Targeting the epigenome: effects of epigenetic treatment strategies on genomic stability in healthy human cells

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Abstract Epigenetic treatment concepts have long been ascribed as being tumour-selective. Over the last decade, it has become evident that epigenetic mechanisms are essential for a wide range of intracellular functions in healthy cells as well. Evaluation of possible side-effects and their underlying mechanisms in healthy human cells is necessary in order to improve not only patient safety, but also to support future drug development. Since epigenetic regulation directly interacts with genomic and chromosomal packaging density, increasing genomic instability may be a result subsequent to drug-induced epigenetic modifications. This review highlights past and current research efforts on the influence of epigenetic modification on genomic stability in healthy human cells.

Keywords Genomic instability · Epigenetics · HDAC inhibitors · Radiosensitivity · DNA methylation · Histone acetylation

Introduction

While referring to the genome as the ‘book of life’, it is the epigenome that forms the annotations, footnotes, and tags necessary to store as well as interpret the raw text. DNA methylation and/or posttranslational histone-modifications build the backbone of the epigenetic code.

Methylation is carried out by a family of DNA methyltransferases (DNMTs) and may be targeted to CpG-rich regions at the genomic DNA level (‘CpG-islands’). If hypermethylation is taking place within the promoter regions of specific genes, this may consequently be associated with transcriptional silencing (Issa 2004). Additionally, tRNA aspartic acid methyltransferase 1 (TRDMT1, formerly known as DNMT2) was shown to methylate with high specificity RNA but not DNA (Goll et al. 2006).

Currently, three DNMT/TRDMT-inhibiting substances are in the focus of clinical trials: 5-azacytidine (5-azaC), 5-aza-2'-deoxycytidine (5-aza-dC) and 5-Fluoro-2'-deoxycytidine, while a fourth substance, zebularine, already showed high toxicity in preclinical studies excluding it from further clinical trials (Issa and Kantarjian 2009). In addition, in 2006, a phase II clinical trial with MG98, a specific antisense oligodeoxynucleotide inhibitor on mRNA for human DNMT1, was stopped due to a lack of significant response (Winquist et al. 2006). Although an updated treatment scheme based on a recently published phase I trial may show better outcome in future trials (Plummer et al. 2009).

While the nucleoside analogue 5-azaC is primarily incorporated into RNA and only a fraction of 5-azaC is secondarily incorporated into DNA, 5-aza-dC directly incorporates into DNA. Incorporation of nucleoside analogues leads to sequestration of DNMTs via covalent bond formation resulting in the inhibition of DNMTs (Stresemann et al. 2006). 5-azaC could recently shown to be also capable of inhibiting TDRMT1 as well (Schaefer et al. 2009). Two drugs have already achieved approval by the FDA: 5-azaC (Vidaza®) in 2004 for treatment of all subtypes of MDS with a recent extension of indications and in 2006 5-aza-dC (Decitabine®), also for the treatment of MDS.
Despite the steadily increasing interest in epigenetic treatment concepts, only little is known about the influence of drug-induced epigenetic modifications in healthy cells. The attention of this review is therefore drawn away from epigenetic treatment effects on malignant cells towards genomic stability in healthy human cells.

**Hypomethylating agents**

Despite the fact that little is known about the consequences of intracellular hypomethylation in the fight against cancer, scientists and clinicians have been testing maximum-tolerable doses (up to 750 mg/m² per application in adults) for the cytosine analogues 5-azaC and 5-aza-dC over many years (Sorm et al. 1964). The use of both agents was limited due to severe cytotoxicity (Haaf 1995). The idea of resetting a deranged epigenetic code is based on the strong hypomethylating qualities of the mentioned cytosine analogues which occur at significantly lower doses, rather than on the cytotoxic potential of these agents (today, 5-azaC is usually applied at a dose of 75 mg/m²). Bone-marrow depression is the most common side-effect in association with hypomethylating agents, while classical cytotoxic side-effects, such as hair loss, mucositis, diarrhoea, and renal failure are rare (Issa and Kantarjian 2009). Nevertheless, although administrated doses have been minimized, three mechanisms with the potential to damage genomic integrity are currently discussed: (1) chromosomal fragility due to DNA hypomethylation, (2) covalent binding of the large DNMT to DNA forming a ‘mechanical barrier’ and (3) decrease in DNA repair capacity (Jackson-Grusby et al. 1997; Kiziltepe et al. 2007; Morales-Ramirez et al. 2008).

