Radiosensitizing Potential of Epigenetic Anticancer Drugs

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Abstract: Over the last few decades, epigenetic tumor changes characterized by promoter hypermethylation and histone modifications have become a topic of intense research.

Of particular interest is the potential reversibility of these processes that has led to the development of epigenetic anticancer drugs such as demethylating agents and histone deacetylase inhibitors (HDAC-I). Besides single agent clinical activity in both hematological and solid malignancies, combinations of both types of epigenetic drugs with classic chemotherapeutics have shown promising results. In addition, as demethylating agents and HDAC-I act synergistically to reverse gene silencing, treatment schedules combining both epigenetic strategies could theoretically enhance tumor response. This assumption has been validated *in vitro* and *in vivo* for several hematological and solid cancer types, and awaits further clinical investigation.

Nowadays, the majority of patients with cancer are treated with radiotherapy. To optimize the results obtained with this treatment modality, efforts are being put in strategies enhancing tumor response selectively in favor of normal tissue response. The combination of epigenetic drugs with radiotherapy is particularly valuable since a drug- and dose-dependent radiosensitizing potential of several classes of HDAC-I has been proven *in vitro* and *in vivo*. The molecular mechanisms underlying this radiosensitization have not been fully clarified yet. In general, higher concentrations of HDAC-I are believed to exert cell cycle redistribution, induction of apoptosis, and downregulation of surviving signals. The radiosensitizing effect of lower, non-toxic doses of HDAC-I has been attributed to, at least in part, acetylation-induced changes leading to altered double strand break (DSB) formation and repair. Although promising so far, further research is needed before HDAC-I administered alone or in combination with demethylating agents will be implemented in the clinic to act as radiosensitizers.

Key Words: Demethylating agents, histone deacetylase inhibitors, anticancer drugs, radiotherapy, radiosensitization, epigenetic.

CANCER AS AN EPIGENETIC DISEASE

Over the last few decades, the classic view on cancer as a result of cumulative genetic abnormalities has evolved towards the idea that this disease is also driven by "epigenetic changes"- heritable changes in gene expression that are not caused by alterations in the gene nucleotide sequence [1].

This epigenetic network encompasses DNA methylation, histone modifications, chromatin remodeling and microRNAs [2]. All together, these processes regulate the delicate homeostasis of gene expression in a normal cell, which is disrupted in cancer.

The genome of a transformed cell shows global hypomethylation together with hypermethylation of CpG dense gene regulatory regions in comparison with its normal counterpart. This altered state leads to chromosomal instability, loss of imprinting, activation of parasitic sequences, inappropriate gene expression, aneuploidy and mutations on the one hand, and transcriptional silencing of tumor suppressor genes (TSG) on the other hand. Gene inactivation by promoter hypermethylation affects a variety of cellular pathways such as DNA repair, cell cycle, apoptosis and cell adhesion. The CpG islands are methylated by DNA methyl transferases (DNMTs) of which the most important isoforms are DNMT1 (considered "maintenance" enzyme), DNMT3a and DNMT3b (putative "*de novo*" enzymes). These enzymes do not act alone in their silencing activities: the gene regulatory machinery harbors methyl-CpG-binding proteins (MBDs), polycomb complexes (PcG), chromatin remodeling factors, histone methyl transferases (HMTs), histone acetyl transferases (HAT) and histone deacetylases (HDAC) [3]. The latter indicate that in addition to methylation changes, histone modifications are also key players in the epigenetic field.

By means of several post-translational modifications, including acetylation, methylation and phosphorylation, histones act as dynamic regulators of gene activity. Loss of monoacetylation of lysine 16 and trimethylation of lysine 20 in the tail of histone H4 are considered as almost universal epigenetic markers of malignant transformation, next to global DNA hypomethylation and CpG island hypermethylation. In general, acetylation of histones is required to maintain chromatin in an open and transcriptionally active state. Deacetylation of histones by HDACs turns the chromatin into a closed formation that is not accessible for transcription factors [4].

Both DNA methylation and histone modifications occur in a higher-order chromatin structure influenced by chromatin remodeling factors and PcGs.

Finally, microRNAs can be considered as epigenetic factors by their ability to participate in the post-transcriptional regulation of gene expression [2].

Of major interest is the potential translational use of epigenetics in cancer: possibilities lie in early detection of tumor cells in bodily fluids, in a role as prognostic or predictive biomarkers, and in targeted therapy. The potential reversibility of the epigenetic processes has indeed led to the development of epigenetic drugs of which the two most important classes are DNA-demethylating agents and HDAC inhibitors (HDAC-I). Based on their observed single and combined antitumor activity in hematological and solid malignancies, these drugs are currently under further clinical investigation.

