression of b4 and b7 integrin chains correlates with tumor invasiveness. In this aspect, it was documented that NaBu inhibited b4 integrin expression in AsPC-1 cells, inhibiting pancreatic tumor invasion. NaBu also reduced the expression of the b7 integrin chain, which was expressed only in the more aggressive pancreatic cancer cell lines^[96]. The cellular morphological characteristics of the PANC-1 cell line treated with NaBu appeared more differentiated, in a dose-dependent manner. The EGFR expression of NaBu-treated PANC-1 cells was decreased in a dose-dependent manner. Ezrin is a cytosolic molecule that cross-links the plasma membrane to actin filaments and has functions related to cell motility, signal transduction, cell-cell and cell-matrix recognition, invasion and metastasis. Both membranous ezrin expression of PANC-1 cells and mRNA expression of ezrin were decreased^[97].

A novel bioconjugate (HA-But) obtained by the esterification of butyric acid (BA) with hyaluronic acid (HA), the main constituent of the ECM, which selectively recognizes transmembrane receptor CD44, was developed. All HA-But treated cell lines, including the pancreatic cancer cell line MiaPaCa, were responsive to the antiproliferative effect of HA-But in a dose-dependent manner with cell growth inhibition higher than that observed in the pres-

ence of BA alone. Like BA, HA-But induced hyperacetylation of histone H4, a dose-dependent overexpression of some G1/S transition-related proteins, including the CdkIs p27 and p21, and the block of cell growth in the G0/G1 phase of the cell cycle^[98]. In another study, HA-But induced a dose-dependent inhibitory effect with an almost complete suppression of MIA PaCa-2 cell growth at the highest concentration used. A decrease in the number of cells in S phase and a concomitant increase of those in G0/G1 or G2/M phase were noted. HA-But decreased cyclin D1 and mut-p53 and increased p27 and p21 protein levels. Additionally, HA-But slightly increased the level of Bax and caspase-7, slightly decreased Bcl-2, but strongly decreased survivin protein levels, providing to be active on both Bcl-2 and survivin-mediated apoptosis pathways^[99].

Benzamides

MS-275: This synthetic benzamide derivative 3-pyridylmethyl-N-{4-[(2-aminophenyl)carbamoyl]benzyl} carbamate inhibits HDACs, and has anti-tumor activity in many preclinical models. The first clinical trial with this agent in 2005 included patients with advanced solid tumors or lymphoma. At high concentrations of MS-275, there is a marked induction of reactive oxygen species, mitochondrial damage, caspase activation and apoptosis. Treatment of sensitive tumor cell lines with MS-275 induces gelsolin, a maturation marker, and produces a change in the cell cycle distribution with a decrease in S phase and an accumulation of cells G₁. The *in vivo* therapeutic efficacy of MS-275 has been demonstrated in a variety of human tumor xenograft models^[100].

The addition of MS-27-275 to cell cultures resulted in the accumulation of hyperacetylated H4 molecules. MS-27-275 transcriptionally induced p21 and gelsolin (tumor suppressor) through acetylation of histones affecting cell cycle progression. In addition, pRb molecules were reduced. The response of Capan-1 cell line, presenting a p53 mutation, to MS-27-275 treatment was moderate, although expression of gelsolin was induced. The gelsolin induction by MS-27-275 seemed to have no correlation with the sensitivity of the cells to the treatment. Additionally, p21 induction was considered crucial for the action of MS-27-275^[101].

TSA, NaBu and MS-275 inhibited the growth of the NET cell lines CM and BON in a dose-dependent manner. In both cell lines, HDAC inhibition resulted in a dose-dependent increase of caspase-3 enzyme activity without affecting cell membrane integrity or exerting immediate necrotic effects. Treatment with HDACi resulted in cell cycle arrest of the NET cells at G₁, thereby decreasing those in the S phase. MS-275 treatment of CM and BON cells resulted in a dose-dependent decrease of Bcl-2, whereas Bax remained unaffected in both cell lines. Cyclin D1 was also downregulated in CM and BON cells by MS-275 treatment, while p21 and p27 were markedly increased^[102].

