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REVIEW PAPER

Epigenetic approach for angiostatic therapy: promising combinations for cancer treatment

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Abstract Cancer cells are often dependent on epigenetic pathways for their survival. Consequently, drugs that target the epigenome, rather than the underlying DNA sequence, are currently attracting considerable attention. In recent years, the first epigenetic drugs have been approved for cancer chemotherapy, mainly for hematological applications. Limitations in single-drug efficacies have thus far limited their application in the treatment of solid tumors. Nevertheless, promising activity for these compounds has been suggested when combined with other, distinctly targeted agents. In this review, we discuss the anti-angiogenic activity of histone deacetylase and DNA methyltransferase inhibitors and their combinations with other targeted (anti-angiogenic) therapeutics in treatment of solid tumors. The role that these inhibitors play in the inhibition of tumor angiogenesis, particularly in combination with other targeted agents, and the advantages they present over broad acting anticancer agents, are critically discussed.

Keywords Anti-angiogenesis · Clinical trials · Combination therapy · Epi-drugs · Histone deacetylase inhibitors · DNA methyltransferase inhibitors · Solid tumors · Tumor vasculature

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Abbreviation

5FD	5-Fluoro-2′-deoxycytidine
Ang-2	Angiopoietin2
AML	Acute myeloid leukemia
AZA	Azacitidine, 5-AZA-CR, 5-azacytidine
bFGF	Basic fibroblast growth factor
FGFR	Fibroblast growth factor receptor
CAM	Chorioallantoic membrane of the chicken embryo
CTCL	Cutaneous T cell lymphoma
CYR61	Cysteine-rich angiogenic inducer 61
DAC	Decitabine, 5-AZA-2′-deoxycytidine, 5-AZA-CdR
DNMT	DNA methyltransferase
E-cadherin	Epithelial cadherin
EC	Endothelial cells
EGCG	Epigallocatechin gallate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase (eNOS)
FAO	Fatty acid oxidation
FSC	Feedback system control
HAT	Acetyltransferase
HDAC	Histone deacetylase
HIF-1α	Hypoxia-inducible factor 1 alpha
HUVEC	Human umbilical vein endothelial cells
HYD	Hydralazine
IC_{50}	The half maximal inhibitory concentration
IGF1	Insulin-like growth factor 1
IL	Interleukin
KP1019	*trans*-[Tetrachlorobis(1*H*-indazole) ruthenate(III)
miRNA	Noncoding microRNA
MMP	Matrix metalloproteinase
MTA1	Metastasis-associated protein 1

mTOR	Mechanistic target of rapamycin
MVD	Microvessel density
NAMI-A	*trans*-[Tetrachloro(dimethylsulfoxide)(imidazole)ruthenate(III)]
NOX4	NADPH oxidase 4
NSCLC	Non-small cell lung cancer
NuRD	Nucleosome remodeling and deacetylase
OS	Overall survival
p300-HAT	p300 histone acetyltransferase
PBA	Phenylbutyrate
PDGF-B	Platelet-derived growth factor subunit B
PDGFR-B	Platelet-derived growth factor subunit B receptor
PFS	Progression-free survival
PVRL2	Poliovirus receptor-related 2
RAPTA-C	Ru(η^6-*p*-cymene)(pta)Cl_2
RAPTA-T	Ru(η^6-toluene)(pta)Cl_2
RCC	Renal cell carcinoma
SAHA	Vorinostat
SAM	S-adenosyl methionine
siRNA	Small interfering RNA
SIRT	Histone deacetylase sirtuins
TGF-β	Transforming growth factor beta
TIMP3	Tissue inhibitor of matrix metalloproteinase 3
Tie-2	Angiopoietin 1 receptor
TKI	Tyrosine kinase inhibitor
TSA	Trichostatin A
TSG	Tumor suppressor gene
TSP-1	Thrombospondin 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VE-cadherin	Vascular endothelial cadherin
VHL	Von Hippel–Lindau
VPA	Valproic acid
WIF-1	Wnt inhibitory factor 1
ZEB	Zebularine

Introduction

The epigenome is at the root of many diseases including neurodegenerative and immune disorders, as well as many cancers. The emerging role of epigenetic regulation in the malignant transformation of cells during carcinogenesis has been extensively investigated in recent years [1], as carcinogenesis does not only depend on genetic alterations but also on gene expression changes that do not alter the primary DNA sequence, i.e., epigenetic pathways. Epigenetics is 'the study on mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence' [2], and consists of changes in DNA methylation, histone modifications and noncoding RNA, such as microRNAs (miRNA) or small interfering RNAs (siRNA). DNA methylation and posttranslational histone modifications are the two best understood epigenetic mechanisms of gene silencing [3]. DNA methylation involves the covalent addition of a methyl group to DNA, a process that is catalyzed by the enzyme family of the DNA methyltransferases (DNMTs). In humans, methylation mainly occurs predominantly at the C5 position of cytosine bases, which precedes a guanine nucleotide, referred to as a 'CpG dinucleotide'. CpG dinucleotides can cluster near the promoter regions of many genes and are then referred to as 'CpG islands' [4]. Such DNA methylation in the promoter region of genes can result in gene silencing through the steric hindrance of transcription factor binding. Alternatively, epigenetic regulation occurs at the level of nucleosomes, where DNA is wrapped around the histone proteins whose amino acid 'tails' protrude from the structure and are available for posttranslational modifications. Various modifications can occur on these histone proteins, the best characterized being the acetylation of histone protein tails. This process is regulated by a crosstalk between histone acetyltransferases (HATs) and histone deacetylases (HDACs) [5]. Acetylation of histone proteins prevents the condensation of chromatin and is associated with open and transcriptionally active chromatin. The removal of acetyl groups from histones by HDACs allows interactions between the DNA and histone proteins to induce chromatin condensation (or the formation of heterochromatin), which blocks the binding sites for transcription factors and results in gene silencing.

Contrary to genetic alterations, epigenetic changes are reversible. Thus, agents that inhibit HDACs or DNMTs (termed epi-drugs) may reactivate previously silenced genes. To date, six epi-drugs (HDAC or DNMT inhibitors) have been FDA-approved for cancer treatment. In 2004, 5-azacytidine (azacytidine or AZA; manufactured by Celgene as Vidaza®) that specifically inhibits DNA methylation and, in 2006, its variant 5-AZA-2′-deoxycytidine (decitabine; manufactured by Eisai as Dacogen®/DAC) were approved both for the treatment of higher-risk myelodysplastic syndromes. Vorinostat (SAHA, Merck)—an HDAC inhibitor—received FDA approval in 2006 for the treatment of cutaneous T cell lymphoma (CTCL) and induced complete response in acute myeloid leukemia (AML) patients. Romidepsin (Istodax®, Celgene), another HDAC inhibitor, showed efficacy in the treatment of CTCL patients [6]. Belinostat (BELEODAQ, Spectrum Pharmaceuticals, Inc.), an HDAC inhibitor, was approved in 2014 for the treatment of peripheral T-cell lymphoma. The potency of these compounds in the treatment of hematological cancers has already been demonstrated and described in detail elsewhere [7]. However, their activity in solid tumors has generally been disappointing [8] suggesting careful consideration of combination treatment strategies.

Besides the epi-drugs mentioned here, there is an increasing interest in the role and development of natural products as a treatment modality for cancer [9, 10] and there are a number of nutrients that have shown to effect epigenetic mechanisms, therefore called epi-nutrients [11, 12].

Generally, cancer cells are frequently associated with genetic instability, global DNA hypo-methylation and a loss in specific histone modifications [1, 13, 14], facilitating the development of drug resistance. Endothelial cells (EC), which compose the inner layer of blood vessels and govern the process of angiogenesis, were originally considered to be genetically stable and homogenic. Thus, the targeting of EC with anti-angiogenic compounds represented an ideal therapeutic approach to minimize the occurrence of resistance and toxic side effects [15, 16]. Unfortunately, clinical data revealed that cancer cells can develop and promote several adaptive mechanisms of resistance to currently available anti-angiogenic treatments, e.g., antagonists of vascular endothelial growth factor (VEGF) [17–20]. Therefore, anti-angiogenic compounds are currently being tested in combination with other agents to overcome their major limitations.

HDAC and DNMT inhibitors have been shown to play a major role in the regulation of a variety of biological processes, including apoptosis induction and cell cycle arrest, DNA damage and repair, and the inhibition of angiogenesis [21]. Their activity in angiogenesis inhibition is of particular interest due to the major clinical role of targeted compounds intervening with various stages of the angiogenic cell-signaling cascade. HDAC and DNMT inhibitors have not only been implicated in the inhibition of angiogenesis indirectly, through the re-activation of tumor suppressor genes (TSGs) in cancer cells, but have also been shown to have direct inhibitory effects through the epigenetic regulation in EC themselves. As such, it has been demonstrated that the reversal of epigenetic modifications can be achieved by DNMT or HDAC inhibitors mediated by the re-activation of angiogenesis-suppressing genes that have been silenced in tumor-conditioned EC [22]. Therefore, apart from the 'standard' modulators of angiogenesis, such as VEGF(R) or endothelial nitric oxide synthase (eNOS), DNMT and HDAC inhibitors may halt or reverse expression levels of certain EC genes and may represent attractive therapeutic targets.

In this review, we discuss the anti-angiogenic activity of HDAC and DNMT inhibitors and their combinations with other targeted therapeutics in cancer treatment. We address the role that epi-drugs play in the inhibition of tumor angiogenesis, particularly in combination with other agents, and the advantages they present over broad acting anticancer agents.

Histone deacetylase inhibitors

To date, the development of histone deacetylase (HDAC) inhibitors has been the focus of epigenetic drug discovery programs. These drugs lead to an increase in chromatin acetylation levels and therefore to an 'open' chromatin state resulting in the expression of previously silenced genes. Histone acetyl transferases (HATs) and HDACs can thus locally alter chromatin structure to regulate translation at specific promoter sites acting as transactional co-activators or co-repressors [23]. HATs have also been shown to interact with non-histone transcription factors [24, 25].