Ionizing radiation is a valuable treatment option for the majority of patients with cancer, frequently resulting in local tumor control or cure. The potential role of these epigenetic agents as radiosensitizers has been investigated *in vitro* and *in vivo* and is promising so far.

The following part of this review will focus on demethylating agents and HDAC-I and their applicability as single or combined agents, with or without radiation.

DEMETHYLATING AGENTS

Thanks to the tumor-specific and reversible occurrence of DNA methylation, this phenomenon is an attractive therapeutic target. Theoretically, as the DNA sequence and protein product of methy-

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lated genes remain unaltered, pharmacologic inhibition of methylation-mediated suppression could reverse the silencing and restore normal gene function. Most DNA-demethylating agents block the action of DNMTs, which usually have moderately elevated expression levels in malignant cells. There are two major groups of demethylating drugs: the nucleoside analogs and the non-nucleoside inhibitors of DNA methylation.

The two best studied members of the nucleoside family are cytidine and 2'-deoxycitidine analogs. Upon incorporation of these drugs into double strand DNA, they inhibit DNMTs by covalent trapping. The binding of the 5-aza-nucleotide forms to DNMTs forces DNA replication to proceed in the absence of DNA methylation, thereby causing genomic DNA hypomethylation.

The first nucleoside analog with discovered demethylating capacity, was 5-azacytidine (Vidaza). This drug is now FDA-approved for the treatment of hematological malignancies but its major drawback is the incorporation into both RNA and DNA. Therefore, interest has moved to the DNA-specific analog 5-aza-2'-deoxycytidine (Decitabine). Despite its DNA specificity and similar to Vidaza, the drug also has several potential drawbacks for clinical use. Firstly, Decitabine can degrade by deamination into a non-active derivate, thereby reducing its half-life. In aqueous solution, the drug is unstable and thus requires frequent preparation in fresh solutions. The differentiating effect of Decitabine in vitro also has a narrow dose window: the drug is cytotoxic at high doses as a result of its incorporation into DNA. Finally, after drug removal, the methylation status returns to its original level both in normal and in tumor cells. However, Decitabine has shown clinical activity as a single agent in hematological malignancies, and its potential is currently investigated in several clinical trials (clinical trials.gov). The most efficient treatment schedules are those based on prolonged low-dose treatments: prolonged to prevent remethylation [5] and low-dose to limit myelotoxicity [6]. At the molecular level, hypomethylation and gene reactivation have been shown and seem to be required for responses [7, 8]. Furthermore, the mechanism may include induction of senescence, differentiation, apoptosis and possibly immune activation. Following its anticancer activity in hematological malignancies, Decitabine has been FDA-approved since 2006 for the treatment of myelodysplastic syndrome. The first clinical studies with Decitabine in solid tumors were disappointing [9]. However, as these older studies did not use optimized treatment schedules, they deserved a second look. Today, there is already some evidence of clinical activity of Decitabine in malignant melanoma [10], and other studies are underway.

Zebularine is a more stable nucleoside analog that can be given orally and has proven demethylating and antitumor effects [11]. Although potentially less toxic than the previously mentioned analogs, the low potency of this drug necessitates high doses, making its use in clinical trials rather difficult.

Because nucleoside inhibitors may be inherently cytotoxic leading to clinical side effects such as neutropenia and thrombocytopenia, non-nucleoside analogs have been developed that inhibit methylation without incorporation into DNA. The drugs procainamide and procaine, FDA-approved for the treatment of cardiac arrhythmias and for use as local anesthetic, respectively, both have DNA hypomethylation activity. Other agents that have been developed include DNMT1 antisense and siRNA. These non-nucleoside inhibitors all have proven *in vitro* demethylating activity with limited clinical experience [12].

Importantly, all existing demethylating agents can also induce expression of genes that are not methylated in their promoter regions. These indirect transcriptional changes may occur by reactivating transcription factors, regulating target genes in an affected signal transduction pathway, and by non-specific changes in gene expression due to biological effects that occur as the result of an upstream gene reactivation event [13]. In support of this theory are data showing that 60% of genes induced by Decitabine treatment of bladder cancer cells do not contain promoter CpG islands [14]. Despite their disperse effects, these drugs exert tumor-specific cell kill while leaving normal tissue toxicity within acceptable limits.

HDAC INHIBITORS

Since it has been recognized that along with DNMTs, HDACs are promising targets for anticancer therapies, HDAC-I have been developed. The idea of HDACs as therapeutic targets results from the aberrant recruitment of HDACs to methylated loci, from their general role as transcriptional corepressor, their ability to deacetylate nonhistone targets, and from altered expression of individual HDACs in tumors.

