LEADING ARTICLE

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Histone Deacetylase Inhibitors

Current Status and Overview of Recent Clinical Trials

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Abstract

Histone deacetylase (HDAC) inhibitors are a new group of anticancer agents that have a potential role in the regulation of gene expression, induction of cell death, apoptosis and cell cycle arrest of cancer cells by altering the acetylation status of chromatin and other non-histone proteins. In clinical trials, HDAC inhibitors have demonstrated promising antitumour activity as monotherapy in cutaneous T-cell lymphoma and other haematological malignancies. In solid tumours, several HDAC inhibitors have been shown to be efficacious as single agents; however, results of most clinical trials were in favour of using HDAC inhibitors either prior to the initiation of chemotherapy or in combination with other treatments. Currently, the molecular basis of response to HDAC inhibitors in patients is not fully understood. In this review, we summarize the current status of HDAC inhibitors, as single agents or in combination with other agents in different phases of clinical trials. In most of the clinical trials, HDAC inhibitors were tolerable and exerted biological or antitumor activity. HDAC inhibitors have been studied in phase I, II and III clinical trials with variable efficacy. The combination of HDAC inhibitors with other anticancer agents including epigenetic or chemotherapeutic agents demonstrated favourable clinical outcome.

In cancer, alteration of some tumour suppressor genes, oncogenes and tumourigenesis-related genes has not been exclusively due to mutations, but rather to inhibition of transcription. DNA methylation and histone modification are mechanisms that have been implicated in transcriptional regulation. In normal cells there is a delicate balance between histone acetylation and deacetylation mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively; however, this balance is impaired in tumour cells.

Recent advances in cancer therapy of haematological malignancies, for example myelodysplastic syndrome (MDS),[1,2] chronic myeloid leukaemia (CML)[1] and acute myeloid leukaemia (AML),[1-4] using newly developed epigenetic treatment strategies have demonstrated favourable clinical outcomes. Encouraging results from these clinical trials have led to the initiation of similar epigenetic treatment strategies in solid tumours, aimed at improving efficacy and lowering recurrence rates.

Epigenetic treatment strategies focus on the development of new agents that could inhibit HDACs and/or DNA methyltransferases (DNMTs). Currently, numerous new agents that promise a new paradigm shift in cancer management are being investigated in phase I, II and III clinical trials.[5] Several HDAC inhibitors were recently investigated in clinical trials as single agents or in combination

therapy with other chemotherapeutic agents for haematological and/or solid tumours.[5] This article focuses on the role of histone deacetylation inhibition in epigenetic cancer therapy and clinical data gathered to date with HDAC inhibitors.

1. Epigenetics in Cancer

Epigenetics is defined as the study of changes in gene expression that do not result from changes in DNA sequence. Epigenetic therapy is mainly associated with the possible reversal of gene silencing observed in tumourigenesis. During tumourigenesis, gene silencing could be attained through two identified molecular mechanisms. Aberrant methylation is one mechanism that is mainly associated with altered regulation of gene expression and could be observed in two patterns. The first is global hypomethylation, characteristic of tumourigenesis, and the second is selective hypermethylation of promoter regions of genes, including tumour suppressor genes.[6,7] Histone deacetylation is the other mechanism that could silence genes, through chromatin modification and deacetylation of histone lysine residues by different classes of HDACs.[8,9] This leads to compacting of the chromatin structure and tight folding of the nucleosome, thus preventing the binding of transcription factors to their respective DNA binding sites, leading to gene silencing.

Recently, epigenetics has gained remarkable attention due to the realization that epigenetic regulation could play an important role in development, X-chromosome inactivation, imprinting and gene transcription, especially in cancer.

1.1 DNA Methylation

Although the main focus of this article is on the role of histone deacetylation inhibition in epigenetic therapy, it should be noted that DNA methylation plays an important role in the epigenetic modulation of tumour response to chemotherapy. This is described in more detail elsewhere.[7,10]

1.2 Histone Acetylation

Histone proteins, non-histone proteins and genomic DNA together make up chromatin structure. Histone modification includes acetylation, deacetylation, methylation, phosphorylation and ubiquitination. Histone acetylation status is controlled by HATs and HDACs. HATs add an acetyl group to the lysine residue of the histone tail, while HDACs remove the acetyl moiety from histones. Histone acetylation and deacetylation affect the structure of chromatin and expression of genes. Histone acetylation, first discovered in the early 1960s,[11] is associated with a more open chromatin structure, which facilitates the binding of transcription factor complexes to the promoter region of genes resulting in activated transcription. Histone acetylation and histone H3 methylation at lysines 4, 36 or 79 are generally associated with gene activation,[12] while histone deacetylation and H3 methylation at lysines 9 and 27 are generally associated with gene silencing.[13] Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 are usually observed with tumour development.[14]

2. Disruption of Histone Acetyltransferase (HAT) and Histone Deacetylase (HDAC) in Cancer

In cancer cells, unlike normal cells, the balance between histone acetylation and deacetylation catalysed by HAT and HDAC is disrupted, a process that has been frequently associated with tumourigenesis.[15]

2.1 Alteration of HAT in Cancer

The alteration of HAT in cancer might result from overexpression, mutation and translocation of HAT genes.[12,16] The first HATs and HDACs were identified in the mid-1990s.[17] In humans, several groups of proteins have been identified to have HAT activity,[11,17,18] such as Gcn5-related N-acetyl transferase (GNAT), cyclic adenosine monophosphate (cAMP) response element binding protein (CREB)-binding protein (CBP)/p300 [CBP/p300], and MYST family including monocytic leukaemia zinc finger protein (MOZ) and Tat interacting protein 60 (Tip60). Of these HAT proteins, p300 has high homology with CBP. CBP/p300 are tumour suppressor-like proteins

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involved in critical tumourigenic pathways, and their inactivation leads to cancer formation.[19] Mutation of p300 and CBP has been widely seen in cancer cell lines,[19] leukaemia,[12] and solid tumours including colorectal, breast, ovarian and gastric tumours.[19] Chromosomal translocation of p300, CBP and MOZ genes have been reported in leukaemia,[20-22] such that the HAT gene might insert into other genes to form a fusion protein, recruiting HAT to specific genes and resulting in the activation of these genes.[23]

2.2 Alteration of HDAC in Cancer

Currently, 18 HDACs have been identified in humans. These HDACs were classified into three main classes based on similarity to yeast HDACs.[24] Class I includes HDAC1, 2, 3 and 8. Class II includes HDAC 4, 5, 6, 7, 9 and 10. HDAC11 is placed in class IV. Class I and class II HDACs contain Zn^{2+} in their catalytic active site. Class III is characterized by sir2-related proteins containing SIRT1 to 7, which cannot be inhibited by compounds that inhibit class I and class II HDACs. HDACs work in concert with co-activators, co-repressors, transcription factors and HATs to change the structure of histones and modulate transcription of genes.[24,25]

Alteration of HDACs has been found in haematological malignancies and solid tumours.[26] Mutations of genes coding for HDACs are rarely found in cancers,[15] but altered expression and aberrant recruitment of HDACs have been reported in tumours. Overexpression of HDAC1, HDAC2, HDAC3, HDAC6 and SIRT7 have been identified in colon, breast, prostate, thyroid, cervical and gastric cancers.[15,27] The aberrant recruitment of HDACs due to chromosomal translocations has a causal role in tumourigenesis. The retinoic acid receptor (RAR) is an important component in the differentiation pathway in myeloid cells. In acute promyelocytic leukaemia (APL), the aberrant promyelocytic leukaemia (PML)-RARα fusion protein generated by chromosomal translocation recruits HDAC to RARα target genes, leading to constitutive repression of these target genes.[15,27,28] In AML, normal AML1 is a transcription factor required for differentiation of haematopoietic cells. The fusion protein AML1-ETO is formed by translocation, recruiting HDACs to AML1 target genes and constitutively repressing their expression.[15,27,28] In non-Hodgkin's lymphoma, a transcription repressor LAZ3/BCL6 was overexpressed in lymphomas, resulting in recruitment of HDACs (such as HDAC2) to target genes, leading to the repression of specific genes like growth regulatory genes.[27,29] Generally, these fusion proteins are transcription regulators that repress their target genes (genes encoding proteins for cell differentiation or tumour suppression) through the aberrant recruitment of HDAC, which eventually leads to tumourigenesis.