MGCD0103: MGCD0103 is an isotype-specific aminophenylbenzamide that inhibits HDAC classes I and IV,

Table 2 Studies of histone deacetylase inhibitors and different pancreatic cancer cell lines in xenograft models

HDAC inhibitor	Pancreatic cancer cell line	Time schedule	Dosage schedule	Results	Ref.
TSA (+ gemcitabine)	T3M4	q28 d	0.25 mg/kg <i>ip</i> , biweekly	50% tumor weight reduction 3-fold H4 increase	[33]
SAHA (+ bortezomib)	L3.6pl	q21 d	50 mg/kg <i>ip</i> , daily	About 70% tumor weight reduction aggresome disruption	[61]
SAHA (+ Zebularine)	Panc-89, YAP C	(1) q7 d or q14 d (2) q7 d	(1) 50 mg/kg <i>ip</i> , daily (2) 50 mg/kg <i>ip</i> , daily	Tumor growth inhibition Upregulation of CK7, CK20 Downregulation of CK8, Vimentin, chromogranin-A	[55]
FK228	CAPAN-1	q14 d-q21 d	1.5 mg/kg <i>ip</i> , biweekly	50% tumor growth inhibition	[108]
MS-275	CAPAN-1	q28 d	(1) 12.3 mg/kg per os (2) 24.5 mg/kg per os (3) 49 mg/kg per os, 5 × weekly	Moderate growth inhibitory effect	[101]
	CAPAN-1, MiaPaca, Panc-1, Panc-15	Not defined	per os once daily (dosage not defined)	Mixed response: moderate growth inhibitory effect/ resistant to inhibition	[109]
NVP-LBH589 (+ gemcitabine)	HPAF-2, L3.6pl	q28 d	25 mg/kg <i>ip</i> , 5 × weekly	63% tumor weight reduction (HPAF-2) About 80% tumor weight reduction (L3.6pl) MIB-1 slight reduction TUNEL slight induction (HPAF-2)	[113]

HDAC: Histone deacetylase; TSA: Trichostatin A; HPAF: Suberoylanilide hydroxamic acid; TUNEL: Transferase dUTP nick end labeling.

with almost no class II effect. MGCD0103 is well-tolerated and exhibits favorable pharmacokinetic and pharmacodynamic profiles, demonstrating target inhibition and clinical responses. It induces cell death and autophagy, synergizes with proteasomal inhibitors and affects non-histone targets, such as microtubules^[103].

A comparative study in order to estimate the pharmacological properties of second generation HDACIs with the hydroxamate and benzamide head group, namely SAHA, LAQ824/LBH589, CI-994, MS-275 and MGCD0103 was carried out. SAHA and LAQ824/LBH589 seemed to behave as quite unselective HDACIs, while the benzamides CI-994, MS275 and MGCD0103 were more selective HDAC1 and HDAC3 inhibitors. All the compounds induced histone H3 hyperacetylation, as well as cell differentiation and apoptosis and inhibited proliferation. A broad cytotoxicity was seen across different tumor cell lines, among them the pancreatic lines AsPC-1, BxPc3 and Panc-1, with LAQ824/LBH589 being the most potent agents^[104,105]. The inhibitory activities of MGCD0103, MS-275 and SAHA were also compared using a panel of cancer cell lines, among them Panc-1. Although the measured IC50 values varied between cell lines, MGCD0103 was always more potent than the comparator molecules in all cases examined, being at least 7-fold more potent than SAHA in PANC-1 pancreatic cancer cells^[106].

IN VIVO EXPERIMENTAL STUDIES

The data available so far regarding the different classes of HDACIs used in *in vivo* animal studies of pancreatic cancer are presented in the following section and are listed in Table 2.

Hydroxamic acids

TSA: *In vivo* studies on xenografts of pancreatic cancer

line T3M4 in nude mice, showed that the combination of TSA (0.25 mg/kg) and gemcitabine (2.5 mg/kg), given biweekly for 4 wk, led to a reduction in the mean tumor weight by about 50% compared to control or single drug treatments. Additionally, TSA treatment resulted in only a 3-fold increase of H4 acetylation levels *in vivo* compared to the 18-fold increase noted *in vitro*. None of the treatments produced any toxicity, as indicated by lack of change in body weight, and none of the animals developed ulcerating tumors^[33]. Finally, TSA exerted an inhibitory effect on the DMBA-induced carcinogenesis model and the growth of pancreatic ductal adenocarcinoma in rats, by upregulating *KiSS-1*, a metastasis suppressor gene located on 1q32, which is considered to exert an important role in inhibiting the invasion and metastasis of pancreatic cancer^[107].