Indeed, HDACs are recruited by DNMTs and by MBDs to form a transcriptionally repressed chromatin state. Deacetylation results in a positive charge associated with the amino group on conserved lysine residues in the histone tails. These histones will then bind tightly to the negatively charged DNA, rendering this DNA less accessible to transcription factors and to other proteins needed for gene transcription.

Changes in acetylation caused by HDACs and HATs are also involved in other processes than methylation-associated gene silencing. It is proposed that histone acetylation levels regulate gene transcription, and that HDACs and HATs function as transcriptional corepressors and coactivators, respectively.

Furthermore, HDAC enzymes can deacetylate numerous non-histone targets such as gene transcription factors and proteins involved in cell cycle progression, DNA repair and cell death.

In addition, overexpression of HDACs and HAT inactivation has been reported in several tumor types. Eighteen HDACs have been identified in humans, and they are subdivided into four classes [15]. Different HDACs have diverse functions and cell-type specific expression patterns. Class I includes HDACs 1, 2, 3 and 8, which are ubiquitously expressed and almost exclusively found in the nucleus. They are presumed to be important in the regulation of proliferation and some authors regard them as the "true" HDACs. Class II HDACs are subdivided into Class IIa, encompassing HDACs 4, 5, 7 and 9 and Class IIb (HDAC6 and HDAC10) enzymes. They display tissue-specific expression and shuttle between the nucleus and the cytoplasm. The third class of HDACs includes the NADH-dependent Sir family of deacetylases which are involved in chromatin-dependent silencing in yeast. This class constitutes a structurally unrelated subfamily and will not be considered here. According to some authors, there is a fourth class of HDACs including HDAC11 as its sole member. This isoform shares sequence similarity with both Class I and Class II enzymes but is not considered to have enough identity to be placed in either class.

The several HDAC-I identified so far inhibit these HDACs in a more or less specific way and form a diverse group of both natural and synthetic compounds [15, 16]. The non-specific HDAC-I include the cyclic and noncyclic hydroxamates (e.g. Trichostatin A (TSA), suberoyl anilide hydroxamic acid (SAHA), LBH589, PCI24781, m-carboxycinnamic acid bishydroxamide (CBHA)), which act in the nanomolar range, and short-chain fatty acids (valproic acid (VPA), sodiumbutyrate (NaB), phenylacetate (PA), phenylbutyrate (PB) and butyric acid prodrugs), which possess HDAC inhibition activity in the millimolar range. All of these compounds have proven *in vitro* activity and a number of them have progressed to phase III clinical trials in cancer patients [17, 18]. SAHA has also recently been approved by the FDA for clinical use in cutaneous T-cell lymphoma.

Despite established clinical activity of the broad spectrum HDAC-I, more specific inhibitors may have a greater therapeutic index than the nonspecific drugs. Examples of HDAC-I inhibiting one or more HDAC isoforms from Class I only include cyclic peptides (depsipeptide (FK228)) and miscellaneous compounds

(MGCD0103), which are potent inhibitors of HDAC activity (in nanomolar range); benzamides (MS275) and electrophilic ketones, which are active in the micromolar range and other synthetic drugs such as SK7041 [19] or PCI34051 [20]. These drugs have also been tested *in vitro* and some are currently undergoing further clinical evaluation [16-19]. An overview of the HDAC-I that will be discussed into further detail in this review is given in Fig. (**1**).

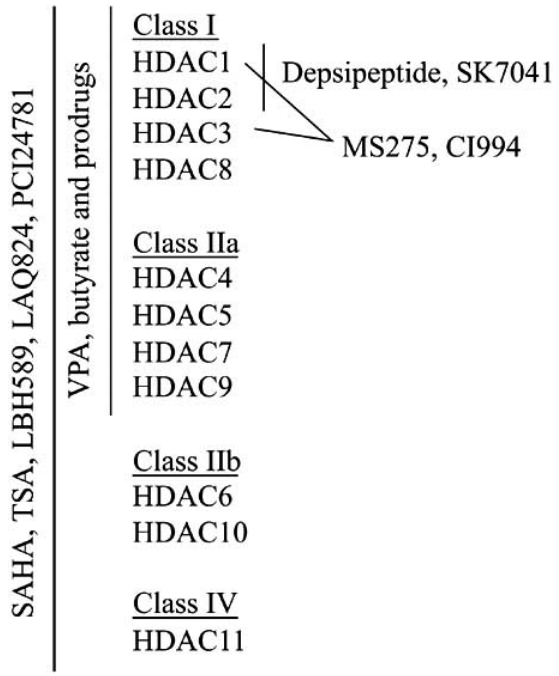

Fig. (1).