3. HDAC Inhibitors

The altered gene expression due to aberrant recruitments of HDACs has been associated with tumourigenesis. Since epigenetic alteration is reversible, histone deacetylases have become an attractive target for epigenetic therapy of cancer. A large number of HDAC inhibitors have been purified from natural sources or have been synthesized. One of the first HDAC inhibitors discovered was butyrate. Trichostatin A was the first natural HDAC inhibitor identified in the 1990s. Suberoylanilide hydroxamic acid (SAHA), structurally similar to trichostatin A, was identified to be an HDAC inhibitor 10 years ago.[17] It was approved by the US FDA in 2006 for the treatment of advanced and refractory primary cutaneous T-cell lymphoma and is marketed as vorinostat (Zolinza®).[30]

HDAC inhibitors can be structurally grouped into at least four classes, e.g. hydroxamate, cyclic peptide, aliphatic acids and benzamide (figure 1).[24,27] Currently, at least 16 HDAC inhibitors have been developed and reached phase I and II clinical trials (table I), with variable reported efficacy and specificity.[24,25,27,31] HDAC inhibitors may be pan- or specific/selective inhibitors of HDAC activities. Most of the HDAC inhibitors, such as vorinostat and trichostatin A, inhibit class I and II HDACs. HDAC inhibitors like valproic acid and sodium phenylbutyrate are selective against class I and IIα HDACs.

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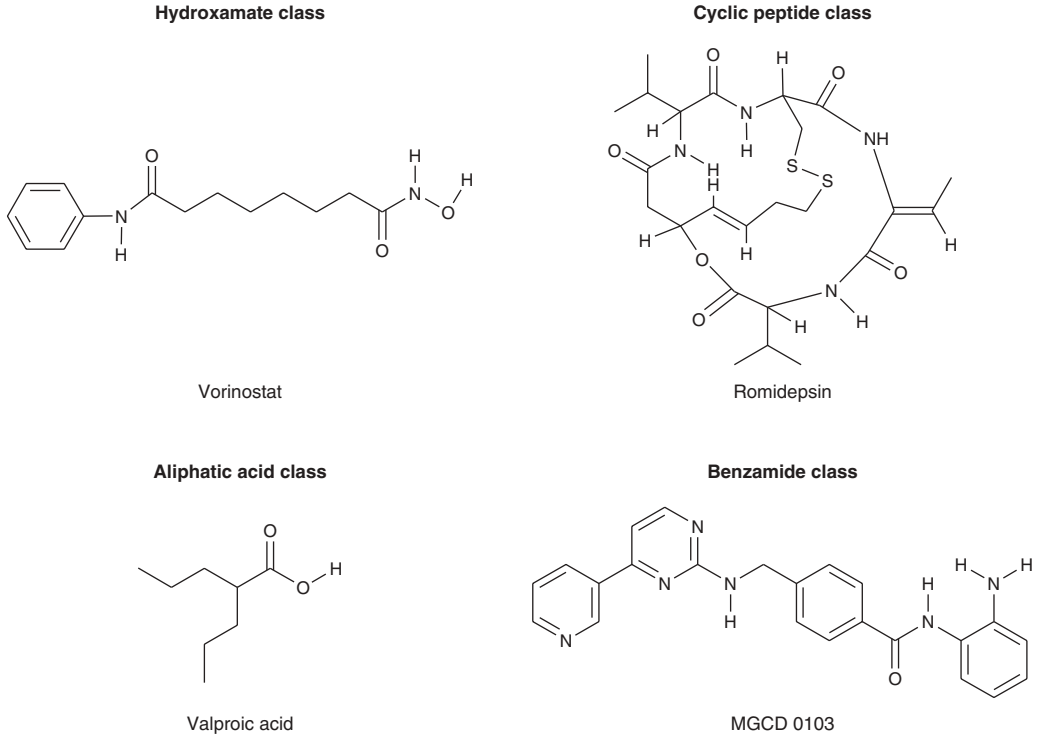

Fig. 1. Structures of the histone deacetylase (HDAC) inhibitor classes studied in clinical trials. Examples are shown of selective and pan-HDAC inhibitors investigated in different clinical trials. Vorinostat (suberoylanilide hydroxamic acid [SAHA]) and valproic acid are two examples of pan-HDAC inhibitors that belong to hydroxamate and aliphatic acid classes of HDAC inhibitors, respectively. Pan-HDAC inhibitors target both class I and class II HDACs (HDACs 1–10), interfering with both histone and non-histone proteins. Romidepsin (depsipeptide) and MGCD 0103 are two examples of selective inhibitors of class I HDACs. Romidepsin and MGCD 0103 belong to cyclic peptide and benzamide classes of HDAC inhibitors, which target HDACs 1, 2 and HDACs 1, 2, 3 and 8, in class I HDACs, respectively.

Romidepsin (depsipeptide, FK 228) specifically inhibits HDAC1 and 2,[24] while entinostat (MS 275) specifically inhibits HDAC1, 2 and 3.[24,26]

4. Mode of Action of HDAC Inhibitors

HDAC inhibitors alter the acetylation status of chromatin and other non-histone proteins, resulting in changes in gene expression (table II), induction of cell death, apoptosis, cell cycle arrest, and inhibition of angiogenesis and metastasis (figure 2). It has also been reported that HDAC inhibitors can induce polyploidy[85] and aberrant mitosis such as mitotic slippage,[86] and premature sister chromatid separation,[87] which can lead to loss of cancer cell proliferation. Transformed cells are much more sensitive to HDAC inhibitors compared with normal cells. The response of transformed cells depends on the type of cancer,[88] the structure and concentration of HDAC inhibitors as well as the exposure time to HDAC inhibitors.

4.1 Induction of the Apoptosis Pathway by HDAC Inhibitors

HDAC inhibitors can activate extrinsic (death-receptor) and (or) intrinsic (mitochondrial) apoptotic pathways. In many transformed cells, HDAC inhibitors treatment can activate transcription of death receptors such as Fas, DR5 and their ligands like tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL).[58] This results in the activation of caspase-8 or caspase-10 and the initiation of the extrinsic apoptotic pathway.[24] When small interfering

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RNA (siRNA) was used to suppress the expression of TRAIL and Fas in APL mice, valproic acid-induced apoptosis was reduced by 50% in the bone marrow and spleen.[58]

HDAC inhibitors typically induce cell death through the intrinsic apoptosis pathway. A number of studies demonstrate that HDAC inhibitors induce the intrinsic apoptosis pathway through inactivation of anti-apoptotic and activation of the pro-apoptotic Bcl-2 family of proteins.[24,27] Anti-apoptotic proteins of the Bcl-2 family, including Bcl-2, Bcl-xL and Mcl-1, were down-regulated by panobinostat (LBH 589), an HDAC inhibitor in lung cancer cell lines.[59] Pro-apoptotic proteins of the Bcl-2 family, including Bak and BH3-only proteins (such as Bik, Bim, Bmf and Noxa), were up-regulated at messenger RNA (mRNA) or protein levels by HDAC