Various HDACs have been shown to play key roles in the regulation of angiogenesis via a number of different mechanisms. HDACs can be divided into four classes based on their functional characteristics and homology to yeast HDACs (Table 1) [26]. Class I, II and IV HDACs have a zinc-dependent active site, whereas Class III HDACs are unique and have NAD+ dependent enzymes called sirtuins [26]. Class I and II HDACs are of particular interest as they have been shown to be directly involved in the suppression of certain anti-angiogenic factors [27].

The activity of HDAC inhibitors results in hyperacetylation, which can induce altered gene regulation (including DNA repair) and the regulation of angiogenesis-related genes leading to apoptosis and cell cycle arrest [21, 28]. With the HDAC inhibitors approved by the FDA for the treatment of cancer, i.e., vorinostat, romidepsin, belinostat and panobinostat, a clear association between HDAC inhibition and the suppression of certain pro-angiogenic factors has been shown, including hypoxia-inducible factor alpha (HIF-1α), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), chemokine (C-X-X motif) receptor 4, angiopoietin, tunica intima endothelial kinase 2 and eNOS [21, 28, 29]. Figure 1 provides a schematic overview of the anti-angiogenic mechanisms involved in HDAC and DNMT inhibition.

The most relevant and well-studied activity of HDACs in the regulation of angiogenesis is their activity in the modulation of hypoxia-induced angiogenesis, most notably through the regulation of HIF-1α. Under normoxia, the deacetylation of HIF-1α is required to regulate oxygen supply and glucose metabolism. Under hypoxia, it regulates angiogenesis and it is overexpressed during tumorigenesis [29, 30]. Hypoxia-induced HIF-1α signaling regulates a variety of angiogenic signaling pathways, particularly through increasing the expression of VEGF [31]. As a consequence, the major direct anti-angiogenic effect of an HDAC inhibitor involves the inhibition of HIF-1α via acetylation [29, 32, 33]. The downregulation of endothelial nitric oxide synthase (eNOS) expression has also been implicated as a mechanism of anti-angiogenic activity in

Table 1 Overview of targeting angiogenesis via HDAC and DNMT inhibition

Target	Class	Drug/molecule	Transcriptional target	Biological effect	References
HDAC1	I	TSA	↓ p53, VHL, HIF-α, VEGF, VEGFR-1, 2, NOX4	↓ EC migration and tube formation ↓ TGFβ1-induced angiogenesis in vivo ↓ Sprouting and tube formation in vivo	[33, 46, 48, 49]
		Romidepsin	↓ HIF-1α, VEGF under hypoxia	↓ MVD and HIF-1 α activity in vivo	[40]
HDAC 1, 3	I	Entinostat	↓ Cyclin D1, VEGF, HIF-1α, IL-6, IL-8, MMP-2, MMP-9	↓ Circulating VEGFR2 + EC, ↓ CD31+/ vWF + blood vessels, ↓ lung metastasis in vivo	[39]
HDAC7	IIa	HDAC7 silencing	↓ MMP10	Failure in endothelial cell–cell adhesion during embryogenesis	[42]
		HDAC7 silencing	↓ PDGF-B, PDGFR-B	↓ EC migration and tube formation	[27]
HDAC9	IIa	HDAC9 silencing	↑ miR-17-92 cluster	↓ EC tube formation and sprouting ↓ Retinal vessel outgrowth	[44]
HDAC6	IIb	HDAC6 silencing	Deacetylation of cortactin	EC migration and sprouting, vessel formation	[45]
HDAC	III	SIRT1	↓ MMP14, p53, CXCR4 ↑ ↓ NF- κB	↓ Tip cell activity, EC senescence, protective effects	[51]
HDAC	III	SIRT6	↓ NF- κB, interleukins	Vascular remodeling	[53]
		SAHA		↓ Tube formation	[49]
		PBA	↑ PPAR-α	↓ MVD in vivo, ↓EC proliferation	[210]
		Dacinostat	H3 acetylation,↑ p21, ↓surviving, Tie-2, Ang-2	↓ EC proliferation, tube formation and invasion ↓ MVD in vivo	[50]
		Resveratrol^{NP}	↓ VEGF	↓ EC proliferation ↓ MVD in vivo	[211]
		Silymarin^{NP}	↓ HIF-α, NOX under hypoxia ↓MMP-2	↓ EC proliferation and EC tube formation, ↑EC apoptosis, ↓tumor vascularity and MVD in vivo	[60, 62–64]
DNMT1		DAC	↑ WIF, miR126, EGFL7, TSP1, JUNB, IGFGP3	↓ EC proliferation, ↓ MVD tumor vessel Development in vivo	[79, 87, 90, 95]
		Guadecitabine	↑ CDKN2A, DLEC1, RUNX3	↓ MVD in vivo	[92]
		Disulfiram	↑ RECK	↓ Pro-angiogenic MMP2 and MMP9	[97, 98]
		Curcumin^{NP}	↓STAT3	↓EC proliferation ↓bFGF stimulated neovascularization	[107, 111]
		EGCG^{NP}	↓ HIF-α, VEGF, NF- κB ↑RECK	↓EC tube formation ↓Capillary density and tumor VEGF expression in vivo	[102, 104, 105]
		ZEB	↑ ICAM1, TSP1, JUNB, IGFBP3	↑ Leukocyte adhesion to EC	[38, 95]
DNMT1, 3a, 3b		HYD		↓ EC tube formation, proliferation and migration ↓VEGF and MVD in vivo	[100, 101]

↓ downregulation/decreased expression/inhibition

↑ upregulation/increased expression/stimulation

AZA azacitidine, *bFGF* basic fibroblast growth factor, *DAC* decitabine, *DNMT* DNA methyltransferase, *EGCG* epigallocatechin gallate, *EC* endothelial cells, *HDAC* histone deacetylase, *HIF-1α* hypoxia-inducible factor 1 alpha, *HYD* hydralazine, *IGF* insulin-like growth factor, *miRNA* noncoding microRNA, *MMP* matrix metalloproteinase, *MVD* microvessel density, *NP* natural product, *NOX4* NADPH oxidase 4, *PBA* phenylbutyrate, P*DGF-B* platelet-derived growth factor subunit B, FK228; *SAHA* vorinostat, *SIRT* histone deacetylase sirtuins, *TGF-β* transforming growth factor beta, *TIMP3* tissue inhibitor of matrix metalloproteinase 3, *Tie-2* angiopoietin 1 receptor, *TSA* trichostatin A, *TSP-1* thrombospondin 1, *VEGF* vascular endothelial growth factor, *VEGFR* vascular endothelial growth factor receptor, *VHL* Von Hippel–Lindau, *VPA* valproic acid, *WIF-1* Wnt inhibitory factor 1, *ZEB* zebularine

EC [34]. In vivo anti-angiogenic activity of the HDAC inhibitor valproic acid (VPA) in the chorioallantoic membrane of the chicken embryo (CAM) and Matrigel plug assays was believed to be associated with reduced eNOS expression [35]. VPA and its various different formulations are currently approved for the treatment of seizures,