At the molecular level, most effects exerted by HDAC-I are related to the hyperacetylation of histones following administration of these drugs. Histone hyperacetylation leads to transcriptional activation or repression of numerous genes, resulting in various effects.

HDAC-I predominantly act by inducing differentiation, apoptosis and cell-cycle arrest with a preferential cytotoxicity for tumor cells [16]. Similar to other anticancer drugs, HDAC-I selectively induce **apoptosis** in tumor cells: *in vitro* studies show that these cells may be tenfold more sensitive to HDAC-I compared with normal cells [21]. Concerning the underlying mechanism arguments have been given for both activation of the extrinsic and intrinsic apoptosis pathway [22]. However, signaling through death receptors for HDAC-I mediated apoptosis remains controversial and more investigations support a role for the mitochondrial apoptotic pathway. Whether this is achieved by a general altered balance in the expression of pro- and anti-apoptotic genes or by a selective activation or induction of BH3-only proteins remains unclear [15]. Other mechanisms contributing to enhanced HDAC-I induced cell death include autophagy, regulation of reactive oxygen species (ROS) production and activity, and downregulation of surviving signals [22]. **Cell-cycle arrest** mediated by HDAC inhibition mainly relies on G1 arrest achieved by induction and repression of several proteins such as induction of p21 and p27 [23, 24]. The mechanisms underlying the less frequently observed G2/M-phase arrest are poorly understood.

Besides inducing cell death and cell-cycle arrest, HDAC-I also have anti-angiogenic, anti-invasive and immunomodulatory activities related with transcriptional changes. The **anti-angiogenic** potential of these drugs has been attributed to the downregulation of pro-angiogenic genes such as vascular endothelial growth factor (VEGF) [25, 26] and chemokine (C-X-C motif) receptor 4 (CXCR4) [27] and to the suppression of endothelial progenitor cell differentiation [28]. Findings on the influence on **invasion and metastasis** are contradictory. On the one hand, HDAC-I seem to downregulate matrix metallo proteinases (MMPs) [29], but other reports describe an increased *in vitro* invasive capacity through activation of urokinase plasminogen activator (uPA) [30]. The impact of HDAC-I on antitumor **immunity** can probably be explained by increased immunogenicity of tumor cells [31] and altered activity of immune cells [32]. However, these effects have not been clarified yet.

The open chromatin structure induced by histone hyperacetylation also renders the chromatin more accessible for **DNA damage** by for example cross-linking agents or ionizing radiation. In addition, this structural change leads to impaired **DNA replication** and **site-specific recombination**.

The observed effects are not only related to histone acetylation, but also to the ability of HDAC-I to acetylate various non-histone targets. These include **transcription factors** such as E2F1 [33], STAT1 [34], STAT3 [35] and NF-κB [36], and several key **regulators of signaling cascades** such as pRB [37], p53 [38] and Hsp90 [39].

A major working mechanism of HDAC-I implies **lowered repair of DNA damage** induced by external agents. This effect is associated with acetylation of proteins involved in DNA repair such as Ku70 [40], Ku80 [41], BRCA1 [42], Rad51 [43] and DNA-PK [41, 43]. These proteins are involved in the two major pathways for repair of double strand breaks (DSB): homologous recombination (HR) and non-homologous end joining (NHEJ). Additional suppression of DNA damage repair by HDAC-I is related to the **direct inhibition of HDAC isoforms**, preventing these from interaction with DNA damage sensor proteins such as ATM (interaction with HDAC1) [44] and 53BP1 (interaction with HDAC4) [45].

Finally, it has been shown recently that HDAC inhibition in itself is also capable to decrease global **methylation** levels and even to induce expression of some- but not all- methylated genes [46, 47]. This effect on gene expression might also add to the anticancer properties of HDAC-I.

All together, these processes are responsible for the pleiotropic effects of HDAC inhibiting drugs (represented schematically in Fig. **2**).

COMBINATIONS OF DEMETHYLATING AGENTS AND HDAC-I

Next to their single agent anticancer potential, demethylating agents and HDAC-I have proven to be valuable in combination schedules.

Both these epigenetic drugs have been successfully combined with classic chemo-or hormonal therapies, *in vitro* as well as in clinical studies. Of particular interest is the combination of demethylating agents with HDAC-I.