Table I. Histone deacetylase (HDAC) inhibitors in clinical trials

Class	Compound	Specificity	Dose range	Development phase	Adverse effects
Hydroxamate	Vorinostat (SAHA)[24]	Class I/II	μmol/L	US FDA approved	Fatigue, nausea, vomiting, diarrhoea, anaemia, anorexia, thrombocytopenia, QTc prolongation[32-37]
	Belinostat (PXD 101)[25,31]	Class I/II	μmol/L	II	Fatigue, nausea, vomiting, diarrhoea, constipation, flushing, QTc prolongation[38]
	LAQ 824[27]	Class I/II	nmol/L	I	Fatigue, nausea, vomiting, diarrhoea, anorexia, constipation, thrombocytopenia, neutropenia, lymphopenia, anaemia, QTc prolongation, ST segment/T-wave changes, headache[39]
	Panobinostat (LBH 589)[24,31]	Class I/II	nmol/L	I/II	Nausea, vomiting, diarrhoea, anorexia, thrombocytopenia, hypokalaemia, QTc prolongation, ST segment/T-wave changes, pericardial effusion[40]
	Pyroxamide[27]	Class I	μmol/L	I	NA
	Givinostat (ITF 2357)[24,26]	Class I/II	nmol/L	I	Fatigue, diarrhoea, thrombocytopenia, leukopenia, neutropenia, QTc prolongation[41,42]
	PCI 24781[24]	Class I/II	nmol/L	I	NA
Cyclic peptide	Romidepsin (depsipeptide, FK 228)[24]	HDAC1, 2	nmol/L	II	Fatigue, nausea, vomiting, anorexia, thrombocytopenia, lymphopenia, leukopenia, neutropenia, anaemia, QTc prolongation, ST segment/T-wave changes, sinus or ventricular tachycardia[43-45]
Aliphatic acid	AN 9 (pivaloyloxymethyl butyrate)[24,25]	NA	μmol/L	II	Fatigue, nausea, vomiting, diarrhoea, anorexia, dysgeusia, fever, hyperglycaemia, hypokalaemia, hepatic transaminase elevation, anaemia[46,47]
	Sodium Phenylbutyrate[24]	Class I/IIα	mmol/L	II	Fatigue, nausea, vomiting, dyspepsia, neutropenia, anaemia, somnolence, confusion, light-headedness[48-51]
	Valproic acid[26]	Class I/IIα	mmol/L	II	Fatigue, nausea, vomiting, leukopenia, thrombocytopenia, neurological toxicities: neurosensory, neurocortical, vertigo, somnolence[52]
	Valproic acid, topical (Baceca®)[24]	Class I	NA	II	NA
	Valproic acid, oral (Savicol™)[24]	Class I	NA	II	NA
Benzamide	Entinostat (MS 275)[24,26]	HDAC1, 2, 3	μmol/L	II	Fatigue, nausea, asthenia, anorexia, anaemia, thrombocytopenia, hypoalbuminaemia, hypophosphataemia, hyponatraemia, headache[53,54]
	Tacedinaline (CI 994)[27]	NA	μmol/L	I/II	Fatigue, nausea, vomiting, diarrhoea, constipation, mucositis, thrombocytopenia[55]
	MGCD 0103[24,25]	Class I	μmol/L	II	Fatigue, nausea, vomiting, anorexia, diarrhoea, dehydration, constipation, abdominal pain, dyspnoea[56,57]

NA = not available; **QTc** = corrected QT interval; **SAHA** = suberoylanilide hydroxamic acid.

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Table II. Alterations in gene expression by histone deacetylase inhibitors[a]

Level	Up-regulated		Down-regulated		References
	transcriptional	translational	transcriptional	translational	
Apoptosis	ND	Fas, DR5, TRAIL, FasL, Bim, Bmf, Bik, Noxa, Bak	XIAP	Bcl-xL, Bcl-2, Mcl-1, XIAP	58-62
ROS-induced cell death	TBP-2	TBP-2	Trx, TrxR	TrxR	60,63,64
Cell cycle arrest	p21	p21, p53	Cyclin B1	Cyclin B1, cyclin D1, cyclin D2, cyclin E	65-72
Angiogenesis	p53, VHL, TSP1, neurofibromin 2	p53, VHL, TSP1	HIF-1α, VEGF, FGF, VEGFR 1, VEGFR2, CXCR4	HIF-1α, VEGF, FGF, CXCR4	73-81
Metastasis	KAI1, RECK, TIMP1	RhoB, RECK, TIMP1	ITGA5	ND	66,82-84

a The expression of studied genes was examined using RT-PCR (XIAP, TrxR, p21, cyclin B1, VHL, HIF-1α, VEGF, FGF, CXCR4, KAI1, RECK), Northern blot (TBP-2, Trx, p53, TSP1, VEGF, VEGFR1, VEGFR2), cDNA microarray (HIF-1α, KAI1, TIMP1, ITGA5), or GEArray (neurofibromin 2, VHL, VEGF), while the protein levels were examined using Western blot (Bcl-xL, Bcl-2, Mcl-1, Noxa, Bim, Bmf, Bik, Bak, XIAP, TBP-2, TrxR, p21, p53, cyclin B1, cyclin D1, cyclin D2, cyclin E , VHL, TSP1, HIF-1α, VEGF, FGF, CXCR4, RhoB, RECK, TIMP1), or flow cytometry analysis (Fas, DR5, TRAIL, FasL).

CXCR4 = CXC chemokine receptor 4; **DR5** = death receptor 5; **FasL** = Fas ligand; **FGF** = fibroblast growth factor; **HIF-1α** = hypoxia-inducible factor-1α; **KAI1** = Kangai 1; **ND** = not detected; **RECK** = reversion-inducing-cysteine-rich protein with kazai motifs; **RhoB** = Ras homologue gene family member B; **ROS** = reactive oxygen species; **RT-PCR** = reverse transcription polymerase chain reaction; **TBP-2** = thioredoxin-binding protein-2; **TIMP1** = tissue inhibitor of metalloproteinases-1; **TRAIL** = tumour necrosis factor-related apoptosis-inducing ligand; **Trx** = thioredoxin; **TrxR** = thioredoxin reductase; **TSP1** = thrombospondin-1; **VEGF** = vascular endothelial growth factor; **VEGFR** = VEGF receptor; **VHL** = von Hippel Lindau; **XIAP** = X-linked inhibitor of apoptosis protein.

inhibitors, including vorinostat, entinostat, panobinostat, romidepsin and CBHA.[27,60,61] The BH3-only pro-apoptotic protein Bid, which is involved in both the extrinsic and intrinsic pathways, was also reported to be activated by vorinostat, romidepsin and oxamflatin.[89] In addition, the X-linked inhibitor of apoptosis protein (XIAP), an anti-apoptotic protein in the intrinsic pathway, was down-regulated after HADC inhibitor exposure in cell lines.[60,62]

In some cancer cell lines, the anti-proliferation activity of HDAC inhibitors is limited due to the induction of genes facilitating tumour growth, e.g. the anti-apoptotic factor nuclear factor kappaB (NF-κB)[90,91] and Mcl-1.[92] However, NF-κB can be effectively down-regulated by the proteasome inhibitor MG 132,[90] the protein kinase inhibitor UCN 01[93] or the NF-κB inhibitor parthenolide.[93] Furthermore, Mcl-1 can be down-regulated by cyclin-dependent kinase (CDK) inhibitors such as roscovitine, NU 6102 and SU 9516.[92] These findings demonstrate that the antitumour efficacy of HDAC inhibitors can be potentiated by combination with other agents that down-regulate anti-apoptotic genes.

HDAC inhibitor-induced cell death is partially mediated by reactive oxygen species (ROS), a cause of caspase-independent cell death. An increase in ROS levels has been detected in transformed cancer cells, but not in normal cells after treatment with HDAC inhibitors.[94] Thioredoxin is an important protein that can scavenge ROS. It can be inactivated by the binding of thioredoxin-binding protein-2 (TBP-2). Vorinostat was found to up-regulate TBP-2 transcription[60,63] and down-regulate thioredoxin transcription,[63] leading to a reduction of ROS scavenging. Another protein involved in redox-regulation, thioredoxin reductase (TrxR), was recently identified to be down-regulated by romidepsin in human lung cancer cells.[64] These findings suggest that redox-sensitive signalling might be a mechanism of HDAC inhibitor-induced cell apoptosis.

Results from a recent study investigating genes responsive to HDAC inhibitors, indicate that HDAC inhibitor-induced apoptosis is associated with aberrant proteasome activity, and this proteasome activity is mediated by HR23B. The human HR23B, a homolog of *Saccharomyces cerevisiae* Rad 23, targets ubiquitylated proteins to the

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proteasome for degradation.[95] In HDAC inhibitor-treated cells, the level of HR23B and its interaction with the proteasome were both increased, while the proteolytic activity of the proteasome was deregulated.[95] Knockdown of HR23B with siRNAs reinstated the proteasome activity but reduced the sensitivity of cells to HDAC inhibitors.[95] These results suggest that proteasome activity is negatively associated with the efficacy of HDAC inhibitors. Proteasome inhibitors (e.g. bortezomib, MG 132 and salinosporamide A [NPI 0052]) in combination with HDAC inhibitors have been reported to synergistically induce apoptosis in different types of cell lines from haematological[96-100] or solid malignancies.[90,101-103] It was observed that treatment of cancer cells with proteasome inhibitors led to the accumulation of misfolded or unfolded proteins and the formation of cytoprotective aggresomes. The stimulated aggresomes were disrupted by HDAC inhibitors, which contributed to enhanced apoptosis and increased endoplasmic reticulum-stress.[104-106] In a phase I clinical trial, the combination of bortezomib and vorinostat has shown significant antitumour activity.[107] In this study, nine of 23 patients with relapsed/refractory multiple myeloma had a partial response and ten had stable disease.