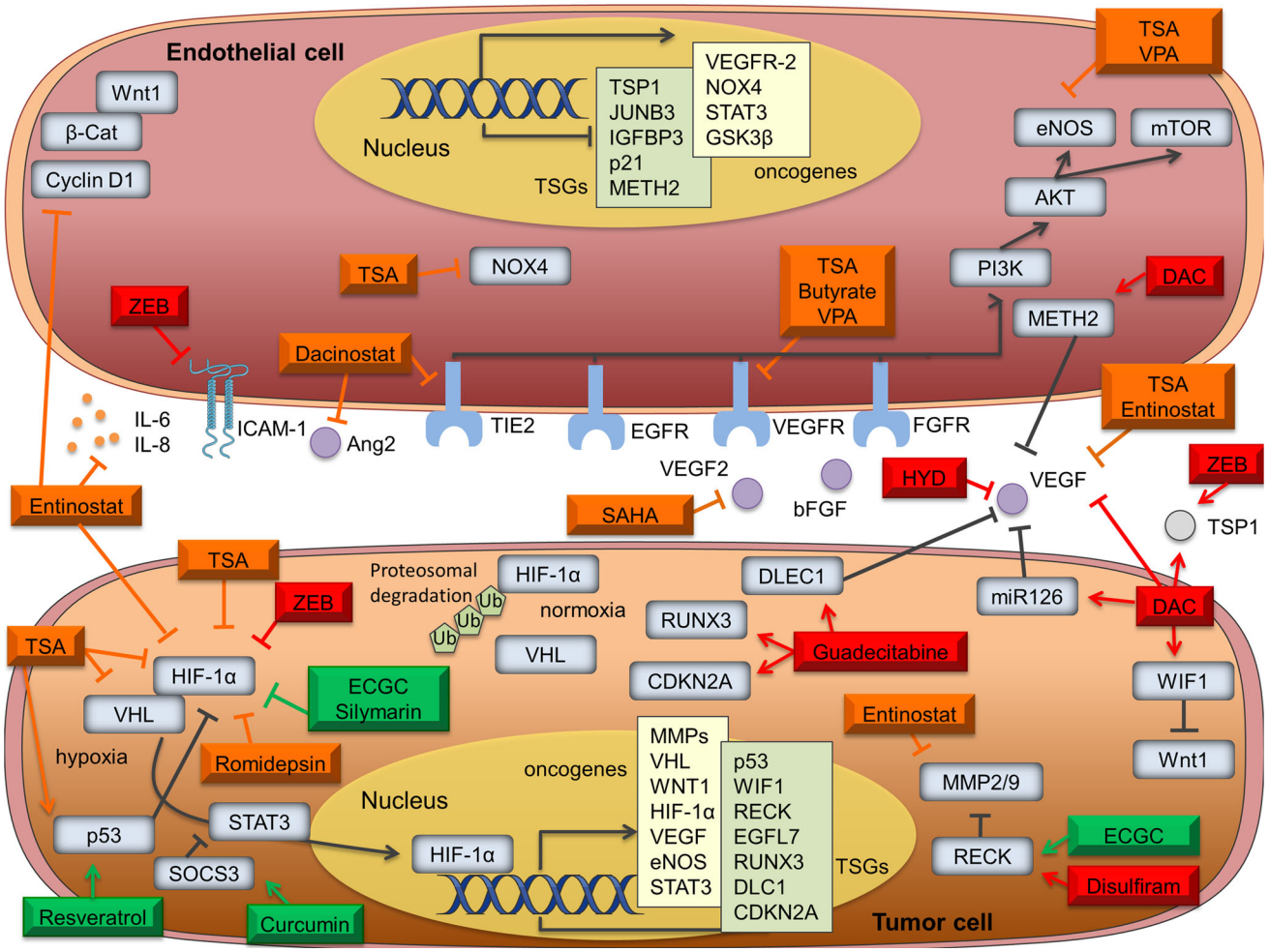

Fig. 1 Schematic overview of HDAC and DNMT inhibitors and their targets. HDAC inhibitors are represented by *orange* boxes, DNMT inhibitors are represented by *red* boxes, and natural products with HDAC or DNMT inhibitor function are represented by *green* boxes. *Arrows* indicate induction and inhibitory lines indicate inhibition, cumulatively leading to decreased expression of proteins and receptors that promote angiogenesis. DNMT inhibitors: *AZA* azacitidine, *DAC* decitabine, *GUA* Guadecitabine, *HYD* Hydralazine, *ZEB* Zebularine, HDAC inhibitors: *SAHA* vorinostat, *TSA* Trichostatin A, *VPA* Valproic acid, *Natural products* Curcumin, *EGCG* Epigallocatechin gallate, *Ang2* angiopoietin 2, *β-cat* β-catenin, *bFGF* basic fibroblast growth factor, *CDKN2A* cyclin-dependent kinase Inhibitor 2A, *DLEC-1* Deleted In Lung And Esophageal Cancer 1, *EGFL7* EGF-like domain-containing protein 7, *EGFR* epidermal growth factor receptor, *eNOS* endothelial nitric oxide synthase, *FGFR* fibroblast growth factor receptor, *HIF-1α* hypoxia-inducible factor alpha, *ICAM11* intercellular adhesion molecule 1, *IL-6/8* interleukin-6/8, *JUNB3* Insulin-like growth factor-binding protein 3, *miR126* microRNA 126, *MMP2/9* matrix metalloproteinases 2/9, *mTOR* mammalian target of rapamycin, *NOX4* NADPH oxidase 4, *PI3 K* Phosphoinositide 3-kinase, *RECK* Reversion-inducing-cysteine-rich protein, *RUNX3* Runt-related transcription factor 3, *SOCS3* Suppressor of cytokine signaling 3, *STAT3* signal transducer and activator of transcription 3, *TSGs* Tumor suppressor genes, *TSP1* thrombospondin 1, *VEGF* vascular endothelial growth factor, *VEGFR* vascular endothelial growth factor receptor, *VHL* Von Hippel–Lindau protein, *WIF1* Wnt inhibitory factor 1

episodes associated with bipolar or manic-depressive disorder, and migraine headache [26] and are also being evaluated for their anticancer potential in various ongoing clinical trials.

Class I HDACs have been implicated in angiogenesis induction via increased expression of HIF-1α with HDAC1 playing a major role in p53 and phosphorylated von Hippel–Lindau (VHL) inhibition [36]. It has also been shown that hypoxic conditions are associated with the increased expression of various Class I HDACs, including HDAC1, HDAC2 and HDAC3. Moreover, the overexpression of HDAC1 has been shown to induce angiogenesis via the suppression of p53 and VHL TSGs [32, 33], resulting in overexpression of HIF-α and VEGF. By inhibiting HDAC1 with trichostatin A (TSA), the expression of p53 and VHL was restored, resulting in decreased expression of HIF-1α and VEGF. Others have proposed that HDAC inhibition-mediated repression of HIF-1α is, in fact, independent of VHL and p53 and results from interactions with the HSP70/HSP90 chaperone proteins [37], or indirectly

through the acetylation of p300 [33]. TSA has also been shown to play a role in the regulation of endothelial cell anergy, a mechanism of immune escape mediated by angiogenesis and resulting in reduced leukocyte–vessel wall interactions [38]. The treatment of tumor-conditioned EC in vitro and murine tumors in vivo with TSA resulted in the re-expression of intercellular adhesion molecule-1 (ICAM1) in EC, the normalization of leukocyte–vessel wall interactions and increased leukocyte infiltration.

Similar to TSA, the HDAC inhibitor entinostat also targeted HIF-1α in breast cancer xenografts, and caused downregulation of angiogenic factors including cyclin D1, VEGF, interleukin (IL) IL-6 and IL-8, matrix metalloproteinase (MMP) MMP2 and MMP9 [39]. Furthermore, treated mice had significantly fewer circulating VEGFR2-positive EC, and immunohistochemical staining for CD31 revealed decreased microvessel density (MVD) of the treated tumors. Another specific HDAC inhibitor, romidepsin (FK228), suppressed HIF-1α activity under hypoxic conditions and demonstrated angiogenesis inhibition in a murine Lewis lung carcinoma model [40].

Class II HDACs have also been shown to directly interact with HIF-1α and are involved in the stabilization of hypoxia-induced HIF-1α via a proteasome-dependent pathway [41]. Class IIa HDACs are involved in maintaining vascular homeostasis. HDAC7 is selectively expressed during embryogenesis in order to maintain vascular integrity by suppressing the expression of MMP10 [42] and has also been shown to play an important role in the regulation of EC migration, possibly via the regulation of platelet-derived growth factor subunit B (PDGF-B) and platelet-derived growth factor subunit B receptor (PDGFR-B) expression [27]. Furthermore, HDAC7 is also involved in the transcriptional regulation of HIF-1α under hypoxic conditions in yeast hybrid models [43]. In this study, HDAC7 migrated from the cytoplasm to the nucleus of HEK-293 cells (a healthy embryonic kidney cell line), where it formed a complex with HIF-1α and p300 resulting in increased transcription of HIF-1α target genes including VEGF. Silencing of HDAC7 in EC by siRNAs resulted in morphological abnormalities and hindered their migration and tube formation without altering cell adhesion, proliferation or apoptosis [27]. Similarly, silencing or deletion of HDAC9 has been shown to have anti-angiogenic effects in vitro on EC tube formation and sprouting, as well as in vivo in retinal vessel outgrowth [44]. Likewise, the expression of Class IIb HDAC, HDAC6, is upregulated in hypoxic conditions via the deacetylation of actin-remodeling protein cortactin in EC and leads to the regulation of migration and sprouting [45].

A recent study demonstrated the role of Class I and II HDACs (particularly HDAC1, −4, −5 and −6) in VEGFR2 expression [46]. Results showed that three HDAC inhibitors, TSA, sodium butyrate and VPA, exhibit anti-angiogenic activity by reducing the expression of VEGFR2 in HUVEC [46]. The reduced expression of VEGFR2 was mediated by vascular endothelial (VE)-cadherin on a transcriptional level. Moreover, administration of HDAC inhibitors also resulted in the reduction of the VEGFR2 protein half-life, via the inhibition of VE-cadherin which has been shown to have an effect on the half-life of VEGFR2 [47]. TSA has also been implicated in angiogenesis inhibition via the reduced expression of NADPH oxidase 4 (NOX4) in EC, resulting in decreased NOX4 protein and H_2O_2 levels. It was shown that the regulation of NOX4 expression was achieved through the ubiquitination of p300-histone acetyltransferase (p300-HAT) and led to inhibition of transforming growth factor beta (TGF-β)-induced angiogenesis in vivo [48]. TSA and SAHA have been shown to inhibit VEGF-stimulated invasion and tube formation in an in vitro type I collagen gel assay [49]. Moreover, the anti-angiogenic activity of these drugs was shown in in vivo models of capillary-like network formation in embryonic bodies, the inhibition of VEGF-induced angiogenesis in the CAM models, and capillary sprouting in a rat aortic ring model. The activity of TSA was associated with the inhibition of VEGF receptors VEGFR1 and 2, as well as neuropilin-1. Dacinostat (LAQ824), a preclinical HDAC inhibitor, was shown to induce acetylation of histone H3 and to result in the upregulation of p21, as well as a reduction in the expression of the anti-apoptotic protein survivin in proliferating EC (but not in tumor cells) [50]. Moreover, dacinostat treatment resulted in the inhibition of the expression of EC-related genes, including Ang-2 and Tie-2 in EC.

Interestingly, only a few studies have described activity of Class III HDACs in relation to angiogenesis. Histone deacetylase sirtuins (SIRT) have been implicated in the regulation of p53 and are therefore of potential relevance in cancer, angiogenesis and aging [51, 52]. SIRT1 was shown to be involved in the transcriptional regulation of nuclear factor-κB (NF-κB), which affects proinflammatory cytokines, adhesion molecules and matrix degrading enzymes known to play a role in endothelial dysfunction during inflammatory disease [53, 54]. Furthermore, another HDAC, SIRT6, was shown to regulate the expression of genes involved in inflammation, vascular remodeling and angiogenesis in HUVEC cells, including IL-1B, IL-6, IL-8, COX-prostaglandin system, MMP2, MMP9, PAI-1, ICAM1, VEGF and FGF2 [53].

Certain natural products, also referred to as epi-nutrients, can influence cancer-associated epigenetic modulation either as HDAC or DNMT inhibitor [55]. These natural products, however, often do not act exclusively via epigenetic modulation but also via various other mechanisms [56]. Previously, it was suggested that angiogenesis

within the tumor microenvironment might be more sensitive to a mixture of natural products in a continuous low-dose administration instead of the conventional single agent drug given intermittently at higher doses [57]. The list of well-studied epi-nutrients is extensive and includes polyphenols from green tea (epigallocatechin gallate; EGCG), curcumin, resveratrol, silymarin, sulforaphane and dihydrocoumarin [56]. The phytoalexin, resveratrol, found in grapes and wine [58], has shown potent anticancer activity. Its IC_{50} values in breast and cervical cancer cells were significantly lower to those seen for the HDAC inhibitor sodium butyrate. Its HDAC inhibitory activity was further confirmed through proteins that were hyper-acetylated at histone H3 with increased expression of the p53 protein and downregulated levels of pERK1/2 [59]. Furthermore, resveratrol has also shown suppression of VEGF in RT-2 glioma cells and reduced MVD in a glioma rat model [60]. Silymarin, a phytochemical derived from milk thistle, showed anticancer activity in several cancers such as colon, lung, bladder, breast, prostate and pancreas, both in vitro and in vivo [61]. In addition, involvement in angiogenesis inhibition was shown via targeting of the VEGF pathway [62–64]. A recent study explored silymarin's activity as an HDAC inhibitor where it was shown to decrease levels of Class I HDACs (HDAC1, HDAC2, HDAC3 and HDAC8) in non-small cell lung cancer (NSCLC) cell lines [65].