Despite the ability of both demethylating agents and HDAC-I to reverse gene silencing on their own, it is generally believed that a combination of these drugs leads to a more efficient re-expression of epigenetically silenced genes [48-51]. The biggest effect on gene expression seems to be obtained with schedules administrating demethylating drugs followed by HDAC-I. Therefore, most preclinical and clinical studies have focused on the potential of these combinational therapies [52], with promising results in several cancer types. Enhanced anti-cancer effects have been described *in vitro* or *in vivo* both in hematological malignancies [53] and in solid tumors such as hepatocellular carcinoma [54], bladder cancer [55], prostate cancer [56], breast cancer [57] and others [48]. These data have suggested that - for the same effect on the methylation level - the dose of Decitabine used in clinical studies could be reduced when used in combination with HDAC inhibitors. The first Phase I/II trials assessing the combination of Decitabine with several HDAC-I (SAHA, Depsipeptide) in hematological malignancies

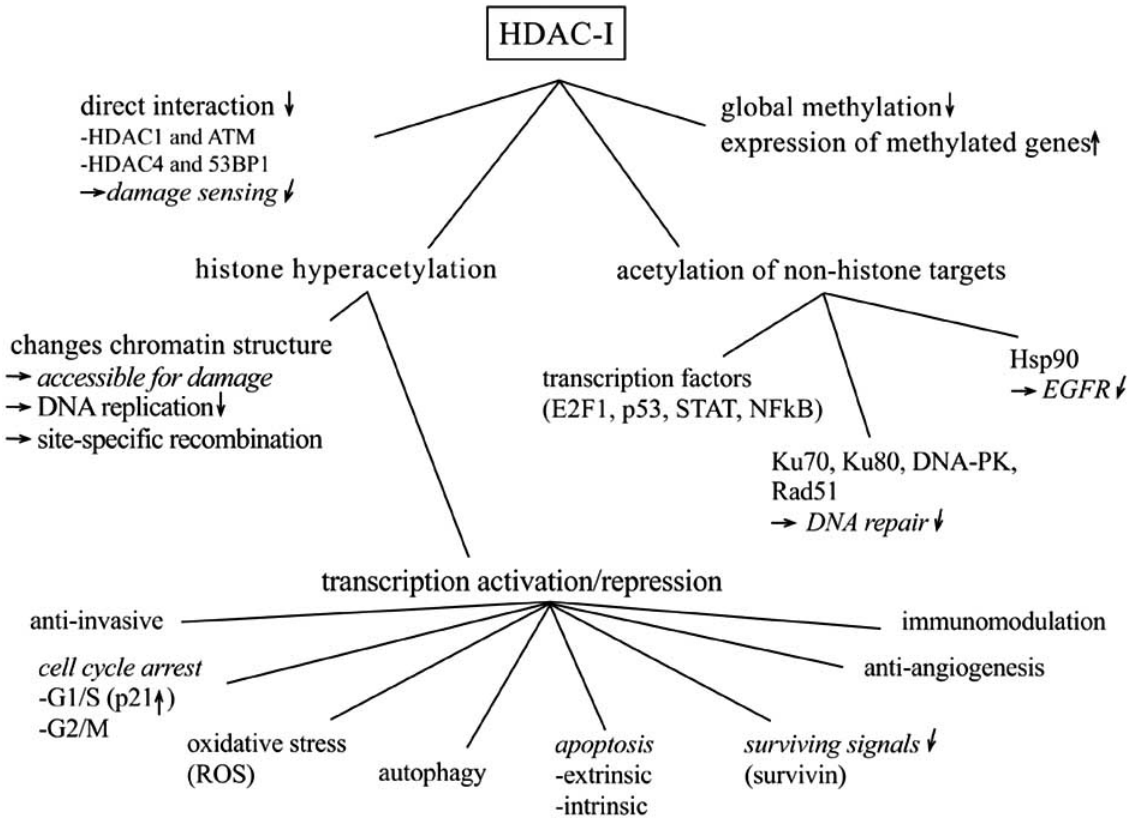

Fig. (2).

have been performed. These combinations seem active and safe and are accompanied by transient reversal of aberrant epigenetic marks [5, 58]. Several other clinical trials, also in solid tumors, are currently ongoing (clinical trials.gov).

RADIOSENSITIZING POSSIBILITIES OF EPIGENETIC ANTICANCER DRUGS

So far, little work has been done on the influence of **demethylating agents** on radiation response.

For some genes involved in response to radiation-induced DNA damage, a relation between methylation of these genes and radiosensitivity has been described. This is the case for ATM, which plays a pivotal role in triggering appropriate cellular response to genome damage. Methylation of the ATM gene correlated with increased radiosensitivity in colorectal [59] and glioma [60] cell lines and this effect was reversed by a treatment with 5-azacytidine. The opposite effect was described for methylation of RUNX3 in esophageal squamous cell carcinoma. RUNX3 was found to increase radiation-induced cell death through activation of apoptosis. Methylation of this gene was associated with radioresistance *in vitro* and in a patient population. The reversal of RUNX3 silencing *in vitro* by 5-aza-2'-deoxycytidine resulted in radiosensitization as demonstrated by colony assays [61].