4.2 Induction of Cell Cycle Arrest by HDAC Inhibitors

A number of studies have demonstrated that almost all HDAC inhibitors can inhibit cell growth by cell cycle arrest at G0/G1 or G2/M checkpoints based on cell type and/or dose of HDAC inhibitor used.[62,65-68] Protein p21 is most commonly reported to be up-regulated by HDAC inhibitors in cancer cell lines.[65-71] Dephosphorylation of pRb was also detected in

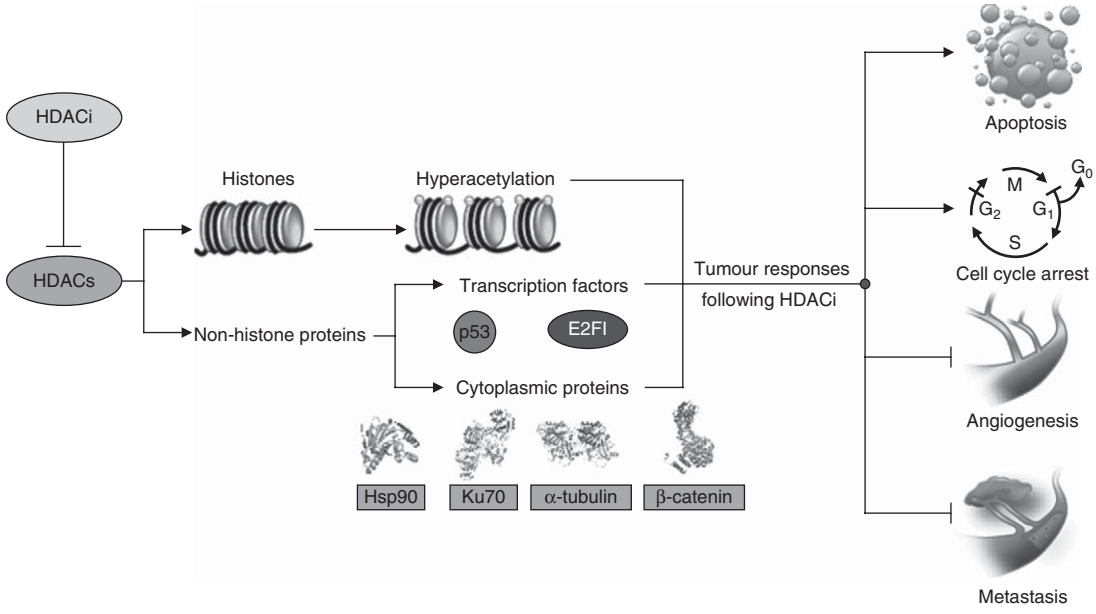

Fig. 2. Anticancer effects of histone deacetylase (HDAC) inhibitors (HDACi). HDAC is a family of proteins that deacetylate histones, leading to compacted chromosome structure and repressed transcription. In addition to histones, non-histone proteins, such as transcription factors (p53, E2F1) and cytoplasmic proteins (Hsp90, Ku70, α-tubulin and β-catenin), can also be deacetylated by HDACs. These non-histone substrates are associated with growth, apoptosis, cell cycle and motility of cancer cells. HDACi represent a group of anticancer agents that can inhibit the enzymatic activity of HDACs, resulting in hyperacetylation of histones and non-histone substrates. Hyperacetylation of histones can relax chromatin structure and facilitate the transcription of genes, including cancer suppressor genes. Hyperacetylation of the non-histone proteins leads to the inhibition of proliferation and motility of cancer cells. HDACi exert their anticancer effect by inducing apoptosis, cell cycle arrest, and inhibition of angiogenesis and metastasis of cancer cells. **Hsp90** = heat shock protein 90.

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human leukaemia cells treated with LAQ 824, an HDAC inhibitor.[62] HDAC inhibitors can also induce the down-regulation of cyclin proteins, such as cyclin B1 (a regulator of G2–M phase and the M phase transition),[65] cyclin D1 and D2 (a regulator of G1/S phase transition)[70,72] and cyclin E,[108] to arrest the cell cycle.

4.3 Inhibition of Angiogenesis by HDAC Inhibitors

The anti-angiogenic and anti-metastatic effects of HDAC inhibitors have been recently investigated *in vitro* and *in vivo*. The genes encoding for proteins involved in angiogenesis, including hypoxia-inducible factor-1α (HIF-1α) and its target vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR)-1 and -2, and CXC chemokine receptor 4 (CXCR4), were down-regulated by HDAC inhibitors,[73,109] whereas genes encoding suppressors for angiogenesis, such as p53, von Hippel Lindau (VHL), thrombospondin-1 (TSP1), and neurofibromin 2 (NF2), were up-regulated by HDAC inhibitors in different cancer and endothelial cells.[74-76]

HIF-1α, a transcription factor, was reported to be down-regulated at mRNA and protein levels by HDAC inhibitors such as vorinostat, romidepsin and panobinostat, in prostate cancer cells, lung carcinoma cells and human umbilical vein endothelial cells (HUVEC).[73,77,78] The gene expression of VEGF and fibroblast growth factor (FGF), another angiogenesis inducer, were suppressed by romidepsin in prostate cancer cells[79] and by valproic acid in colon cancer cells.[80] The VEGF-induced transcription of VEGFR1 and VEGFR2 in endothelial cells could be inhibited by trichostatin A; VEGF-induced angiogenesis was also inhibited by trichostatin A and vorinostat.[81] Another protein required in angiogenesis, the endothelial chemokine receptor CXCR4, was repressed by panobinostat at mRNA and protein levels.[73] In addition, HDAC inhibitors inhibited angiogenesis and tumour growth *in vivo*.[73,110] In mouse models with xenografts developed from prostate carcinoma cells, microvessels in tumours were reduced following treatment with panobinostat or valproic acid.[73,110]

HDAC inhibitors also induce the expression of anti-angiogenic factors, such as p53, VHL, TSP1 and NF2. The tumour suppressors p53 and VHL can inhibit angiogenesis by promoting the degradation of HIF-1α and inhibiting the gene transcription activated by HIF-1α in liver carcinoma cells. Following trichostatin A treatment, the gene expression of p53 and VHL were up-regulated at transcription and protein levels, and hypoxia-induced angiogenesis was inhibited.[74] TSP1, an extracellular matrix glycoprotein, is a natural inhibitor of angiogenesis inhibiting endothelial cell growth, adhesion and motility. The gene expression of TSP1[75] and another anti-angiogenic factor NF2[76] in Hela cells was recently reported to be up-regulated by trichostatin A and romidepsin, respectively.

4.4 Inhibition of Metastasis by HDAC Inhibitors

In cancer cells, HDAC inhibitors could up-regulate the expression of those genes encoding metastatic suppressors such as Kangai 1 (KAI1), Ras homologue gene family, member B (RhoB), reversion-inducing-cysteine-rich protein with kazal motifs (RECK) and tissue inhibitor of metalloproteinases-1 (TIMP-1), while those genes encoding for proteins that promote metastasis such as matrix metalloproteinases (MMPs), integrin-α5 and collagen proteins are down-regulated. The expression of KAI1 was found to be down-regulated in many cancer cells, but could be induced by sodium butyrate.[82] RhoB, a small guanosine triphosphatase that negatively regulates tumour metastasis,[111] was detected to be low in both lung cancer tissues and in cell lines, and found to be restored by trichostatin A in lung cancer cell lines.[83] RECK encodes a membrane glycoprotein that suppresses tumour metastasis and angiogenesis. Trichostatin A treatment up-regulated RECK, which in turn inhibited the activity of MMP-2, and suppressed the invasiveness of lung cancer cells.[84] TIMP-1 was shown in many previous investigations to be a metastasis suppressor, with its expression being increased by sodium butyrate[82] and belinostat.[66] Belinostat actively inhibited the metastasis of prostate tumour xenografts. In this experiment,

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about half of the mice not treated with belinostat were detected to have lung metastases; however, lung metastases were not detected in mice treated with belinostat.[66]

Using cDNA microarray, sodium butyrate was identified to down-regulate the expression of MMPs, integrins (e.g. ITGA5) and collagens in human lung carcinoma cells.[82] Inhibition of α5-integrin encoded by ITGA5 in ovarian cancer xenografts with its specific antibody significantly reduced the number of metastases and increased survival.[112]

4.5 Hyperacetylation of Non-Histone Proteins by HDAC Inhibitors

In addition to histone proteins, some non-histone proteins are also substrates of HDACs.[113-119] HDAC inhibitor treatment could lead to hyperacetylation of these non-histone proteins, including transcription factors and proteins in the cytoplasm.