Chromosomal breaks occur more frequently within undercondensed chromatin regions

Chromosomes or chromosome fragments which are not incorporated into daughter nuclei during mitosis can be excluded from the cell nucleus, thus forming ‘micronuclei’ within the cytoplasm. The scoring of micronuclei is still a popular method in the determination of chromosomal instability (Geigl et al. 2008). In the 1970s and 1980s the formation of “pulverized” (Schmid et al. 1984) and over-segmented chromosomes (Viegas-Péquignot and Dutrillaux 1976) subsequent to high doses of 5-azaC ($\geq 10^{-4}$ M) was reported, while low doses inhibited the formation of constitutive heterochromatin in human lymphocytes (effects can be even found at very low doses of $5 \times 10^{-9}$ M, (Haaf 1995)). Epigenetics was still in its infancy when in 1994 the formation of micronuclei was reported after incubation with 5-azaC in human lymphocyte cultures as an expression of chromosomal instability (Guttenbach and Schmid 1994).

Most interestingly, chromosomes 1, 9, 15, 16 and Y have been reported to be undercondensed and thus particularly sensitive to 5-azaC-treatment (at a dose of $\geq 5 \times 10^{-7}$ M), which subsequently results in DNA strand breakage and thus in the exclusion of micronuclei (Guttenbach and Schmid 1994; Satoh et al. 2004), although later participation of chromosome 15 couldn’t be confirmed (Fauth et al. 1998). Additionally, analysis of 5-azaC-induced undercondensation in the pericentromeric region of human fibroblast chromosomes gave no evidence of whole-chromosome aneuploidy, but of chromosomal aberrations including the classical DNA satellite regions (Cimini et al. 1996). In contrast to these findings, Kiziltepe et al. using 5-azaC at similar doses did not find any significant cytotoxicity against normal donor PBMCs and BMSCs, but against multiple myeloma (MM) cells (Kiziltepe et al. 2007).

**HDAC inhibitors**

Another fundamental regulation of gene expression takes place at the nucleosome level: Acetylation and deacetylation of N-terminal histone tails allows for the posttranslational modification of gene activity. The acetylation of specific lysine residues is mediated by the enzymatic activity of histone-acetyl-transferases (HATs; following an approach for a new nomenclature they may be better called K-acetyltransferases KATs, see Allis et al. (2007)) and is associated with the formation of transcriptionally active euchromatin. Conversely, the removal of such acetyl groups from specific lysine amino acid residues and substitution by positively charged amino groups at the N-terminal histone tails results in transcriptional gene silencing (see Table 1 for an overview over known histone deacetylases (HDACs)). A steadily increasing number of naturally occurring or synthetically produced HDAC inhibitors with heterogeneous chemical structures allow—at least in vitro—the reactivation of genes, which may include both, dormant tumour-suppressor genes and potentially also oncogenes (Mahlknecht and Hoelzer 2000; Mei et al. 2004; Purrucker et al. 2010; Villar-Garea and Esteller 2004). Until today, SAHA (suberoylanilide hydroxamic acid, Vorinostat, Zolinza®) is the only HDAC inhibitor that has been approved by the FDA. For further information on HDAC inhibitors mentioned in this review, please refer to Table 2.

Radiosensitization of healthy human cells

Numerous in vitro studies have been published on a potential HDAC-inhibitor-mediated tumour-radiosensitization. However, only few and quite controversial data are available on radiosensitization effects in normal human cells. At sites of radiation-induced DNA double-strand breaks (DSBs)
immediate phosphorylation of the histone variant H2AX takes place, forming distinct foci, which can be visualized e.g. through immunofluorescence techniques such as the γH2AX-focus analysis (Sedelnikova et al. 2002). The loss of ionizing radiation-induced γH2AX foci correlates with clonogenic survival (MacPhail et al. 2003). DSBs are repaired following two major pathways: Homologous recombination utilizes a complementary template provided by the sister chromatid during DNA replication, whereas non homologous end-joining does not restore the original sequence and is not bound to a specific phase of the cell cycle making it the major pathway of DSB repair in mammalian cells (Lieber 2008; Pandita and Richardson 2009).