Concerning radiosensitization induced by DNA demethylating agents, Dote et al described enhancement of tumor cell radiosensitivity *in vitro* and *in vivo* by Zebularine [62]. A 48-hour treatment of glioblastoma, pancreatic and prostate carcinoma cells with this drug resulted in maximum loss of methylation and an increase in radiosensitivity with dose enhancement factors of >1.5. The observed effect was accompanied by an increase in γH2AX foci 24 hours after irradiation, suggesting the presence of unrepaired DNA double strand breaks. The radiosensitizing mechanism did not include an effect on cell cycle or apoptosis.

The radiosensitizing potential of **HDAC inhibitors** is being studied more extensively and seems promising. Theoretically, the combination of HDAC inhibitors and radiation could result in additive cell death due to the different cytotoxic mechanisms associated with each modality. Both modalities could also act synergistic because of the capacity of HDAC inhibitors to modulate chromatin structure and to regulate gene expression. Indeed, compaction of chromatin into higher order structures protects DNA from radiation-induced double strand breaks (DSBs) [63] and non-transcribing genes are generally less sensitive to the effects of ionizing radiation [64].

Several *in vitro* studies have reported on the radiosensitizing capacity of HDAC inhibiting drugs. The earliest observations showing enhancement of radiation sensitivity by sodium butyrate were already published about two decades ago [65-67]. Some years later, TSA was also described to have radiosensitizing potential [68]. Since then, several compounds have been tested successfully *in vitro* in several tumor types, covering most classes of HDAC-I. For some of the HDAC-inhibiting drugs, the radiosensitizing effects have been confirmed in animal models. To our knowledge, only two clinical trials assessing the combination of HDAC inhibition and radiotherapy are currently ongoing: one phase I trial on SAHA and palliative radiotherapy, and one phase II study on the combination of VPA, temozolomide and external beam radiotherapy (www.cancer.org). Although they are potent radiosensitizers in vitro, clinical use of some of the investigated HDAC-I (e.g. TSA, M344) is limited because of drug toxicity and instability. All preclinical studies showing radiosensitization with HDAC-I are listed in Table **1**.

Radiosensitization by HDAC-I has been explained by modulation of cell-cycle regulation [69-71], enhancement of radiation-induced apoptosis [43, 70, 72-81] and downregulation of surviving signals [82]. In some cases, the observed radiation-induced cell

Table 1. Preclinical Studies with HDAC-I in Combination with Radiotherapy

Drug Class	Drug	Tumor Type	*In vitro/in vivo*	References
hydroxamates	TSA	leukemia	*In vitro*	[72, 95, 96]
		medulloblastoma	*In vitro*	[73]
		glioblastoma	*In vitro*	[89]
		melanoma	*In vitro*	[41]
		breast	*In vitro*	[42, 97]
		squamous cell	*In vitro*	[42, 90]
		epidermoid	*In vitro*	[82]
		pancreatic	*In vitro*	[98]
		colon	*In vitro*	[68-70]
		lung	*In vitro*	[70]
	SAHA	NSCLC	*In vitro*	[75]
		medulloblastoma	*In vitro*	[73]
		squamous cell	*In vitro*	[90]
		melanoma	*In vitro*	[74]
		colon	*In vitro*	[69]
		glioma	*In vitro*	[43]
		prostate	*In vitro*	[43]
	LBH589	NSCLC	*In vitro/in vivo*	[76]
	PCI24781	cervix	*In vitro*	[84]
		colon	*In vitro*	[84]
	LAQ824	NSCLC	*In vitro/in vivo*	[77]
	M344	squamous cell	*In vitro*	[90]
	CBHA	lung	*In vitro/in vivo*	[85]
		squamous cell	*In vitro*	[83]
		gastric	*In vitro*	[81]
		colorectal	*In vitro*	[81]
short-chain fatty acids	VPA	leukemia	*In vitro*	[78, 99]
		brain	*In vitro/in vivo*	[87]
	NaB	colon	*In vitro*	[65, 66, 100]
		medulloblastoma	*In vitro*	[73]
		endometrium	*In vitro*	[101]
		melanoma	*In vitro*	[41]
	PB	nasopharyngeal	*In vitro*	[102]
		cervix	*In vitro*	[103]
		prostate	*In vitro*	[26, 71]
		breast	*In vitro*	[71]
		colon	*In vitro*	[71]
		brain	*In vitro*	[71, 88]
	PA	cervix	*In vitro*	[103]
		prostate	*In vitro*	[71]
		breast	*In vitro*	[71]
		brain	*In vitro*	[71]
		colon	*In vitro*	[71]
	Tributyrin	melanoma	*In vitro*	[41]
	AN1	glioma	*In vitro*	[79]
	AN113	glioblastoma	*In vitro*	[80]