As presented above, most HDACs, mainly of Classes I and II, have been shown to play a critical role in regulation processes in angiogenesis. Moreover, anti-angiogenic properties have been described for various HDAC inhibiting compounds in both in vitro angiogenesis assays and in tumor models (Table 1).

DNA methyltransferase inhibitors

Unlike histone acetylation, DNA methylation is capable of silencing genes through methylation of promoter regions occurring at DNA cytosine/guanine sites (so-called CpG islands) [66]. DNA methylation can affect transcription directly by preventing the binding of transcription factors to DNA or indirectly by attracting HDACs or methyl-CpG-binding proteins [67–70]. DNA methyltransferases (DNMTs) catalyze the transfer of a methyl group from the methyl donor S-adenosyl methionine (SAM) to DNA. There are three different DNMTs active in humans, i.e., DNMT1, DNMT3a and DNMT3b. DNMT1 binds hemi-methylated DNA, whereas DNMT3a/b can bind both hemi-methylated and unmethylated DNA [71]. Additionally, DNMT3a and b were shown to affect gene expression independently of promoter methylation, suggesting a broad mechanism of action [22]. In contrast, DNMT3a and b are mainly known to be important for de novo methylation during genomic imprinting in gametogenesis and in embryonic development [72]. DNMT1 is believed to not only be crucial in embryonic development, but also required for propagating methylation patterns during DNA replication in mitotic cell divisions [73–75].

DNMTs have a clear role in the regulation of angiogenesis as they have been demonstrated to affect gene expression in EC [76]. Therefore, it is not surprising that the deregulation of DNMTs contributes to tumor angiogenesis [22]. As such, the therapeutic intervention of DNMT function represents an area of particular interest in the search for anti-angiogenic cancer therapies. Currently, there are two FDA-approved DNMT inhibitors AZA and DAC. Various other DNMT inhibitors are in development or are currently undergoing clinical trials, including: guadecitabine, hydralazine (HYD), disulfiram and zebularine (ZEB). All of these DNMT inhibitors target DNMT1 (Table 1).

Deregulation of DNMTs during carcinogenesis can cause promoter hypermethylation and may contribute to angiogenesis through the suppression of important genes involved in the regulation of angiogenesis [77] including thrombospondin-1 (TSP1) [78], EGFL7 [79], ICAM1 [38] and RECK [77]. Furthermore, promoter hypermethylation at the promoter region of the WIF1 TSG (encoding for the Wnt protein antagonist WIF1) has been observed in various cancers [80–84] and stimulation of the Wnt pathway has been correlated with angiogenesis induction [85, 86].

DNMT inhibitors are able to reverse the hyper-methylated status of these silenced genes and restore their expression (Fig. 1). In cervical cancer, for example, treatment with the DNMT inhibitor DAC resulted in a significant increase in WIF1 mRNA expression. Re-expression of WIF1 led to a decrease of CD31, Wnt1 and VEGF, as well as angiogenesis inhibition in cervical cancer xenografts [87]. In colorectal cancer models, DAC treatment resulted in the re-expression of the TSG EGFL7, the host gene of miR-126 [79]. miR-126, which is suppressed in several cancers [79, 88], can inhibit angiogenesis by interfering with the translation of VEGF mRNA resulting in decreased VEGF formation [79, 89]. In melanoma cells, DAC was shown to restore the expression of another TSG, i.e., TSP1, by directly reducing DNMT1 expression in these cells [90]. TSP1 is an endogenous angiogenesis inhibitor known to inhibit EC motility and stimulate EC apoptosis, to affect vascular endothelium remodeling and to block neovascularization [91]. In addition, another study showed that in mice bearing A375 melanoma tumors, DAC treatment resulted in a significant reduction in tumor growth and a decrease in tumor vascularization, most likely due to DNMT1 inhibition leading to TSP1 re-expression [90]. Furthermore, guadecitabine, which is a dinucleotide

antimetabolite of DAC, was shown to have anti-tumor activity in hepatocellular carcinoma via demethylation of several TSGs, including CDKN2A, DLEC1 and RUNX3 [92]. Of these TSGs, DLEC1 is often silenced in hepatocellular carcinoma and expression of DLEC1 was reported to be associated with a decrease in VEGF and HIF-1α [93]. Expression of RUNX3 also inhibits angiogenesis as it was shown to result in a decrease of VEGF accounting for the inhibition of metastasis formation and angiogenesis in vivo. Moreover, guadecitabine was able to reduce tumor growth and inhibit angiogenesis in a HepG2 xenograft model [92].

Assessment of the anti-angiogenic activity of another DNMT inhibitor, zebularine (ZEB), was initiated following the discovery that ICAM1 expression was reduced in tumor-conditioned HUVEC as compared to quiescent HUVEC [38]. Suppression of the adhesion molecule ICAM1 is reported to be the result of pro-angiogenic factors leading to an immune escape [94]. Treatment with the DNMT inhibitor ZEB led to a significant increase in ICAM1 expression [38]. Furthermore, it was shown that that leukocyte adhesion to EC was restored after ZEB treatment in vitro and in vivo in mice bearing B16/F10 melanoma [38]. In addition to modulation of ICAM1 expression, another study demonstrated that ZEB mediated inhibition of tumor vascularization in vivo, leading to a reduction of melanoma and colon carcinoma growth [95]. The angiostatic activity of ZEB was further supported by data showing the inhibition of EC (HUVEC/bEND5) proliferation and re-expression of the anti-angiogenic genes TSP1, JUNB and IGFBP3. However, silencing of these genes was not due promoter hypermethylation, indicating that ZEB and other DNMT inhibitors may have methylation-independent or off-target effects resulting in the re-expression of anti-angiogenic factors and angiogenesis inhibition [95].

In the context of the re-activation of angiogenesis related to TSGs by DNMT inhibitors, the TSG RECK is of interest since its expression results in anti-angiogenic activity through the inhibition of the MMP2 and MMP9 [96]. Low expression of the RECK gene has been correlated with higher expression of VEGF and poor clinical outcome [96]. Re-activation of the RECK gene may be possible with the DNMT inhibitor disulfiram as it was reported to result in a decrease in MMP2 and MMP9 [97, 98]. In fact, out of 34 bioactive compounds that induce RECK expression, disulfiram was found to be the most potent suppressor of spontaneous lung metastasis in vivo in nude mice bearing RM72 human fibrosarcoma tumors [98].

Finally, another compound, HYD, was originally discovered as an anti-hypertensive drug and was later found to have effects on DNMTs [99, 100], resulted in the inhibition of VEGF in Hep2G cells and tube formation of CRL2480 EC. Further evidence from the CAM and Hep2G xenograft models treated with HYD confirmed inhibition of tumor angiogenesis and tumor growth [101].

In addition to drug-based intervention of epigenetic processes, so-called epi-nutrients, that are found in common foods and beverages, are also known to affect epigenetic regulation [55]. These epi-nutrients can act as DNMT inhibitors, and two well-studied epi-nutrients, the green tea phenol epigallocatechin gallate (EGCG) and curcumin, have shown anti-angiogenic potential [57]. EGCG was suggested to exhibit anti-angiogenic activity in NSCLC where it blocks IGF1-induced HIF-1α and abrogated the expression of VEGF in a A549 lung carcinoma xenograft model [102]. However, in this study, no change in the level of mRNA expression of HIF-1α was found, suggesting that EGCG may block HIF-1α through a posttranscriptional mechanism [102]. In another study, treatment with EGCG caused the suppression of angiogenesis via the upregulation of the angiogenesis inhibitor endostatin and the subsequent inhibition of VEGF in A549 cells leading to tumor growth inhibition in vivo [103]. Anti-angiogenic activity of EGCG has also been demonstrated in breast cancer [104]. EGCG treatment of C57BL/6 mice bearing E0771 mouse breast adenocarcinomas resulted in the inhibition of tumor growth, along with a reduction in tumor MVD and VEGF expression [104]. Similar to the DNMT inhibitor disulfiram, EGCG also induced expression of the RECK gene and subsequent downregulation of MMP2 and MMP9 in oral squamous cell carcinoma cells [105].

Another well-studied epi-nutrient, curcumin, is the primary component of turmeric. Curcumin exhibits significant in vivo anti-tumor activity in a wide-variety of cancers including hematological-, gastrointestinal-, pancreatic- and breast cancers [106]. It was also discovered that curcumin inhibited angiogenesis in vivo partially accounting for tumor reductions in colon and skin cancer [107]. Later it was shown that curcumin acted through the inhibition of DNMT1 [108, 109]. It was suggested that epigenetic regulation might be responsible for curcumin-mediated differential gene expression [110]. For example, the transcription factor STAT3 is a target of curcumin [111] and is often involved in promoting angiogenesis during carcinogenesis [112, 113]. STAT3 also mediates DNMT1 activation and thereby represses many TSGs, including SOCS3, a negative feedback regulator of STAT3 [114, 115]. SOCS3 protein has been described as an endogenous inhibitor of angiogenesis [116]. Both EGCG and curcumin are under investigation in clinical trials. Currently, EGCG is being tested in an ongoing phase II trial in patients with non-metastatic bladder cancer (NCT00666562). Furthermore, a phase II trial for curcumin including patients with pancreatic cancer was conducted by Dhillon et al. where it showed biological activity

(NCT00094445) [117]. Another phase II trial including curcumin is currently ongoing in patients with colorectal cancer (NCT01490996).

Overall, DNMT inhibition results in the re-expression of TSGs that are hyper-methylated in many cancer types. The data discussed above present a compelling rationale for the use of DNMT inhibitors to target epigenetically stimulated tumor angiogenesis.