Table 1 Overview over histone deacetylase inhibitors and their properties cited in this review

| HDAC-inhibitor | Full name | Synonyms/brand names | Chemical class | Substrate | Clinical trials | Number of clinical trialsa (A =active, NA=not active) |
|----------------|-----------|----------------------|----------------|-----------|----------------|-----------------------------------------------|
| AN-1           | Butyroyloxymethyl butyrate | – | Short-chain fatty acid | N/A | – | – |
| AN-9           | Pivaloyloxymethyl butyrate | Pivanex® | Short-chain fatty acid | N/A | Phase I, II (CLL, MM, NSCLC) | n=3 (NA 3) |
| NaB            | Sodium-butyrate | – | Short-chain fatty acid | Class I, IIa | – | – |
| PB             | Phenylbutyrate | – | Short-chain fatty acid | Class I, IIa | Phase I, II (AML, MDS, NSCLC, colorectal cancer) | n=9 (NA 9) |
| VA             | Valproic acid | Depakote®, Convulex®, Orfiril®, Stavzor® | Short-chain fatty acid | Class I | Phase I, II (hematologic neoplasias, solid tumours) | n=45b (A 23, NA 22) |
| AAHA           | Azelaic-1-hydroxamate-9-anilide | – | Hydroxamate | N/A | – | – |
| ABHA           | Azelaic bishydroxamic acid | – | Hydroxamate | N/A | – | – |
| CBHA           | m-Carboxycinnamic acid bis-hydroxamide | – | Hydroxamate | N/A | – | – |
| PCI-24781      | – | – | – | – | n=5 (A 2, NA 3) |
| SAHA           | Suberoylanilide hydroxamic acid | Vorinostat®, Zolinza® | Hydroxamate | Class I, IIa, b | Phase I, II (hematologic neoplasias, sarcoma) | n=159 (A 78, NA 81) |
| SBHA           | Suberic bishydroxamate | – | Hydroxamate | N/A | – | – |
| TSA            | Trichostatine A | – | Hydroxamate | Class I, IIa, b | – | – |
| MS275          | SNDX-275, Etinostat | – | Benzamide | Class I, IIa | Phase I, II (hematologic neoplasias/solid tumours) | n=9 (A 3, NA 6) |

Some data obtained from (Eot-Houllier et al. 2009; Rasheed et al. 2007; Witt et al. 2009)

Abbreviations: AML acute myeloid leukaemia, CLL chronic lymphocytic leukaemia, MM malignant melanoma, NSCLC non-small cell lung cancer, MDS myelodysplastic syndrome

a As listed on http://clinicaltrials.gov

b Only studies in which drug was used alone or in combination for treatment of neoplasias were included
Both, increased induction of DSBs as well as delayed or inhibited repair increases the risk of genomic rearrangements. Incubation with HDAC inhibitors prolongs the expression of γH2AX foci significantly in a great number of tumour cell lines and reduces their clonogenic survival, suggesting a decrease in DNA repair capacity (Camphausen and Tofilon 2007). Using the method of premature-chromosome-condensation, Stoilov et al. recognized that incubation with NaB (sodium-butyrate) inhibits the repair of chromosome breaks in G0 human lymphocytes, while it has no effect on the initial level of radiation-induced DNA damage (Stoilov et al. 2000), which would have been a sign of direct HDAC-inhibitor-induced genomic instability. Munshi et al. (Munshi et al. 2005) found NaB-induced radiosensitization in two melanoma cell lines without changes in DNA repair capacity in healthy human lung fibroblasts.