(Table 1) Contd…

Drug Class	Drug	Tumor Type	*In vitro/in vivo*	References
cyclic peptides	FK228	squamous cell	*In vitro*	[83, 90]
		lung	*In vitro*	[85]
		colorectal	*In vitro*	[81]
		gastric	*In vitro/in vivo*	[81]
benzamides	MS275	prostate	*In vitro/in vivo*	[86, 104]
		glioma	*In vitro*	[86]
		gastric	*In vitro*	[81]
		colon	*In vitro*	[69, 81]
	CI994	glioma	*In vitro*	[91]
		squamous cell	*In vitro*	[90]
other	SK7041	colon	*In vitro*	[70]
		lung	*In vitro*	[70]

death was connected to hyperacetylation-associated increased (Bim [81], Bmf [83]) and decreased anti-apoptotic (Bcl-XL [26, 81]) protein expression. While these are important mechanisms underlying radiation sensitization at high doses of HDAC-I, lower doses have also been shown to modulate the sensitivity of cells to ionizing radiation. Indeed, radiosensitization occurs at doses that are non-toxic and do not induce cell-cycle arrest, but are potent enough to alter histone acetylation status. Several explanations underlie this effect. First, HDAC-I render the chromatin into an open structure that is more accessible to external damage. Upon HDAC inhibition, ionizing radiation can more efficiently induce potentially lethal DSBs. In addition, normal cellular responses to DSBs include detection and repair of these lesions by DNA damage sensor proteins and DNA repair proteins, respectively. HDAC-I disrupt both processes: they disrupt association of HDAC enzymes with the sensor proteins ATM and 53BP1, and they alter the acetylation status of proteins involved in both the HR (BRCA1, Rad51, Rad50) [42, 43, 74] and NHEJ (Ku70, Ku80, DNA-PK) [26, 41, 43, 74] repair pathways. The above-mentioned mechanisms result in an increase in the number of DSBs combined with a decrease in the repair of these lesions. Phosphorylation of H2AX to form γH2AX is one of the earliest events following the induction of DSBs and plays an important role in recruiting repair factors to nuclear foci following DSB formation. Several investigations have shown an increase in the number [80, 84-86] and a prolonged expression [41, 76, 77, 87] of γH2AX foci upon treatment with ionizing radiation.

Besides influencing repair pathways, HDAC-I can also change the acetylation status of other proteins such as Hsp90. Some authors have attributed the observed radiosensitization to dissociation of EGFR from the Hsp90 complex, resulting in receptor degradation [43]. Another protein that can be altered upon HDAC inhibition is p53, for which the role in the radiosensitization has not been clearly established [69, 70, 81, 85, 88]. These indirect effects may at least be partly responsible for the effect on radiation response.

Today, there is no firm agreement on the optimal time schedule for combinations of HDAC-I with radiotherapy. Most investigations have shown a beneficial effect when the epigenetic drug is administered for 18h to 24h and removed before irradiation [41, 43, 70, 72, 74, 76, 85, 89, 90]. Others have claimed more effective radiosensitization in case of concomitant radiation and drug application: in these cases the drug is administered before and after irradiation [80, 86-88, 91]. For PB, Goh *et al.* have described enhancement of radiation-induced apoptosis by administering the drug for 72h following irradiation [26]. One report has mentioned a reduction in radiation sensitivity by means of drug pretreatment times of 24h, while an increase was reported after 72h of drug pretreatment [71].

These considerations have to be taken into account when moving from *in vitro* to *in vivo* and eventually, clinical studies. *In vivo* experiments have shown radiosensitization with HDAC-I administered pre- and post-irradiation, with acceptable normal tissue toxicities [91]. Whether the presence of drug in the post-irradiation period is also necessary when treating patients remains to be elucidated.

Interestingly, HDAC-I not only act as tumor-selective radiosensitizers, but also as protectors of radiation-induced normal tissue damage. Topical applications of TSA, VPA and NaB were shown to protect from radiation-induced injury in an animal model of skin radiation syndrome. Although the mechanisms behind this radioprotection remain to be clarified, an HDAC-I-mediated decrease in the inflammatory cytokine tumor necrosis factor alpha (TNF-α) and transforming growth factors (TGF)-β1 and TGF-β2 [92, 93] could be partly responsible.