SAHA: Effects of bortezomib and SAHA in orthotopic human pancreatic tumors (cell line L3.6pl) were investigated. Tumors were treated biweekly with 1 mg/kg bortezomib, daily with 50 mg/kg SAHA, or a combination of the two agents for 21 d. *In vivo* data showed aggresome disruption by the combination and reduction of pancreatic tumor weight, with minimal toxicity noted, what there was being related to bortezomib^[61]. Another experiment in mice presented tumor suppression when treated with SAHA and the DNA methyltransferase inhibitor Zebularine. The first experimental setting included a single intraperitoneal (*ip*) bolus injection with SAHA (50 mg/kg), Zebularine (1 g/kg) or the combination of both agents, and the animals were sacrificed after 7 or 14 d. For daily treatment, animals received the same doses of drugs over a time-course of 1 wk. Gemcitabine as a control therapy was administered *ip* every fourth day. The hypermethylated cell line Panc-89 was more susceptible to SAHA than YAP C. No adverse effects were noted and all animals

survived. Furthermore, the expression of CK7, which is a marker associated with pancreatic cancer cell differentiation, was higher in Zebularine and combined Zebularine/SAHA-treated xenografts, as well as that of the glandular differentiation marker CK20. The expression of CK8, Vimentin and chromogranin-A was lower or constant in Zebularine and Zebularine/SAHA-treated animals^[55].

Cyclic peptides

FK228 (FR901228, depsipeptide): More than 90% of human pancreatic cancers are associated with oncogenic mutations of RAS, in particular K-RAS at codon 12. The Tyr-kinase inhibitors, PP1 and AG, block the RAS mutation-induced activation of PAK1, which is the Rac/CDC42-dependent Ser/Thr kinase. PAK1 is essential for RAS-transformation. Based on these data, the therapeutic potential of either FK228, the combination of these two Tyr-kinase inhibitors or GL-2003, a water-soluble derivative of AG 879, on human pancreatic cancer (Capan-1) xenograft in mice, was examined. Capan-1 cells were injected sub-cutaneously (*sc*) into several groups of nude mice. Each group was treated *ip* with either FK228 (1.5 mg/kg), GL-2003 (20 mg/kg), a combination of PP1 and AG 879 (20 mg/kg of each drug), a combination of PP1 and GL-2003 (20 mg/kg of each drug), or vehicle alone (0.1 mL of 1% DMSO in PBS) as the control, twice a week for 2-3 wk. No adverse effects were detected. The most effective combination was that of GL-2003/PP1 that suppressed cell growth by around 80%, while FK228 alone showed only 50% inhibition. The synergy between GL-2003 and PP1 in blocking the RAS-induced PAK1 activation was not observed *in vitro*^[108].

Benzamides

MS-275: MS-275 administered orally, once daily, 5 d per week for 4 wk, strongly inhibited the growth in 7 out of 8 tumor cell lines implanted into nude mice, although most of these did not respond to 5-FU. Tumors were passaged several times before starting *in vivo* antitumor testing, a tumor lump (2-3 mm in diameter) was transplanted *sc* into the flank of a nude mouse, and the therapeutic efficacy of MS-27-275 was examined. MS-27-275 at 49 mg/kg showed a moderate effect against the only pancreatic Capan-1 tumor. The drug at 24.5 mg/kg and 12.3 mg/kg also showed significant effects against these tumors. As the dose of 49 mg/kg was the maximum tolerated one in this administration schedule, and apparent signs of toxicity, such as weight loss and poor appearance, were reported. The maximum dose of the drug was lowered to 24.5 mg/kg, at which no gross weight loss was observed^[101]. Additionally, the pancreatic carcinoma cell lines Capan1, MiaPaCa, Panc1 and Panc15 were grown as xenografts in nude mice and afterwards, treated per os once daily with MS-275. For most cell lines used, antitumor activity and dose-dependent response of MS-275 *in vivo* were observed. While various tumor cell lines from other malignancies showed an almost complete response, in pancreatic cell lines a mixed response was achieved,

with half of the models tested to present either a moderate growth inhibitory effect or resistance to treatment^[109].