4.5.1 Transcription Factors

The transcription factors p53 and E2F play an important role in cell growth and survival. The activity of p53 and E2F was shown to be modified by both acetylation and deacetylation.[113-115] Acetylation of p53 improved its stability and activity, while deacetylation mediated by the HDAC1 complex promoted its degradation.[120] In prostate cancer cells, acetylation of p53 at specific sites was stabilized by HDAC inhibitors, which promoted the assembly of p53 transcriptional complex on the promoter of p21 and induced p21 transcription.[121]

E2F transcription factors are important regulators of cell cycle and apoptosis, aberrant expression of which is related to tumourigenesis. E2F1, a member of the E2F family, has been shown to be acetylated/activated by CBP/p300 acetylase and deacetylated/repressed by Rb-associated histone deacetylase.[115] The binding of E2F1/4 in complex with HDACs was negatively associated with the transcription of tumour suppressor gene ARHI (Ras homologue member I, DRAS3).[122] In breast[122] and ovarian[123] cancers, the expression of E2F1 and E2F4 was found to be up-regulated; however, the expression of ARHI was markedly down-regulated. Trichostatin A treatment increased promoter activity of ARHI by increasing acetylation of E2F[124] and reducing its binding to ARHI.[122]

4.5.2 Cytoplasmic Proteins

Cytoplasmic proteins, such as heat shock protein (Hsp)90, Ku70, α-tubulin and β-catenin, can be deacetylated by HDACs which are associated with growth, apoptosis and motility of cancer cells.[116-119] Hyperacetylation and functional disruption of these proteins were detected following HDAC inhibitors treatment in human cancer cells.[59,119,125,126]

Hsp90 is a known target of HDAC6. Panobinostat treatment could increase the acetylation of Hsp90, resulting in the impairment of its chaperone function with epidermal growth factor receptor (EGFR), Akt and signal transducer and activator of transcription 3 (STAT3) in human lung cancer cell lines, leading to blockade of cell growth.[59] Romidepsin was recently found to increase acetylation and impair the function of Hsp70, which is required for the Hsp90-client protein complex formation.[127]

Ku70 is a DNA repair protein and has a carboxy-terminal domain to bind DNA and Bax.[128] The class III HDAC SIRT1 has been shown to deacetylate Ku70 and increase the DNA repair ability of cells when subjected to radiation.[117] HDAC inhibitors (trichostatin A, vorinostat, entinostat and AR 42 [OSU-HDAC42]) can increase the acetylation of Ku70 and reduce its DNA binding affinity, leading to the reduced ability of prostate cancer cells to repair drug-induced DNA damage.[125] In addition, the carboxy-terminal of Ku70 interacts with Bax and suppresses the mitochondrial translocation of Bax.[128] Trichostatin A treatment of neuroblastoma cells increased acetylation of Ku70, released Bax to mitochondria and induced cell apoptosis.[129]

α-Tubulin is the known substrate of HDAC6, and the deacetylation of α-tubulin is associated with cell motility[118] and transforming growth factor-β_1-induced epithelial-mesenchymal transition (EMT).[130] Inhibition of HDAC6 by its inhibitors (trichostatin A or tubacin) can increase

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acetylation of α-tubulin[118,126] and decrease motility of carcinoma cells,[126] suggesting the potential of HDAC inhibitors as an anti-metastatic therapeutic agent.

β-Catenin, a non-histone substrate of HDAC6, is an important component of Wnt signalling for cell proliferation. In most colorectal carcinomas, the Wnt signalling pathway is constitutively active. Inhibition of deacetylation of β-catenin by trichostatin A has recently been found to block EGF-induced nuclear translocation of β-catenin and consequent activation of c-Myc, leading to inhibition of tumour cell proliferation.[119]

5. HDAC Inhibitors in Clinical Trials

5.1 HDAC Inhibitors as Single Agent Therapy

The antitumour efficacy of HDAC inhibitors has been extensively demonstrated *in vitro* (cancer cell lines) and *in vivo* (animal models). Over the past several years, many HDAC inhibitors have been studied in clinical trials either as single agents (table III) or in combination with other antitumour agents (table IV). In most clinical trials thus far, HDAC inhibitors have shown biological or antitumour activity. Information about HDAC inhibitors investigated in clinical trials sponsored by the National Cancer Institute is available through the website http://www.cancer.gov/clinicaltrials. Several examples of HDAC inhibitors that have been examined in these clinical trials are presented in this section.

5.1.1 Hydroxamates

Vorinostat is the first HDAC inhibitors approved by the FDA[30] for the treatment of cutaneous T cell lymphoma. Vorinostat has also been investigated in phase I and II clinical trials in other haematological malignancies and solid tumours.[1,34-37,139-141] It was demonstrated that vorinostat could be orally administered with a maximum tolerated dose (MTD) of 400 mg once daily or 200 mg twice a day for continuous daily dosing, or 300 mg twice a day for solid tumours for 3 consecutive days per week in a 4-week cycle.[37] In this study, vorinostat was well tolerated, and had both biological and antitumour activity. An accumulation of acetylated histones was noted in the peripheral blood mononuclear cells (PBMCs) after the administration of vorinostat. One of the 73 patients had a complete response, three had partial responses, two had unconfirmed partial responses and 16 had stable disease.[37] Another phase I clinical trial also showed that vorinostat is active in patients with advanced leukaemias and MDS.[1] In contrast, in some phase II clinical trials in patients with solid tumours,[34-36,139-141] vorinostat as a single agent had limited effect, possibly due to the limited number of patients or drug exposure.

Other hydroxamates, including belinostat[38,142] and panobinostat,[40] have been investigated in clinical trials. Belinostat was intravenously administered to 46 patients with advanced solid tumours with the MTD being 1000 mg/m^2/day. H4-hyperacetylation was detected in the PBMCs of all patients after drug infusion, and stable disease was achieved in 18 patients (39%).[38]

5.1.2 Cyclic Peptides

Phase I[3] and II[43-45,143] clinical trials of romidepsin have been conducted in patients with leukaemia, lymphoma and solid tumours including neuroendocrine tumours and lung cancer. Romidepsin is usually administered by infusion at a dose of 10–22 mg/m^2/day. Fatigue, nausea, anorexia and vomiting are common adverse events, but serious cardiac adverse events have occurred in some patients with metastatic neuroendocrine tumours.[43] Although clinical efficacy remains under investigation, romidepsin-mediated biological activity has been detected in lung cancer cells in which histone H4 acetylation and the expression of p21 are increased.[45]

5.1.3 Aliphatic Acids

Sodium phenylbutyrate,[48-51] valproic acid,[52] AN 9 (pivaloyloxymethyl butyrate)[46,47] and other aliphatic acids have been evaluated in patients with advanced or refractory solid tumours and recurrent malignant gliomas. Sodium phenylbutyrate is safe even with prolonged infusions and has been shown to have therapeutic activity. In one of the clinical trials,[50] 1 of 23 patients with recurrent malignant gliomas treated with

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Table III. A summary of clinical trials using histone deacetylase (HDAC) inhibitors as a single agent in the treatment of cancer

Agent	Phase	No. of pts	Tumour type	Administration regimen	Adverse events (no. of pts)	Clinical trial outcome (no. of pts)
Vorinostat (SAHA)[34] [a]	II	27	Epithelial ovarian or peritoneal carcinoma	Oral dose 400 mg/day in a 21-day cycle until disease progression or unacceptable toxicity	Grade 4 toxicity (2): neutropenia and leukopenia respectively; grade 3 toxicity (14): constitutional, gastrointestinal, neutropenia, thrombocytopenia, metabolic abnormalities, neurological complaints, pain	PR (1), SD (9), PD (14)
Vorinostat[35]	II	16	Solid tumours	Oral dose 200, 300, 400 mg bid for 14 days, followed by a 7-day rest until disease progression or unacceptable toxicity	DLT in 3 patients each with 300 mg and 400 mg twice daily doses, respectively, no DLT with 200 mg twice daily; drug-related AEs: anorexia, fatigue, nausea, vomiting, diarrhoea, thrombocytopenia and weight loss	No confirmed CR or PR, SD (1) in breast cancer, SD (1) in colorectal cancer, SD (6) in non-small cell lung cancer, PD (2)
Vorinostat[36]	II	13	Head and neck cancer	Oral dose 400 mg once daily every 4 weeks, treatment continued until disease progression	Grade 3–4 toxicities (7): thrombocytopenia, anorexia, dehydration	No confirmed PR or CR, unconfirmed PR (1), SD (3), PD (7)
Vorinostat[37]	I	73	Solid tumours (50 patients) or haematological malignancies (23 patients)	Oral doses of 200, 400 and 600 mg once or 200, 300 and 400mg twice daily on a continuous basis or 300 and 400 mg twice daily for 3 days/week every 4 weeks	Solid tumours: DLT (10); haematological malignancies: DLT (8); DLT: anorexia, dehydration, diarrhoea and fatigue	Solid tumours: PR (2), unconfirmed PR (2), SD (12), haematological malignancy: CR (1), PR (1), SD (4), 22 (30%) remained on study for 4–37+ days
Belinostat (PXD 101)[38]	I	46	Advanced solid tumours	30 min IV infusion on days 1–5 every 21 days as a cycle, dose 150–1200 mg/m^2/day, 6 dose levels, 158 cycles	DLT (7): grade 3 fatigue, diarrhoea and atrial fibrillation, grade 2 nausea and vomiting	No CR or PR, SD (18) [39%], including 15 treated for ≥4 cycles
Romidepsin (depsipeptide)[43] [a]	II	15	Neuroendocrine tumours	4-hour IV infusion on days 1, 8 and 15 every 28 days, dose 14 mg/m^2	Most common AEs: nausea, anorexia, vomiting, fatigue, grade 4 lymphopenia (1), grade 5 sudden death (1)	No CR or PR, PD (3)