In order to assess the anti-proliferative activities of the HDAC inhibitors AN-1 (butyroyloxyxymethyl butyrate) and AN-9 (pivaloylomethyl butyrate) against glioma cell lines, Entin-Meer et al. (Entin-Meer et al. 2005) also performed tests on human primary astrocytes and found a strong cell-line-specific and dose-dependent increase in apoptosis in all glioma cell lines (apoptosis rates: 50–90%), but not in primary astrocytes, where apoptosis rates were only around 20%. In order to determine potential radiosensitization effects, cell lines were treated with AN-1 and AN-9 prior to irradiation with doses of 3, 6 or 10 Gy. After irradiation, apoptosis rates were determined by flow cytometry. Unfortunately, a direct comparison between cell lines is complicated due to differences in the administration of radiation as well as drug doses. While combination-treatment schemes (AN-1/9 plus irradiation) led to increased apoptosis in all tumour cell lines when compared with irradiation alone, there was no similar increase in mortality in primary astrocytes.

In 2007, Banuelos et al. (Banuelos et al. 2007) reported the radiosensitization of cervical and colon carcinoma cells subsequent to exposure to the HDAC inhibitor PCI-24781, a hydroxamate which is undergoing first clinical trials. Survival was determined using a clonogenic survival assay, with cells exposed to drug 20 h prior until 4 h post-irradiation. Similarly, in this study radiosensitization of human skin fibroblasts was also assessed, however human skin fibroblast survival was assessed subsequent to irradiation with a single dose of 3 Gy, while carcinoma cell lines were irradiated with 2, 4, 6 and a maximum of 8 Gy, respectively. The authors’ documentation of only a minimal radiosensitization in human noncycling fibroblasts upon treatment with PCI-24781 therefore is not very convincing and requires further evaluation.

In recently published experiments, we examined the possible effects of HDAC inhibition on DNA repair in human skin fibroblasts. For this purpose, we exposed cells in vitro to the HDAC inhibitors SAHA, MS275, NaB and VA before radiation with 0.5 Gy. Survival was determined using a clonogenic survival assay. By first using the γH2AX-focus analysis, we found a significant decrease in DSB-repair capacity for SAHA, MS275 and NaB, with up to 3.3 times higher persisting foci rates after 24 h. Consequently, clonogenic survival was reduced as well (Purrucker et al. 2010) (for results on VA please refer to the special case of VA’ below).

Effects on proliferation and cell viability

As reported, HDAC inhibitors carry effects on both, genomic stability as well as on the regulation of gene expression. Since both processes may result in decreased proliferation and impaired cell viability, it is worth to take a closer look on the following publications. In 1997, an Australian research group began to investigate potential anti-proliferative effects of the HDAC-inhibitor azeleic bishydroxamic acid (ABHA). Cell growth in neonatal foreskin melanocytes and fibroblasts (NFF) was not decreased, but proliferation of human cervical (HeLa) and ovarian tumour cell lines as well as human melanoma (MM418c1, MM96 L, Mel-SV) and transformed keratinocytes (HaCat) was reduced (Parsons et al. 1997). Later, the same research group published somewhat controversial data by reporting a G2/M growth arrest of NFF when treated for 24 h with ABHA (Qiu et al. 2000), but this may be explained by the use of tenfold higher doses of ABHA in this study.

In general, HDAC inhibitors may block proliferation by up-regulating the expression of the cdk (cyclin dependent kinase) inhibitor p21Waf1/Cip, which leads to a G1 phase arrest (Mahlknecht and Hoelzer 2000). Tumour cells frequently lack proper checkpoint function, which leads to aberrant mitosis, transmission of mutated genes, fractured chromatin and eventually cell death. Accordingly an observed increase in cell death in MM96L and HeLa cells upon treatment with ABHA may be explained by cells attempting to undergo mitosis in an inappropriate state, resulting in the non-congression of condensed chromosomes and missegregation during cytokinesis (Qiu et al. 2000). Qiu et al. demonstrated NFF cells to own a functional ABHA-sensitive G2 checkpoint which allows cells to switch into G2/M arrest. The authors did however not comment on whether the growth arrest was reversible or irreversible after drug removal, an important factor of possible toxicity in rapidly proliferating normal cells in vivo. Furthermore, results seemed to be drug specific for ABHA, since Qiu et al. had reported earlier a significant killing of MM96L and HeLa cells after treatment with ABHA and AAHA (azeleic-1-hydroxamate-9-anilide), with
AAHA going along with an increased proliferation of fibroblasts (Qiu et al. 1999). However, the hydroxamate trichostatin A (TSA) caused more killing in fibroblast cultures than in MM229 and HeLa tumour cells (Qiu et al. 1999). This result is most remarkable in so far as it is one of the rare reports on stronger toxicity in normal human cells when compared with transformed cells.