Ruthenium-based compounds impacting epigenetics and angiogenesis

In recent years, ruthenium-based compounds have been attracting attention as potential alternatives to platinum-based chemotherapeutic compounds [118]. They represent an interesting group of agents with anticancer, anti-metastatic and/or anti-angiogenic activity together with improved toxicity profiles compared to cisplatin and other platinum-based drugs [119, 120]. Moreover, ruthenium-based complexes are usually not affected by platinum-induced resistance mechanisms [121]. The mechanism of action of two of the most clinically advanced ruthenium compounds, NAMI-A, *trans*-[tetrachloro(dimethylsulfoxide)(imidazole)ruthenate(III)], and KP1019, a salt of *trans*-[tetrachlorobis(1*H*-indazole)ruthenate(III)], has been linked to the formation of adducts at histone protein sites and/or to DNA modulation [122]. While both these ruthenium(III) complexes can bind to DNA, this binding is not believed to be relevant for their biological activity. Moreover, compounds that target DNA tend to be more systemically toxic than those with cancer-specific targets. Both NAMI-A [123, 124] and KP1019 [125, 126] have undergone phase I and II clinical trials. A phase I/II trial of NAMI-A in combination with gemcitabine for the treatment of NSCLC showed that the combination is less active than treatment with gemcitabine alone [123]. However, at low NAMI-A doses, the drug combination treatment was promising, but low-dose treatment conflicts with usual clinical practices where maximum tolerated doses are usually administered. It should be noted that NAMI-A also exhibits some intrinsic anti-angiogenic properties in vitro [127, 128] and in vivo [129].

The search for new ruthenium-based compounds is actively ongoing with some alternative agents already showing promising properties. Since a common mechanistic characteristic of NAMI-A and KP1019 is believed to be that they are activated by reduction to more active ruthenium(II) species in the low-oxygen environment of solid tumors, several Ru(II)-based complexes have been evaluated at the preclinical level for their anticancer activity. Although the mechanism of action of these compounds is poorly understood, their activity has been associated with specific molecular targets and epigenetic regulation. Indeed, a plethora of Ru(II) arene complexes have been prepared and evaluated in vitro for their anti-proliferative effects [130–132], and Ru(II) arene complexes with the amphiphilic 1,3,5-triaza-7-phosphaadamantane (pta) ligand are the most extensively studied [133]. In particular, Ru(η^6-toluene)(pta)Cl_2, RAPTA-T, and Ru(η^6-*p*-cymene)(pta)Cl_2, RAPTA-C, originally introduced as anti-metastatic agents [134], were subsequently shown to inhibit the growth of primary tumors with favorable clearance properties [135] (Fig. 2a). Recently, the anti-angiogenic potential of these complexes was also demonstrated in preclinical studies [135, 136].

The action of RAPTA-C and RAPTA-T on EC is quite profound. Interestingly, the sensitivity of EC toward these compounds was found to be higher than that of tumor cells [136]. With its low cell viability inhibitory potential, i.e., IC_{50} values of >300 μM in both normal and cancer cell lines [134, 136], RAPTA-C would fail regular drug screening (cytotoxicity) assays [137]. Remarkably, RAPTA-T normalizes tumor blood vessels at clinically relevant doses without significantly inducing anti-angiogenic activity and vessel pruning. Thus, it may offer similar clinical benefits as VEGF and VEGFR inhibitors, but potentially without the translational difficulties presented by these compounds, including reduced perfusion of tumor tissue resulting in increased tumor hypoxia and tumor cell invasiveness. In a murine pleural mesothelioma model (a model of an incurable cancer), RAPTA-T pretreatment followed by treatment with cisplatin led to a significantly improved treatment outcome mediated through higher cisplatin uptake into the formerly chemoresistant tumor.

RAPTA-C not only causes a reduction in proliferation, migration and tube formation in EC but also stimulates apoptosis in cancer cells. At very high doses, RAPTA-C causes G2/M cycle arrest leading to cell death and this effect could correspond with its binding potential to chromatin-associated proteins [138]. In the CAM [139], the inhibition of vessel formation was observed following RAPTA-C treatment [136]. Moreover, in preclinical tumor models, the tumor growth inhibition was driven by both anticancer and anti-angiogenic mechanisms [135]. The mild growth inhibition seen on primary tumors in vivo may be explained by binding of RAPTA-C to chromatin (Fig. 2b). The combined anti-angiogenic and anti-metastatic properties of RAPTA-C appear due to interactions with the cell membrane [140].

While the mechanism of action of RAPTA compounds remains to be fully elucidated, the formation of adducts in histone proteins in chromatin might be involved [138]. RAPTA-C binds to the nucleosome core of chromatin forming adducts at specific histone sites; approximately 5% of intracellular ruthenium content was found to be

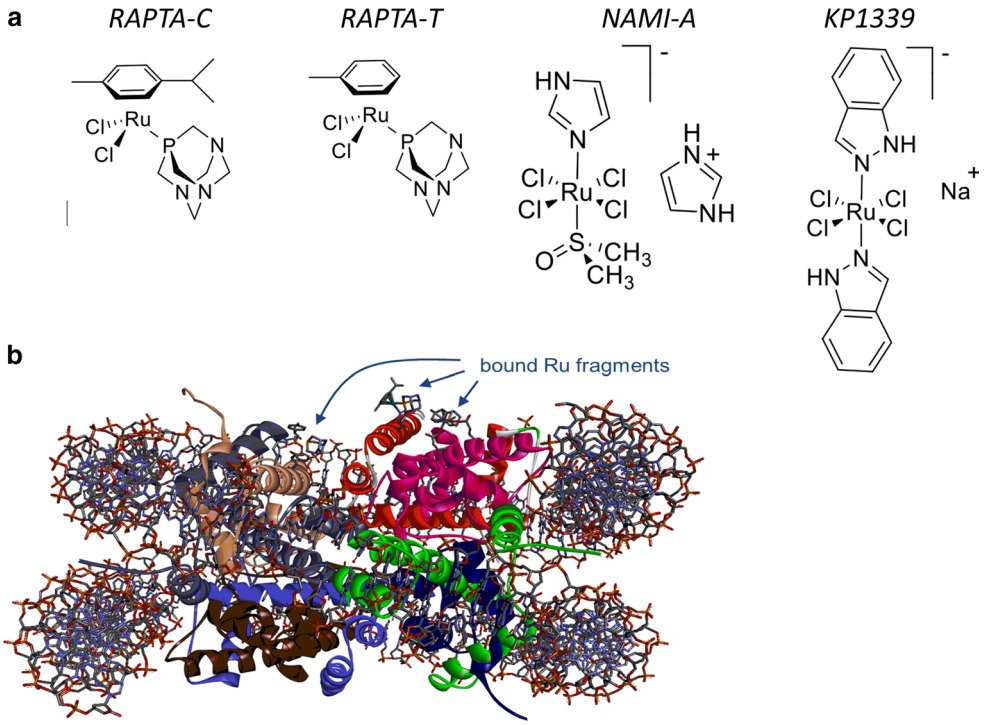

Fig. 2 Ruthenium-based compounds. **a** Chemical structures of RAPTA-C, RAPTA-T, NAMI-A and KP1339. **b** Crystal structure showing the binding of RAPTA-C to the nucleosome on the histone core (*top*) and the dominant histone protein adduct formed with RAPTA-C following substitution of the two chloride ligands by two glutamate residues (*bottom*)

associated with chromatin in RAPTA-C-treated cancer cells [141]. RAPTA-T was recently found to bind to the same histone sites [142]. Since RAPTA-C has a particular affinity to accumulate adducts at chromatin-associated proteins, it was suggested that histone lesions may contribute to the efficacy of this agent [138]. These unique properties support the hypothesis that RAPTA-C may act at the epigenetic level rather than at the genetic level. The ability of RAPTA-C to recognize not only chemical, but also structural features of nucleosomes, may lead to possible targeting of cancer cells via their epigenetic imperfections.

The activity of RAPTA-C in efficient inhibition of tumor growth can be significantly augmented when applied in combination with other drugs presenting complementary mechanisms of action. Finding a synergistic drug combination is not trivial due to enormous parametric space and is often based on data from prior clinical studies. Combinatorial drug screening methods, such as a phenotypic streamline feedback system control technique (i.e., cell viability screen combined with in silico data modeling), have been used to combine multiple agents. This technology, compared to other available methods [143], identifies low-dose synergistic drug combinations with only minimal experimental effort as it is based on statistical design [144]. We have used this technique for the first time, to optimize the combination of numerous anti-angiogenic agents [145]. An optimal drug combination containing three agents, i.e., RAPTA-C, an EGFR inhibitor and an mTOR inhibitor, was identified by in vitro screening and further successfully translated to in vivo tumor growth inhibition. In two tumor models, the synergistic tumor growth inhibition was driven via anti-angiogenic processes. It may very well be that RAPTA-C provokes so-called epi-sensitization [146] of the cell to other compounds in the optimized combination, which was consequently responsible for the observed synergism.

Summarizing, the above-mentioned findings indicate that ruthenium-based compounds that do not target DNA directly seem to be promising alternatives for platinum compounds in cancer treatment. Their epigenetic modifications on signaling pathways, especially in combination with other anticancer drugs, show considerable promise. It should be noted that the ruthenium compounds described here were not designed as inhibitors of epigenetic pathways, and other non-epigenetic pathways are probably also in play. However, a number of metal-based drugs that act as HDAC inhibitors have been reported. These compounds covalently link vorinostat to various metal units, such as ferrocene [147, 148], and it is not unreasonable to assume that broad acting ruthenium-based compounds connected to organic inhibitors of HDACs could have interesting clinical applications.