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Table III. Contd

Agent	Phase	No. of pts	Tumour type	Administration regimen	Adverse events (no. of pts)	Clinical trial outcome (no. of pts)
Romidepsin[44] [a]	I	24	Solid tumours	4-hour infusion weekly for 3 weeks of a 28-day cycle, dose 10–22 mg/m^2, 4 dose levels	DLT (3): reversible asymptomatic T-wave inversions; DLT (1): sick sinus syndrome, hypocalcaemia	No objective tumour responses, SD (3)
Romidepsin[45]	II	19	Lung cancer	4-hour infusions on days 1 and 7 of a 21-day cycle, dose 17.8 mg/m^2	DLT (1): myelosuppression; grade 3–4 AEs: hypoxia, anaemia, neutropenia and thrombocytopenia; no significant cardiac toxicities	No objective responses, transient SD (9), PD (14)
AN 9 (pivaloyloxymethyl butyrate)[46]	I	28	Solid tumours	6-hour IV infusion daily for 5 days every 3 weeks, dose 0.047–3.3 g/m^2/day	No DLT, moderate nausea, vomiting, hepatic transaminase elevation, hyperglycaemia, fever, fatigue, anorexia, injection site reaction, diarrhoea, visual complaints	PR (1), SD (6) for 4–10 months as their best response
AN 9[47]	II	47	Non-small cell lung cancer	6-hour IV infusion for 3 days every 21 days until disease progression, dose 2.34 g/m^2/day	Grade 4 anaemia (1), grade 4 episode hypersensitivity (1), grade 3 thrombocytopenia (1), grade 3 fatigue and hypokalaemia (2)	PR (3), SD (14) for ≥12 weeks, overall median survival 6.2 months, 1-year survival 26%
Sodium phenylbutyrate[48] [a]	I	24	Solid tumours	120-hour infusion every 21 days, dose 150–515 mg/kg/day, 6 dose levels, 89 cycles	DLT (2): neurocortical accompanied by hypokalaemia, hyponatraemia, hyperuricaemia; other mild toxicities: fatigue and nausea	No CR or PR, SD (2) remained on therapy, other patients (3) remained on therapy
Sodium phenylbutyrate[49]	I	28	Solid tumours	Oral dose 9–45 g/day, 5 dose levels, until disease progression	DLT (4): hypocalcaemia, nausea and vomiting, fatigue, neurocortical toxicity; most common toxicity: grade 1–2 dyspepsia and fatigue	No CR or PR, SD (7) for >6 months, PD (11)

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Table III. Contd

Agent	Phase	No. of pts	Tumour type	Administration regimen	Adverse events (no. of pts)	Clinical trial outcome (no. of pts)
Sodium phenylbutyrate[50] a	I	23	Gliomas	Oral dose 9–36 g/day, 4 dose levels of a 28-day cycle until disease progression	DLT (2) at 36 g/day: fatigue, somnolence; DLT (1) at 27 g/day: grade 3 fatigue	CR (1) for 5 years, no PR, SD (5), PD (13), median survival 5.4 months
Sodium phenylbutyrate[51] a	I	21	Solid tumours	Infusion 60–360 mg/kg/day, 5 dose levels, 2 consecutive days a week for 2 weeks every month	DLT: short-term memory loss, sedation, confusion, nausea and vomiting; most common toxicities: grade 1 nausea/vomiting, fatigue, lightheadedness	No CR or PR, SD (3) without tumour progression for 4, 5, and 7 months, respectively
Valproic acid[52]	I	26	Solid tumours	1-hour infusion daily for 5 consecutive days of a 21-day cycle, dose 30–120 mg/kg/day, 5 dose levels	DLT (8): neurocognitive impairment, most common toxicity: neurological toxicity, no grade 3 or 4 haematological toxicity	No objective responses, SD (2) lasting 3 and 5 months, respectively
Entinostat (MS-275)[53] a	I	22	Solid tumours and lymphoid malignancies	Oral dose 2–8 mg/m^2, 4 dose levels, once weekly for 4 weeks of a 6-week cycle	DLT (4): grade 3 hypophosphataemia, hyponatraemia, hypoalbuminaemia; other AE: myelosuppression	No CR or PR, SD (1) for >8 months
Entinostat[54] a	I	27	Solid tumours or lymphomas	Oral dose 2–6 mg/m^2 once every 2 weeks; or 2 mg/m^2 twice weekly for 3 weeks followed by 1 week of rest; or 4 and 5 mg/m^2 once weekly for 3 weeks followed by 1 week of rest	DLT: hypophosphataemia and asthenia on the weekly and twice-weekly treatment schedule; no DLT on every other week schedule	No CR, PR (2), SD (6) lasting for 45 days to 10 months
MGCD 0103[56]	I	38	Solid tumours	Oral dose 12.5–56 mg/m^2/day, 6 dose levels, 3 times per week for 2 weeks of a 3-week cycle, 99 cycles	DLT (5): grade 3 fatigue, nausea, vomiting, anorexia, dehydration	No objective tumour responses, SD (5) for ≥4 cycles

a Sponsored by a grant from the National Cancer Institute.

AE = adverse events; **CR** = complete response; **DLT** = dose-limiting toxicity; **IV** = intravenous; **PD** = progressive disease; **PR** = partial response; **pts** = patients; **SAHA** = suberoylanilide hydroxamic acid; **SD** = stable disease.

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Table IV. A summary of the clinical trials using histone deacetylase inhibitors in combination with other agents in the treatment of cancer

Agent	Combination drugs	Phase	No. of pts	Tumour type	Clinical trial outcome (no. of pts)
Valproic acid[131]	Epirubicin	I	48	Solid tumours	PR (9), SD (16)
Magnesium valproate[132]	Hydralazine	II	17	Solid tumours	PR (4), SD (8), PD (3)
Valproic acid[133]	Azacitidine	I	55	Solid tumours and others	No CR and PR, SD (14; 25%) lasting 4–12 months
Vorinostat (SAHA)[134]	Carboplatin, paclitaxel	I	28	Solid tumours	PR (11), SD (7)
Tacedinaline (CI 994)[135]	Carboplatin and paclitaxel	I	30	Solid tumours	CR (2), PR (5)
Phenylbutyrate[136]	Fluorouracil	I	9	Colorectal cancer	SD (3) lasting 12+, 25 and 54 weeks, PD (1)
Tacedinaline[137]	Capecitabine	I	54	Solid tumours	PR (1), SD (19)
Tacedinaline[138]	Gemcitabine	II	86	Pancreatic cancer	No CR, PR (8), response rate 12%, median survival: 194 days, decrease in pain

AE = adverse events; **CR** = complete response; **DLT** = dose limiting toxicity; **PD** = progressive disease; **PR** = partial response; **pts** = patients; **SAHA** = suberoylanilide hydroxamic acid; **SD** = stable disease.

sodium phenylbutyrate had a complete response for 5 years. The overall response rate in this trial was 5%, with a median survival time of 5.4 months. This study defined the MTD and recommended a dose of phenylbutyrate 27 g/day for a phase II clinical trial.

The therapeutic activity of valproic acid has been studied in haematological malignancies[144] and advanced solid tumours.[52] In patients with advanced solid tumours, the MTD of valproic acid was 60 mg/kg/day and the most common toxicity was neurological. Histone hyperacetylation was induced and HDAC2 was downregulated in the peripheral blood lymphocytes of patients.[52] No objective responses were noted in this study, but 2 of 18 evaluable patients had stable disease.