CI-994: CI-994 or N-acetyldinaline[4-(acetylamino)-N-(2-amino-phenyl) benzamide] is a novel oral compound with a wide spectrum of antitumor activity in preclinical models. The mechanism of action may involve inhibition of histone deacetylation and cell cycle arrest. CI-994 is currently undergoing clinical trials. Although several changes in cellular metabolism induced by the drug have been characterized, the primary molecular mechanism of its antitumor activity remains unknown^[110].

CI-994 was previously identified as having cytotoxic and cytostatic activity against several murine and human xenograft tumor models. CI-994 had activity against 8/8 solid tumors tested among them pancreatic adenocarcinoma 02 and 03^[111]. Notably, CI-994 was active against a Pan-02 pancreatic tumor of C57BL/6 mouse origin^[112].

Other HDACIs

Pancreatic tumors were induced in nude mice by *sc* injection of HPAF-2 and L3.6pl cells. Animal groups received either NVP-LBH589 (25 mg/kg, 5 × weekly) or gemcitabine (5 mg/kg, 1 × weekly) or a combination of both (NVP-LBH589 at 25 mg/kg, 5 × weekly plus gemcitabine at 5 mg/kg, 1 × weekly) *ip*, whereas the control group received placebo only, for 28 consecutive days. Three days after commencement of NVP-LBH589 or combination treatment, HPAF-2 cell tumors showed a significantly reduced volume compared with the control. Combination therapy was significantly more efficient than gemcitabine treatment alone and significantly more efficient than NVP-LBH589 therapy alone, in both cell lines. Regarding side effects, weight loss was 6% and 25% for the combination treatment in HPAF-2 and L3.6pl cell tumor bearing mice, respectively. Treatment with NVP-LBH589 and the combination slightly reduced proliferation (Ki-67 index) and slightly induced apoptosis markers in HPAF-2 cell bearing mice, whereas proliferation was not decreased and apoptosis only slightly increased in L3.6pl cell bearing mice^[113].

CONCLUSION

In this review, the recent advances in the understanding and clinical development of HDACIs were discussed. The exact mechanism and the molecular basis for the antitumor effects of these new drugs are complex and not completely understood. HDACIs regulate the acetylation of histones and many non-histone proteins that are involved in gene expression, cell proliferation, migration and death. HDACIs have been shown to induce differentiation and cell cycle arrest, activate the extrinsic or intrinsic pathways of apoptosis and to inhibit invasion, migration and angiogenesis in different cancer cell lines. Normal cells are relatively more resistant to HDACIs-induced cell death. Although not completely elucidated, the main mechanisms by which HDACIs act in pancre-

atic cancer are common. The cdk inhibitor p21 is one of the most commonly induced genes in various pancreatic cancer cell lines by all HDACIs tested. Transcriptional induction of p21 is associated with G1 cell cycle arrest and growth inhibition. Cell cycle arrest and growth inhibition is also correlated with transcriptional activation of other cell cycle regulatory genes such as p16, p27, cyclin E and gelsolin, while inhibition of cyclins A, B1, D1 and D2 were also noted in many cancer cell lines. The pro-apoptotic proteins Bax, Bad and Bim were upregulated, among others, whereas anti-apoptotic proteins, such as Bcl-2, Bcl-XL and survivin, were downregulated. HDACIs reduced the expression of angiogenic factors, such as VEGF receptors -1 and -2, and affected the expression of a panel of metastasis promoting genes (Table 1).

Additionally, these drugs exhibited antiproliferative effects in cancer animal models. Various pancreatic carcinoma cell lines were grown as xenografts in nude mice and treated with HDACIs (Table 2). For most lines used, anti-tumor activity and dose-dependent response were observed, with reduction of cell proliferation (Ki-67 index) and induction of apoptosis (transferase dUTP nick end labeling test). The reduction of pancreatic tumor weight was achieved with minimal toxicity.

These results support the efficiency that HDACIs presented *in vitro*; however, more studies and well-controlled experiments are required to obtain stronger *in vivo* data. Furthermore, phase II / III trials including patients with pancreatic cancer are needed to determine the clinical efficacy of these new drugs.

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