Links between epigenetic mechanisms and relevance to combination strategies

Mechanisms involved in epigenetic regulation are linked, i.e., the two best understood mechanisms, histone deacetylation and DNA methylation, intricately regulate

and affect one another. DNA methylated by DNMTs, preferentially at CpG dinucleotide sites, can be recognized by methyl-CpG-binding proteins (e.g., MeCP, MBD1, MBD2, MBD3 and MBD4). These can, in turn, recruit HDACs and other chromatin remodeling proteins to facilitate chromatin condensation resulting in long-term suppression of gene expression [149–153] (Fig. 3). Thus, besides DNA methylation directly mediating the suppression of gene expression, it can silence gene expression indirectly through HDAC activity [152]. HDACs have also been reported to directly promote DNA methylation with histone deacetylase HDAC6 being able to interact with DNMT MET1 and HAT FLD [154]. Further evidence that HDAC inhibitors affect DNA methylation and that DNMT inhibitors affect histone acetylation has also been reported. For example, DNA demethylation of p16 affected surrounding histone acetylation [155] and the HDAC inhibitor TSA caused hypo-methylation in specific genomic regions [156]. One explanation for this behavior might be through cross-targeting of inhibitors, as pathways of HDAC and DNMT degradation and stability can overlap [157]. However, it has also been suggested that this interplay can also be caused by epigenetic activity induced by modification patterns [158, 159], e.g., hypo-acetylation causing an induction in DNMT activity. In this example, miscommunication between HATs and HDACs caused loss of histone acetylation, thereby loosening the chromatin structure and attracting *de novo* DNMTs to activate DNA methylation at CpG sites on promotor regions. This results in a definitive repressed state of the promotor region and the silencing of important TSGs including those involved in the inhibition of angiogenesis [160].

HDACs and DNMTs have both been implicated in the regulation of tumor angiogenesis, either via directly modulating the tumor cells or by affecting the tumor-EC [22, 27]. As such, it was not unexpected that anti-angiogenic activity was reported for both HDAC and DNMT inhibitors in cancer treatment [33, 49, 95] and this provides a rationale for exploring strategies of combining epigenetic HDAC and DNMT inhibitors [161]. An overview of clinical trials exploring HDAC/DNMT inhibitor combination therapy is provided in Table 2. Cameron et al. were one of the first to suggest synergistic interactions between HDAC and DNMT inhibitors and showed that the hyper-methylated TSGs such as MLH1, TIMP3, CDKN2B (INK4B, p15) and CDKN2A (INK4, p16) could not be reactivated by administration of the HDAC inhibitor TSA alone in CRC cells in vitro. However, when TSA treatment was combined with a low dose of the DNMT inhibitor DAC, the silenced TSGs were robustly re-expressed [162]. Of

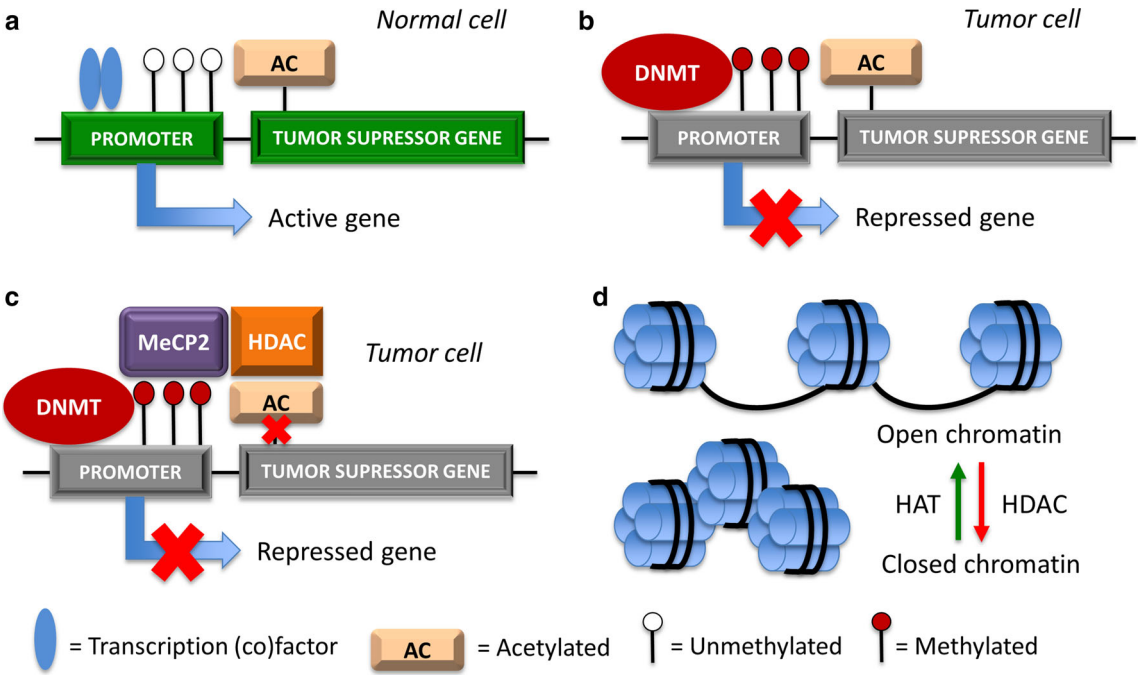

Fig. 3 Epigenetic silencing of tumor suppressor genes can be mediated via DNMT and HDAC interaction. **a** In normal cells, the tumor suppressor gene has an unmethylated promoter region and active chromatin marked by histone acetylation. **b** During tumorigenesis, a DNA methyltransferase (DNMT) causes hypermethylation of CpG islands within the promoter region which can directly prevent transcription factor binding. **c** Methyl-CpG-binding proteins such as MeCP2 recognizes and binds the methylated region which in turn attracts a histone deacetylase (HDAC), resulting in deacetylation followed by epigenetic silencing of the tumor suppressor gene. **d** Chromatin marked by acetylation and unmethylated genes is considered open and active chromatin. HDAC-mediated deacetylation causes condensation of chromatin and suppression of gene expression

Table 2 Overview of clinical trials on epi-drug combination therapies in patients with solid tumors

Drugs	Target	Trial phase	Trial status	Patients	NCI identifier
DAC	DNMT1	I	Completed	NSCLC	NCT00084981
VPA	HDAC1,4-6				
DAC	DNMT1	I/II	Completed	Melanoma	NCT00925132
Panobinostat	HDAC1,2,4				
Temozolomide	Chemotherapy				
DAC	DNMT1	I	Completed	Solid tumors, lymphomas	NCT00275080
SAHA	HDAC				
DAC	DNMT1	I	Completed	Lung malignancies	NCT00037817
Romidepsin	HDAC				
AZA	DNMT1	I	Completed	Solid tumors	NCT00496444
VPA	HDAC1,4-6	I	Completed	Solid tumors	NCT00529022
AZA	DNMT1	I/II	Recruiting	Lymphoma	NCT01537744
Romidepsin	HDAC7	I	Active	Liposarcoma, viral-derived tumors	NCT01998035
AZA	DNMT1	I/II	Completed	NSCLC	NCT00387465
Entinostat	HDAC	II	Completed	CRC	NCT01105377
		II	Active	Breast cancer	NCT01349959
AZA	DNMT1	I	Active	Nasopharyngeal cancer	NCT00336063
SAHA	HDAC	I/II	Completed		NCT01120834
AZA	DNMT1	I	Completed	Solid tumors	NCT00005639
PBA	HDAC	II	Completed	Prostate cancer, NSCLC	NCT00006019
HYD	DNMT1,3a,3b	I	Completed	Lung cancer	NCT00996060
VPA	HDAC1,4-6				
HYD	DNMT1,3a,3b	II	Completed	Solid tumors	NCT00404508
Valproate	HDAC	III	Unknown	Cervical cancer	NCT00532818
		III	Unknown	Ovarian cancer	NCT00533299
$EGCG^{NP}$	DNMT	II	Unknown	Lymphoma	NCT00455416
$Resveratrol^{NP}$ and other NPs	HDAC				

AZA azacitidine, *CRC* colorectal cancer, *DAC* decitabine, *DNMT* DNA methyltransferase, *EGCG* epigallocatechin gallate, *HDAC* histone deacetylase, *HYD* hydralazine, *NCI* National Cancer Institute, *NP* natural product, *NSCLC* non-small cell lung cancer, *PBA* phenylbutyrate, *SAHA* vorinostat, *TSA* trichostatin A, *VPA* valproic acid

these TSGs, tissue inhibitor of matrix metalloproteinase 3 (TIMP3) and p16 are known to play an important role in inhibiting tumor angiogenesis indicating the anti-angiogenic potential of the DAC-TSA drug combination. Protein p16 is known to form a complex with HIF-1α, thereby repressing HIF-1α-induced VEGF activation. Moreover, p16 was found to inhibit angiogenesis in breast cancer and lung cancer models [163, 164]. The TIMP3 is an endogenous inhibitor of MMPs that is found in the extracellular matrix (ECM) and is a potent inhibitor of angiogenesis and tumor growth [165]. TIMP3 also inhibits angiogenesis by interfering in the binding of VEGF to VEGFR2 [166]. Others have further evaluated the (anti-angiogenic) potential of the combination of DAC and TSA showing that the TSGs TSP1, JUNB and IGFBP3 can be reactivated in tumor-conditioned EC where these genes were silenced [22, 167]. Of note, the re-activation of these genes is independent of promotor CpG island methylation, but instead is correlated with DAC/TSA-mediated histone H3 deacetylation and loss H3 lysine 4 methylation. Interestingly, successful re-activation of TSGs in these tumor-conditioned EC also indicates that although they can be heavily modified epigenetically, they have little, if any, genetic aberrations in contrast to tumor cells [22]. In addition, the combination of DAC and TSA could result in strong, synergistic inhibition of proliferation in pancreatic cancer and human leukemic cell lines in vitro [168, 169]. To date, there are no reports on the clinical evaluation of this combination.

Another HDAC inhibitor and DNMT inhibitor combination with anti-angiogenic activity is the combination of phenylbutyrate (PBA) and DAC. EGFL7 gene is responsible for miR-126 expression and was found to be hypermethylated in colon, bladder and prostate cancers [79, 88]. It was shown that when bladder, cervical and breast cancer cells were treated with PBA and DAC, there was a

synergistic increase in miR-126 levels which may directly account for a decrease in VEGF [88, 89]. No in vivo results have been reported, and the clinical evaluation of the DAC–PBA drug combination has yet to be conducted (Table 2). Treatment with DNMT and HDAC inhibitors alone showed only limited clinical benefit in patients with solid tumors [170, 171], but combination treatment with DNMT and HDAC inhibitors could result in robust re-expression of epigenetically suppressed genes and significant clinical responses [172, 173], although contradicting results have also been reported [174]. A promising example of early phase trials of HYD combined with magnesium valproate have led to phase III trials for cervical and ovarian cancers (Table 2).