The aliphatic acid AN 9, a prodrug of butyric acid, has been shown to inhibit proliferation and differentiation and to induce apoptosis. AN 9 was investigated in phase I and II clinical trials for the treatment of patients with solid tumours such as non-small cell lung cancer.[46,47] AN 9 was tolerated and exhibited antitumour activity as a single agent in a phase II clinical trial.[47] A total of 47 patients with non-small cell lung cancer were intravenously administered AN 9 at a dose of 2.34 g/m^2/day for 3 days in a 21-day cycle until disease progression.[47] Observed toxicity included grade 1–2 fatigue, nausea and dysgeusia. Partial responses were achieved in three patients, and 14 patients had stable disease for over 12 weeks. The overall median survival was 6.2 months.

5.1.4 Benzamides

Entinostat, a novel HDAC inhibitor, has been used to treat patients with leukaemias, lymphomas or solid tumours in phase I and II clinical trials.[53,145-147] Entinostat appeared to have limited antitumour activity in patients with solid tumours.[53,146,147] In a phase I clinical trial, 21 patients with solid tumours and one patient with a lymphoid malignancy were treated with entinostat with an MTD of 6 mg/m^2. The dose-limiting toxicities were hypophosphataemia, hyponatraemia and hypoalbuminaemia. Acetylation of proteins in PBMCs was increased following treatment, but no complete or partial response was observed except for disease stabilization achieved in one patient.[53] Recently, a phase I clinical trial was conducted in patients with refractory solid tumours and lymphomas, and entinostat was shown to have antitumour activity.[54] Entinostat was administered orally with three treatment schedules and proved to be well tolerated up to 6 mg/m^2 once every other week or 4 mg/m^2 once weekly for 3 weeks in a 28-day cycle. Two of 27 patients had confirmed partial responses and six patients had stable disease ranging from 45 days to 10 months.[54]

Another agent in the benzamide group, MGCD 0103, is currently being investigated in patients with advanced solid tumours.[56] Thirty-two

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of 38 patients enrolled in this study were assessable. Five of these 32 assessable patients had stable disease during treatment for four or more cycles (3-week cycle).

5.2 HDAC Inhibitors in Combination with Other Agents

HDAC inhibitors have been shown to have varying antitumour activity in both preclinical and clinical trials. However, in some solid tumours the efficacy of HDAC inhibitors as single agents did not result in favourable outcomes.[34-36] Tumourigenesis and progression is a complex process and may be due to several different mechanisms. The combination of HDAC inhibitors with other antitumour agents may be feasible and effective as a treatment approach. The combination of HDAC inhibitors with other epigenetic therapy or chemotherapeutic agents has been demonstrated to be safe and have antitumour activity.[131,132,134-137] HDAC inhibitors have been studied clinically in patients with solid tumours in combination with the topoisomerase II inhibitor epirubicin,[131] or the DNA methylation inhibitors hydralazine[132] or azacitidine.[133] Various cytotoxic chemotherapy agents have also been used in combination with HDAC inhibitors, such as carboplatin, paclitaxel,[134,135] fluorouracil[136] or its oral prodrug capecitabine,[137] or other antimetabolites, e.g. gemcitabine (table IV).[138] These examples are described in the following sections.

5.2.1 HDAC inhibitors in Combination with Epirubicin

The anticancer effect of the topoisomerase II inhibitor epirubicin has been demonstrated to be potentiated by valproic acid both *in vitro*[148] and *in vivo*.[149] In a phase I trial, combination therapy of valproic acid followed by epirubicin has been shown to be effective in patients with solid tumours.[131] The MTD for valproic acid was 140 mg/kg/day for 48 hours and for epirubicin 100 mg/m^2. Epirubicin-related toxicity was not observed to be exacerbated in this setting. Of the 44 assessable patients in this study,[131] partial responses were observed in nine patients in whom histone H4 acetylation was at least 2-fold increased. 16 patients had stable disease for over 12 weeks. In this study, pre-exposure to valproic acid was beneficial and was associated with the relaxation of chromatin structure, thereby facilitating the binding of epirubicin to substrate DNA.

5.2.2 HDAC inhibitors in Combination with Hydralazine

Hydralazine is a weak non-nucleoside DNA methylation inhibitor. Encouraging antitumour activity was observed in an earlier phase II clinical trial where combination therapy, including magnesium valproate (salt of valproic acid) and hydralazine, was administered a week before chemotherapy and until the last day of the final chemotherapy cycle to patients with refractory solid tumours.[132] In this clinical study a total of 27 patients signed informed consent. Three were ineligible and 7 patients were non-compliant. Seventeen patients were evaluable for toxicity. Fifteen patients were assessable for response. Four of these 15 assessable patients had partial responses and eight had stable disease. This study showed that the combination of the DNA methylation inhibitor and the HDAC inhibitor could overcome chemotherapy resistance, achieving high clinical benefit (80%).

5.2.3 HDAC Inhibitors in Combination with Azacytidine

Azacitidine is a DNAMT inhibitor approved by the US FDA in 2004 for the treatment of MDS.[150] In a phase I clinical trial, the HDAC inhibitor valproic acid was used in combination with azacitidine to treat 55 patients with advanced cancers, including colorectal cancer (n = 11), melanoma (n = 10) and breast cancer (n = 4).[133] Azacitidine at various doses from 20 to 94 mg/m^2 was administered daily to patients for 10 days and valproic acid was administered orally once daily every 28 days until progression of disease or serious toxicity occurred. This clinical trial showed that the combination of valproic acid and azacitidine 75 mg/m^2 was safe. Global DNA methylation and histone acetylation of PBMCs from patients were analysed on days 1 and 10 of each treatment cycle. Global DNA methylation showed

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a small reduction on day 10; however, it did not reach statistical significance. Conversely, hyperacetylated histone H3 was increased 2-fold with a higher frequency in patients having stable disease. No complete or partial responses were observed.[133]

5.2.4 HDAC Inhibitors in Combination with Carboplatin and Paclitaxel

Paclitaxel is often administered in combination with carboplatin to treat breast, ovarian and lung cancer. Carboplatin is a platinum analogue that exerts its cytotoxicity through the formation of platinum adducts with DNA, thus inducing inter- or intra-DNA cross-links. While paclitaxel binds to and inhibits depolymerization of tubulin, reports indicate that its antitumour activity was increased *in vivo* when combined with trichostatin A[151] and *in vitro* when combined with valproic acid.[152] Recently, in phase I clinical trials of vorinostat[134] or tacedinaline (CI 994)[135] in combination with paclitaxel and carboplatin, these drugs have demonstrated promising antitumour activity in patients with advanced solid tumours. In one trial, vorinostat was administered orally once daily for 2 weeks or twice daily for 1 week in a 3-week cycle. Paclitaxel and carboplatin infusion were administered once in one cycle. Of the 25 assessable patients, 11 patients had partial responses and seven had stable disease.[134] In the other phase I study, tacedinaline was orally administered in combination with carboplatin and paclitaxel. Coadministration of carboplatin and paclitaxel did not affect the absorption and disposition of tacedinaline. Lymphocyte histone H3 acetylation level was associated with disease response. Complete responses were seen in two of 30 patients and partial responses were seen in five patients.[135] These clinical trials demonstrate that combination therapy of HDAC inhibitors with other agents is feasible, with promising outcome in solid tumours.

5.2.5 HDAC Inhibitors in Combination with Fluorouracil

HDAC inhibitors have also been examined in combination with traditional chemotherapeutic drugs such as fluorouracil or its prodrug capecitabine.[136,137] Fluorouracil is typically used to treat breast, colorectal, and various other aerodigestive tract cancers. It has several biochemical effects including inhibition of thymidylate synthase activity, and disruption of DNA and RNA synthesis. HDAC inhibitors have been shown to enhance fluorouracil cytotoxicity by downregulating thymidylate synthase in human cancer cells.[153] In a phase I clinical trial, patients with metastatic colorectal cancer were treated with an infusion of fluorouracil over 24 hours and sodium phenylbutyrate administered over 120 hours weekly until unacceptable toxicity.[136] The combination of fluorouracil followed by sodium phenylbutyrate was well tolerated and three of nine patients had stable disease. Although the number of patients was limited, this study indicated the potential activity of combination therapy.