To our knowledge, so far only one group has reported on the radiosensitizing potential **demethylating agents combined with HDAC-I**. Indeed, Arundel *et al.* [66] have shown a greater than additive effect when adding 5-azacytidine/Decitabine in combination with NaB to irradiation in colon tumor cells. They attributed this effect partly to a decrease in potentially lethal damage repair.

CONCLUSION

The recent acknowledgement of cancer as an epigenetic disease has resulted in several initiatives implementing epigenetics in cancer prevention and treatment. Next to a potential role of promoter hypermethylation and histone modifications as molecular biomarkers, the reversibility of these processes has led to the development of so-called epigenetic drugs. In that scope, both demethylating and histone acetylating drugs have been recognized as potential therapeutics.

Several reports so far have reported on the *in vitro* and *in vivo* anticancer potential of these epigenetic drugs as single agents. These positive results have been extended to clinical trials, with emphasis on hematological malignancies.

Besides their activity as single agents, demethylating drugs and HDAC-I have also proven to be valuable in combinational therapies. Both epigenetic drug types have been successfully combined with classic chemo- and hormonal therapeutics.

Because of their additional effect on gene silencing reversal, the combination of demethylating agents with HDAC-I is of special interest. Schedules combining these drugs - mostly administering the demethylating drug in advance, followed by HDAC inhibition - have shown anticancer activity *in vitro* and *in vivo* in several hematological and solid tumor types. Clinical studies in hematological

malignancies appear optimistic, and trials in solid cancer are underway.

Most of this review, however, focused on the radiosensitizing potential of epigenetic drugs. Concerning the combination with radiation, most work so far has concentrated on HDAC inhibition alone. Radiosensitization has been reported *in vitro* and *in vivo* for several classes of HDAC-I. Whether these drugs could also be implemented in the clinic to enhance the effect of ionizing radiation remains unclear and warrants further investigation. Interestingly, HDAC-I could also act as radiation protectants of normal tissue. Based on preclinical reports, Demethylating drugs as single agents also hold potential as radiosensitizers. Clinical investigations on demethylating agents in combination with irradiation are lacking but seem promising. One step further is the combination of demethylating agents + HDAC-I + irradiation. Theoretically, demethylating agents and HDAC-I will affect the expression of multiple genes and this might result in opposing effects on radiosensitivity. However, as radiation resistance is considered to be a polygenic process, there may also be advantages to such a multitargeted approach. To our knowledge, this was only investigated by one group in the eighties, showing radiosensitization of colon cancer cells by combining 5-azacytidine or Decitabine with NaB [66].

Translating this knowledge to the clinic, one has to realize that patients' response to epigenetic therapies as radiosensitizers will be heterogeneous. An important challenge lies in the identification of methylation patterns that could reliably predict response to treatment. There is a clear need for a clinically applicable tool to determine these methylation profiles and for pharmacodynamic markers to monitor *in vivo* administrations of epigenetic drugs [94].

In conclusion, the field of epigenetics has evolved dramatically over the last few decades. Besides the potential of epigenetic alterations to act as biomarkers for cancer detection and treatment, their reversibility has led to the development of several drugs aiming to restore a normal epigenetic balance. These drugs can act as single agents but also seem successful in combinational therapies. Their radiosensitizing potential *in vitro* and *in vivo* is encouraging, but as always, clinical trials have to be set up to correctly evaluate their radiosensitizing potential.

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TSA: Trichostatin A; SAHA: suberoylanilide hydroxamic acid; CBHA: m-carboxycinnamic acid bishydroxamide; VPA: valproic acid; NaB: sodium butyrate; PB: phenyl butyrate; PA: phenyl acetate.

LIST OF ABBREVIATIONS

CXCR4 = chemokine C-X-C motif receptor 4
DNMT = DNA methyl transferase
DSB = double strand breaks
HAT = histone acetyl transferase
HDAC = histone deacetylase
HDAC-I = histone deacetylase inhibitors
HMT = histone methyl transferase
HR = homologous recombination
MBD = methyl-CpG-binding proteins
MMP = matrix metallo proteinase
NaB = sodium butyrate
NHEJ = non homologous end joining
PcG = polycomb complexes
PA = phenyl acetate
PB = phenyl butyrate
SAHA = suberoyl anilide hydroxamic acid
TNF-α = tumor necrosis factor alpha
TGF = transforming growth factor
TSA = trichostatin A
TSG = tumor suppressor gene
VEGF = vascular endothelial growth factor

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