Taken together, these data demonstrate a clear link between histone deacetylation and DNA methylation and support combination therapy with HDAC and DNMT inhibitors as a treatment strategy with anti-tumor and anti-angiogenic effects.

Epigenetic regulation combined with targeted agents

As epi-drugs affect a broad range of mechanisms, the currently available drugs have also been limited by a lack in selectivity and by off-target toxicity [175]. Therefore, to overcome these limitations, epi-drugs have been combined with numerous treatment modalities, such as immunomodulators [176, 177], differentiation agents [178], radiotherapy [179], chemotherapy [8], photodynamic therapy [180] or angiogenesis inhibitors, in an attempt to improve their efficacy and selectivity.

The contribution of single anti-angiogenic agents to the improvement of cancer therapy is modest, and several constrains account for this limited success, including severe dose-restricted toxicities, patient and tumor heterogeneity and the occurrence of acquired resistance [181]. Many combination studies have therefore been performed, investigating the potential of angiogenesis inhibitors with other treatment modalities [182–184] showing varying degrees of success. One of such proposed combination strategies in cancer treatment is the addition of epi-drugs to angiogenesis inhibitors, and results obtained at (pre)clinical level are available and are discussed below.

The HDAC inhibitor dacinostat, having clear anti-angiogenic effects (through downregulation of HIF-1α, Ang-2, Tie-2 and survivin), was combined with the VEGFR tyrosine kinase inhibitor PTK787/ZJ222584 (PTK) [50]. This combination showed superior activity compared to single drugs in reducing EC viability, tube formation and invasion, whereas in vivo tumor growth inhibition of breast and prostate carcinomas was clearly correlated with a decrease in tumor MVD, suggesting angiogenesis inhibition as part of the mechanism of action.

VPA, a HDAC inhibitor, has been tested in combination with various angiogenesis inhibitors in solid tumor models, as well as in clinical trials (Table 3). VPA in combination with the anti-angiogenic peptide derivative of the natural angiogenesis inhibitor TSP1 peptide ABT-510 was administered in a pediatric neuroblastoma tumor model. This combination significantly inhibited tumor growth, as compared to monotherapies. This effect was linked to blood vessel normalization, and a decrease in MVD was associated with a robust induction of TSP1-mediated anti-angiogenic effects, including inhibition of pro-angiogenic factors such as VEGF, bFGF and IL-8 [185, 186]. VPA was also combined with pazopanib, a multi-kinase inhibitor of c-KIT, PDGFR, FGFR and VEGFR, currently approved for sarcoma and RCC treatment. This combination showed robust anti-tumor efficacy in in vivo models. Interestingly, this combination strongly suppressed mTOR expression, suggesting an anti-angiogenic potential, since mTOR complexes are known to regulate HIF-1α and promote the release of pro-angiogenic factors [187].

The combination of targeted anti-angiogenic agents and ruthenium-based epigenetic modulators has also been investigated. For example, sorafenib has been shown to synergistically interact with the ruthenium-based drug KP1339 (see chapter 4 and Fig. 2a) in the inhibition of hepatoma cancer both in vitro and in vivo [188]. Various mechanisms for their synergistic interactions were investigated, including the increased intracellular accumulation of both compounds and the blocking of p38 activation by sorafenib, which acts a protective pathway against G2/M cell cycle arrest induced by KP1339.

Available results from completed clinical trials do not give clear answers on the success of these combinations. Phase I dose-escalation study of DAC with a fixed dose of genistein, a natural isoflavone, to treat advanced solid tumors was followed by a phase II study in advanced lung cancer patients (NCT01628471). The combination therapy was well tolerated but activity was rather modest in the phase II cohort of heavily pretreated NSCLC patients [189].

Bevacizumab was tested versus bevacizumab plus vorinostat in a phase I/II randomized trial in adults with recurrent glioblastoma (NCT01266031). The mixture of bevacizumab and vorinostat neither improved PFS or OS nor reduced symptom burden compared to bevacizumab alone in these patients [190].

In another trial, bevacizumab, everolimus and panobinostat were tested in combination for the treatment of advanced solid tumors. At the lowest proposed doses, the combination did not have an acceptable safety and

Table 3 Overview of clinical trials on epi-drugs/targeted agent combination therapies in patients with solid tumors

Drugs	Target	Trial phase	Trial status	Patients	NCI identifier
DAC	DNMT1	I/II	Completed	NSCLC	NCT01628471
Genistein	VEGF				
DAC	DNMT1	I	Completed	Melanoma	NCT00791271
PEG-INFα-2b	INFα				
Panobinostat	HDAC	I	Completed	NSCLC	NCT00738751
Erlotinib	EGFR			H&N cancer	
Panobinostat Bevacizumab	HDAC	I	Completed	Advanced solid tumors	NCT01055795
Everolimus	VEGF				
	mTOR				
DAC	DNMT1	I/II	Recruiting	Pediatric relapsed or refractory malignancies	NCT02499861
Genistein	VEGF				
Guadecitabine	DNMT1	I/II	Recruiting	mCRC	NCT01896856
Regorafenib	VEGFR1/2				
Irinotecan	Chemotherapy				
TAS-102	Chemotherapy				
Hydroxychloroquine	Autophagy Inhibitor	I	Recruiting	Advanced cancers	NCT01266057
Sirolimus	mTOR				
Vorinostat	HDAC				
Vorinostat	HDAC	I/II	Ongoing	Recurrent glioblastoma	NCT01266031
Bevacizumab	VEGF				
Sirolimus	mTOR	I	Ongoing	Advanced cancers	NCT01087554
Vorinostat	HDAC				
Vorinostat	HDAC	I	Recruiting	EGFR mutant lung cancer	NCT02151721
Gefitinib	EGFR				
Bevacizumab	VEGF	I/II	Ongoing	Advanced sarcomas	NCT01106872
Gemcitabine	Chemotherapy				
Docetaxel	Chemotherapy				
Valproic acid	HDAC				
Pazopanib	TKI	I	Ongoing	Advanced cancer	NCT01339871
Vorinostat	HDAC				

CRC colorectal cancer, *DAC* decitabine, *DNMT* DNA methyltransferase, *EGCG* epigallocatechin gallate, *EGFR* epidermal growth factor receptor, *H&N* head and neck, *HDAC* histone deacetylase, *HYD* hydralazine, *INF* interferon, *NCI* National Cancer Institute, *NP* natural product, *NSCLC* non-small cell lung cancer, *mTOR* mammalian target of rapamycin, *PBA* phenylbutyrate, *SAHA* vorinostat, *TKI* tyrosine kinase inhibitor, *TSA* trichostatin A, *VEGF* vascular endothelial growth factor receptor, *VPA* valproic acid

tolerability profile and did not consistently inhibit HDAC activity. Therefore, the investigators did not recommend further evaluation of this combination (NCT01055795). Disappointing clinical results with some of these combinations may also be linked to patient heterogeneity, where the variability of enzymes and/or gene signatures between tumor types and patients, as well as the duration of exposure to the inhibitor combination, may limit efficacy and require the identification of biomarkers for patient populations that will respond favorably to treatment [191].

On the other hand, when VPA was combined with bevacizumab, in a phase I clinical trial in advanced cancers, it showed activity in patients with colorectal, gastroesophageal and prostate cancers, and treatment was considered safe [192]. Interestingly, patients that developed any grade of hypertension, a known side effect of bevacizumab treatment, had improved OS. This study was initiated without any supporting preclinical data and was based on promising in vitro and in vivo results on the combined HDAC and VEGF pathway inhibition by other drugs (dacinostat/PTK combination therapy) [50].

The fact that many phase I or I/II clinical trials in solid tumors were initiated in recent years using angiostatic targeted therapy in combination with epi-drugs clearly underscores the expected promise for this combination (Table 3). As is the case for many combination therapy

strategies, the angiostatic drugs of choice were most often targeting VEGF(R), mTOR or EGFR. The results of many trials are still pending and will provide important information on the tolerability and efficacy of these combination therapies.

Taken together, studies investigating the activity of epigenetic modulators in combination with targeted angiogenesis inhibitors show significant activity in various angiogenesis and tumor models, as well as in certain clinical trials. Although the anti-angiogenic mechanism of action of the mentioned single drugs is often well described, the exact mechanism of action of combined epigenetic drugs and angiogenesis inhibitors remains to be further elucidated.

Since acquired resistance to anti-angiogenic therapy remains a major limitation to its use [19], many research groups have focused on ways to reverse this process. It was previously shown that epigenetic drugs are able to reverse acquired resistance to chemotherapy [193, 194], and it was hypothesized that this may also hold true for the reversal of acquired drug resistance to anti-angiogenic therapies. The synergistic activity of erlotinib in combination with RAPTA-C (described above) applied at low doses was recently reported [195]. This drug combination was shown to target angiogenesis in in vitro bioassays and in human ovarian carcinoma tumor models. It is worth mentioning that its activity was also pronounced in cis-platinum-resistant human ovarian carcinoma model and led to cell senescence. The latter result suggests a therapeutic potential of erlotinib and RAPTA-C mixture in the treatment of chemoresistant tumors.

A combination comprising the multiple receptor tyrosine kinase inhibitor AEE788 with HDAC inhibitors (TSA, LBH589 or LAQ824) has been reported [196]. This study demonstrated synergistic cytotoxicity in a broad spectrum of cancer cell lines, including cis-platinum-resistant ovarian adenocarcinoma cells. These results suggested further preclinical and possible clinical studies for a broad spectrum of cancers, also in cases where chemoresistance already appeared.