5.2.6 HDAC Inhibitors in Combination with Capecitabine

Capecitabine is approved in the US for the treatment of breast and colorectal cancer. In order to achieve additive antitumour effect, tacedinaline in combination with capecitabine was orally administered to patients with solid tumours in a phase I clinical trial.[137] The pharmacokinetics of tacedinaline were not affected by capecitabine. The MTD recommended for the phase II clinical trial was 6 mg/m^2 (or 10 mg) for tacedinaline in combination with capecitabine administered at 2000 mg/m^2/day for 2 weeks in a 3-week cycle. The principal dose-limiting toxicity observed in patients was thrombocytopenia, with the most common adverse events being anorexia, diarrhoea, nausea and vomiting. The combination treatment showed a moderate anticancer effect; one of 54 patients had a partial response and 19 patients had stable disease.

5.2.7 HDAC Inhibitors in Combination with Gemcitabine

Gemcitabine is reasonably well tolerated and has been used in the treatment of patients with pancreatic cancer. Gemcitabine alone demonstrates a relatively low response rate in these

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patients. Other agents have been used in combination with gemcitabine in an attempt to increase its anticancer effect. Recently, a phase II clinical trial using gemcitabine in combination with tacedinaline was conducted in 86 patients with pancreatic cancer.[138] A control group (88 patients) were administered gemcitabine and placebo capsules. Tacedinaline 6 mg/m^2/day was administered orally on days 1–21, and gemcitabine was administered as an infusion of 1000 mg/m^2/day on days 1, 8 and 15 in each 28-day cycle. Grade 3 and 4 leukopenia, anaemia, thrombocytopenia and asthenia were the main toxicities. The response rate assessed by the individual investigators was 12%, whereas it was 1% based on the central radiologist's assessment. In this study, the combination therapy of gemcitabine plus tacedinaline did not increase patients' survival or response rate and seemed to have no advantage over gemcitabine alone in treating patients with pancreatic cancer.

6. Conclusions

Studies thus far using HDAC inhibitors, either alone or in combination with other epigenetic therapy or chemotherapeutic agents, have raised more questions than answers. Several of these questions remain to be addressed by clinical investigators, while even more need to be addressed by basic science researchers. Addressing these questions will clarify some of the unexplained observations noted in clinical trials using HDAC inhibitors.

The first of these observations highlights the increased susceptibility of transformed cells to HDAC inhibitors. Although histone acetylation occurs in normal and transformed cells, it is poorly understood why transformed cells are more sensitive to HDAC inhibitors. Many mechanisms have been suggested to explain the selective preference of HDAC inhibitors for transformed cells; one such mechanism proposes that HDAC inhibitors (e.g. valproic acid) selectively up-regulates the expression of death receptors DR5, Fas and death ligands TRAIL and FasL, which in turn induce cell apoptosis in fully transformed leukaemia cells, but not in normal cells.[58] Another mechanism implicates the accumulation of ROS in transformed cells treated with HDAC inhibitors (e.g. vorinostat and entinostat).[94] Additionally, it was reported that vorinostat in combination with an estrogen derivative, 2-medroxyestradiol, can cause an increase in ROS, activation of caspase, and apoptosis in human leukaemia cells but not in normal cells.[154]

The second observation is the selective preference of HDAC inhibitors for tumour cells with certain molecular expression patterns/signals. For example, HDAC inhibitors, including panobinostat, were found to be more effective in promoting apoptosis in EGFR-mutated cancer cells[59] or in tumour cells with high E2F1 activity[155] compared with cancer cells without these molecular signatures, indicating that depletion of EGFRs and oncogenic E2F1 pathway may be involved in HDAC inhibitor-induced apoptosis. Also, valproic acid selectively inhibited the invasive characteristics of bladder cancer cells, but the invasiveness of prostate cancer cells in this study was not affected after valproic acid treatment, suggesting that different types of cancer cells might exhibit different molecular expression signatures, and thereby different invasion mechanisms.[88]

The third observation is the controversy about which of the two HDAC inhibitor approaches is better: the use of selective HDAC inhibition (e.g. romidepsin and entinostat) or pan-HDAC inhibition (e.g. vorinostat and belinostat). No convincing clinical or experimental evidence is currently available to support the use of either type of HDAC inhibitor. However, evidence from some clinical trials[43,44,53] suggests that blocking one or two signalling pathways through inhibition of one or two HDACs by specific/selective HDAC inhibitors might not be sufficient in achieving inhibition of tumour growth.

The fourth observation indicates that the use of HDAC inhibitors in combination treatment regimens requires a better understanding of the mode of action of each administered agent, in addition to the molecular profile of treated patients. HDAC inhibitors were administered concurrently and sequentially in different clinical

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trials with no clear hypothesis, or rational or documented molecular studies to support either approach. In cancer cells, both concurrent and sequential treatments with trichostatin A and fluorouracil showed a synergistic effect,[153] but in some trials HDAC inhibitors were initially administered, then followed by other agents to achieve a synergistic effect. A good example is epirubicin (topoisomerase II inhibitor), the DNA binding of which is facilitated by the presence of a relaxed chromatin structure. Thus, in a phase I clinical trial of the combined treatment regimen of valproic acid and epirubicin, valproic acid was administered first followed by epirubicin, which resulted in an active combination.[131] Additionally, the combination effect of HDAC inhibitors with other epigenetic agents or cell cycle-dependent agents was also studied. Baylin and colleagues reported that the administration of a HDAC inhibitor (e.g. trichostatin A) following decitabine (5-aza-2′deoxycytidine) was able to effectively restore the expression of hypermethylated/silenced genes in cancer.[156] Pretreatment of cancer cells with decitabine is required for the transcriptional activation of genes by trichostatin A. In a recent preclinical study, vorinostat was combined with a cell cycle-dependent agent, cytarabine (cytosine arabinoside, ara-C), to treat acute leukaemias. Cytotoxic synergism was observed only when vorinostat was followed by cytarabine with a vorinostat-free interval, while concurrent treatment resulted in cytotoxic antagonism.[157] This antagonism was attributed to cell cycle arrest caused by vorinostat in the G1 or G2 phase, which reduced the availability of cells in the S-phase, thereby limiting the cytotoxic action of cytarabine. This experimental evidence suggests that mechanism-based sequence of drug administration is crucial for an effective combination treatment.

The fifth observation notes that in most combined treatments including two or three anticancer agents, HDAC inhibitors are commonly administered at several dose levels, while the doses of the other agents are kept relatively fixed. Usually, two or three dosing regimens are used followed by dose modification, which is based on the occurrence of toxicities and finally selection of the optimal dose and administration schedule for the next phase of clinical trials. This underscores the importance of filling the information gap between clinical phenotype, translational research, and the patient's molecular profile ('blueprint').

The sixth observation relates to the possible contribution of dietary and nutritional factors that might influence the activity of HDAC inhibitors. Natural foods like garlic and broccoli have HDAC inhibitory activity.[17] Although a high-fat meal was reported to slightly increase the extent of absorption of vorinostat,[158] in the absence of clinical and translational evidence, the question of whether food has an effect on HDAC inhibition remains to be addressed.

7. Future Directions

HDAC inhibitors are a relatively new group of epigenetic agents that have multiple substrates including histones and non-histone proteins, suggesting that HDAC inhibitors may be involved in multiple cellular processes. However, the precise mode of action of HDAC inhibitors and their influence on cell signalling pathways, long and short term consequences on the molecular profile of patients, and the use of different doses and routes of administration in combination treatments, remain to be fully elucidated. Unlike colorectal cancer patients in some clinical trials, where patients are genetically stratified into different arms according to their V-ki-ras2 Kirsten rat sarcoma viral oncogene homologue (KRAS) and V-raf murine sarcoma viral oncogene homologue B1 (BRAF) mutational status, epigenetic molecular stratification for patients is currently unavailable. However, in future clinical trials it might be possible to initiate epigenetically pre-stratified prospective clinical trials using methylation and acetylation marks in genes relevant to the administered class of HDAC inhibitor and combination treatment regimens. These trials might provide valuable information to: (i) further support the clinical utility of HDAC inhibitors either as single agents or in combination treatments; (ii) assist in treatment design; and (iii) aid in drug selection based on the mode of action of the HDAC inhibitors and the molecular sig-

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nature associated with different cancer types. Thus, to permit the realization of personalized effective epigenetic therapy, it might not be premature to start screening patients for epigenetic alterations before the initiation of a cancer treatment regimen containing an epigenetic agent, whether it is an HDAC inhibitor, a DNAMT inhibitor or both.

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