Resistance to EGFR inhibitors used to treat NSCLC, such as erlotinib and gefitinib (both possessing strong anti-angiogenic activity), and the possibility of reversing this process with epigenetic drugs has been the topic of recent research efforts. For instance, the sensitivity to gefitinib was evaluated in a panel of NSCLC cell lines, and six were found to be gefitinib-resistant [197]. Further analysis showed that expression of E-cadherin in the resistant cell lines was undetectable, which differed from the sensitive cell lines where E-cadherin was highly expressed. This difference indicates that gefitinib sensitivity might be dependent on the presence of E-cadherin. Loss of E-cadherin expression was found to contribute to tumor initiation and metastasis and to induction or enhancement of angiogenesis [198, 199]. In the gefitinib-resistant NSCLC cells, the expression level of the regulator of E-cadherin, Zeb1, was found to be higher than in gefitinib-naïve cells. Zeb1 inhibits E-cadherin expression via the recruitment of histone deacetylases. Strikingly, treatment of resistant NSCLC cell lines with the HDAC inhibitor entinostat increased E-cadherin expression and sensitized these cells to gefitinib therapy, resulting in similar efficacy of gefitinib as compared to the efficacy observed in nonresistant cell lines [197]. In addition, a synergistic interaction was found between entinostat and gefitinib in a sequential treatment schedule resulting in enhanced cell viability inhibition and increased cell death.

Similar results were observed with the HDAC inhibitor MPT0E028. This novel HDAC inhibitor combined with erlotinib resulted in synergistic activity in vitro and in vivo in erlotinib-resistant lung adenocarcinoma cells and tumors, thereby overcoming initial resistance to erlotinib therapy [200].

Recently, second (e.g., BIBW2992 or afatinib) and third generations (e.g., WZ4002) EGFR TKIs have been developed in an attempt to overcome acquired resistance to EGFR-targeted therapies. In lung adenocarcinoma cells resistant to EGFR inhibitors (caused by T790 M mutation), the combination of SAHA with a second or third generation EGFR inhibitor led to significantly enhanced antiproliferative activity, induction of apoptosis and autophagy, and resulted in enhanced tumor growth inhibition in vivo [201].

A randomized phase II clinical trial was conducted evaluating erlotinib treatment alone and in combination with entinostat in patients with advanced NSCLC. In terms of progression-free survival, the combination treatment did not outperform single erlotinib treatment [202]. Molecular analysis of tissues from patients that did benefit from the combination treatment revealed that approximately 40% of these patients exhibited higher E-cadherin expression. E-cadherin was therefore suggested as a potential biomarker in NSCLC for increased sensitivity to the erlotinib/entinostat combination treatment, as was shown in a combined gefitinib/entinostat study [197]. Another clinical study further confirmed the interaction between HDAC inhibitor and EGFR inhibitors and the relation between high E-cadherin levels and clinical outcome. In a phase I trial of panobinostat and erlotinib in advanced NSCLC and head and neck cancer patients, a high E-cadherin level was positively correlated with PFS, independent of EGFR mutation status [203] (Table 3).

The above-mentioned studies show that HDAC inhibition can induce the reversal of resistance to EGFR-targeting therapies, providing a strong rationale for combining HDAC inhibitors with EGFR inhibitors. Furthermore,

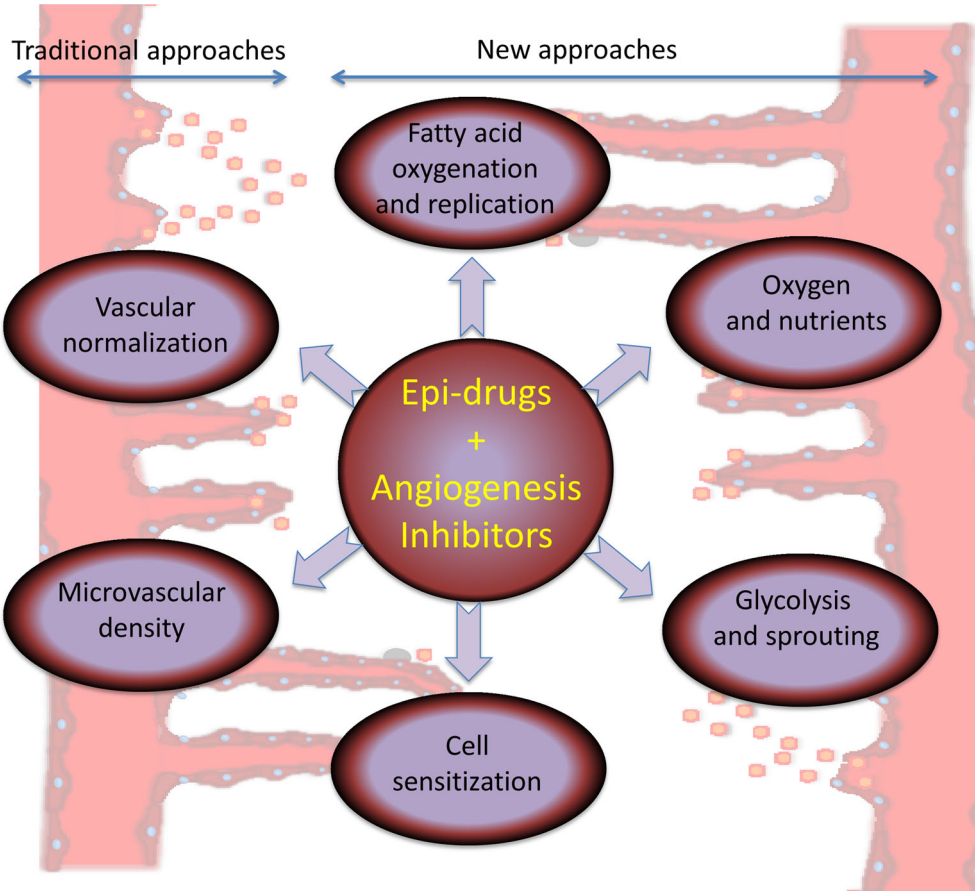

Fig. 4 Overview of the variety of biological effects induced by epi-drugs when combined with angiogenesis inhibitors

expression levels of E-cadherin were shown to correlate with positive response to therapy and may serve as a potential biomarker. Randomized controlled trials and the definition of specific patient populations are warranted for future clinical applications.

Conclusions

Angiogenesis inhibition as a strategy for the treatment of cancer is currently a well-studied therapeutic modality. Moreover, therapies intervening with the epigenome by altering DNA methylation and histone deacetylation are increasingly being studied in the field of angiogenesis. Indeed, there is strong emerging evidence that HDAC and DNMT inhibitors affect angiogenesis in relation to cancer development. In addition, several natural products referred to as epi-nutrients have been shown to act as HDAC or DNMT inhibitors resulting in similar anti-angiogenic activity. Interestingly, ruthenium-based compounds are being developed as alternatives to conventional platinum-based chemotherapeutics and showed very promising epigenetic activity. Some of these drugs have been suggested to act via epigenetic mechanisms by forming histone adducts in chromatin [138]. The approved epi-drugs are not very selective, i.e., HDAC inhibitors tend to inhibit the entire family of HDAC enzymes to some extent, and their broad action causes side effects; hence, more selective inhibitors could further improve treatment outcomes.

While selective, targeted, small molecule drugs interacting with a variety of key modulators of angiogenesis (such as VEGF(R), FGF(R), PDGF(R), eNOS) are continuously being developed, it has become increasingly clear that several limitations hamper their clinical success, including severe toxicities and drug resistance [204, 205]. Combining different treatment modalities to achieve more efficient and robust angiogenesis inhibition is attractive due to several advantages including increased efficacy, the use of reduced drug doses (as a result of synergistic drug interactions) and the ability to overcome drug-acquired resistance [144, 145, 206]. According to a new paradigm, not only traditional angiogenesis inhibition approaches, i.e., the administration of TKIs or growth factor inhibitors, but also other potential innovative routes can be combined with epi-drugs (Fig. 4). One may think of targeting metabolic pathways in EC such as glycolysis or the inhibition of fatty acid oxidation (FAO), which renders proliferating EC quiescent or sensitized to other drugs [207]. Moreover,

environmental factors such as oxygen or nutrition/diet can regulate the expression of genes via epigenetic mechanisms such as the modification of DNA and its associated histones, thereby also influencing angiogenesis [207].

The rationale of drug combinations and careful patient stratification based on clinically relevant modification in epigenetic enzymes will likely lead to the identification of new treatment strategies and beneficial effects in patients. As the identification of optimal synergistic drug combinations is not trivial due to the large search space, a feedback system control (FSC) approach has been developed to rapidly identify highly synergistic low-dose drug combinations [144, 145, 208]. This methodology allows—with minimal experimental effort—the identification of synergistic low-dose drug combinations without a prerequisite for knowledge of the mechanism. Of note, the above-mentioned combination of erlotinib and RAPTA-C was discovered using the FSC approach [195]. The optimization search for drug combinations consisting of HDAC inhibitors and targeted therapies is currently being performed. As the clinical value of epi-drugs is often limited by their cytotoxicity, it will be of critical importance in the future to take the therapeutic window of malignant and non-malignant cells into consideration and this might increase the translation value of such combinations for clinical use.

There is a general consensus, in both the fields of tumor angiogenesis and epigenetics that combination therapies possess major advantages over single-drug treatments. Therefore, several studies on combining HDAC and DNMT inhibitors, as well as on combining these drugs with targeted therapies, have been carried out. Combinations of HDAC and DNMT inhibitors were shown to re-express silenced TSGs involved in angiogenesis inhibition [162] and, for example, increase miR-126 expression leading to a decrease in VEGF [88]. Furthermore, combinations of both HDAC and DNMT inhibitors with targeted therapies have shown strong evidence for enhanced anti-angiogenic activity in various in vitro and in vivo models [50, 209]. Combined HDAC inhibition and EGFR-targeting therapies were shown to overcome acquired resistance to these EGFR inhibitors [197, 200]. The re-sensitization of EGFR-targeting therapy was shown to be correlated with E-cadherin expression [197], and E-cadherin expression was found to be associated with clinical responses, thereby possibly serving as future biomarker [202, 203].

In conclusion, epigenetic modulators exert a variety of anti-angiogenic effects and there is a strong suggestion that combining these compounds, either with each other or with targeted therapies, may result in superior activity in terms of anti-tumor and anti-angiogenic efficacy. Although it is clear that HDAC and DNMT inhibitors can augment the activity of a broad range of anticancer or anti-angiogenic drugs, their underlying molecular mechanisms and pharmacology remain to be elucidated. Research on the ability to overcome acquired resistance with epigenetic and targeted therapies together with understanding disease-specific targets and the development of appropriate biomarkers might bring significant future clinical benefits.

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