Again in 1999, the same Australian institute published effects of NaB and TSA on growth and differentiation of human keratinocytes (HKCs) and squamous carcinoma cells (SCC25 and SCC15) (Saunders et al. 1999). Proliferation was measured by [3H]-thymidine incorporation. Both NaB and TSA were reported to induce growth arrest, which was irreversible in the case of TSA, where a significant proportion of keratinocytes did not resume DNA synthesis. Consistent with this, mRNA-levels of cdk1 were reduced. Even though proliferation was strongly inhibited, both HDAC-inhibitors were not substantially cytotoxic, since cell viability remained high (EC50 NaB; of 0.5 mM; EC50 TSA: 120 nM). Interestingly, the authors report similar effects on cell viability in squamous carcinoma cell lines, with the exception that mRNA expression of the differentiation specific gene TG1 (transglutaminase type I) was induced in HKC cells, while it was decreased in squamous carcinoma cell lines. Additional in vitro and in vivo studies will be needed to determine whether the therapeutic index in this context will be sufficient or not. Currently, there is no clear evidence of a direct effect of HDAC inhibition on genomic stability as the cause of the observed growth inhibition. However, it needs to be considered particularly in view of the fact that the observed time frame was only 48 h and cells were not specifically exposed to DNA damaging agents, such as ionizing radiation as one of the most frequent sources of iatrogenic DNA damage (Brenner and Hall 2007; Heilig et al. 2010).

In 2001, Brinkmann et al. compared growth inhibition of normal keratinocytes (nHKCs) and dermal fibroblasts (HDFs) to transformed keratinocytes (SCC25) by exposing cells to various HDAC inhibitors (suberic bishydroxamate SBHA, NaB, phenylbutyrate and its enantiomeric forms R-PB/S-PB and AAHA (Brinkmann et al. 2001). Hexamethylene bisacetamide (HMBA), which is not an HDAC-inhibitor, but belongs to the same chemical group of hybrid polar compounds together with SAHA and CBHA, was also tested in this study (Brinkmann et al. 2001; Richon et al. 1998; Xu et al. 2005). The authors reported a growth inhibition in normal and transformed keratinocytes, but not in normal dermal fibroblasts, although direct comparison seems to be difficult, since IC50 values were only reported for nHKC vs. SCC25 cells, and not HDFs. Growth inhibition was reported to be drug specific, as SBHA showed higher IC50 levels for nHKC than for SCC25, although with a high standard error of the estimate. In contrast, inhibition of DNA synthesis was compared in nHKC vs. HDFs, and again, results seemed to be drug and not substance-class specific: Interestingly, HDAC inhibitors NaB, R-PB, AAHA and SBHA increased DNA synthesis in HDFs and decreased synthesis in nHKCs, while in the case of HMBA it is the other way round. Apoptosis was not detected in nHKCs or SCC25 cells, making it difficult to contribute observed growth inhibition directly to an increase in genomic instability.

Additionally, accumulation of hyperacetylated histones per se is not sufficient to cause growth inhibition (Brinkmann et al. 2001), endorsing the hypothesis that growth inhibition due to HDAC inhibitors is a complex process based upon direct modified gene expression and contributing effects of chromosomal or genomic instability.

Combined treatment concepts

As well as sensitizing the human genome to radiation, HDAC inhibitors could be thought of as drugs capable to untighten the chromatin DNA in order to make it more accessible for classic chemotherapeutic agents, like DNA cross-linkers or alkylating agents. Again, studies comparing cytotoxic effects on both malignant and normal cells are rare.

No increased genomic susceptibility to common anticancer drugs was found for normal breast (MCF-12) and intestinal epithelial cells (FHs 74 int) (Kim et al. 2003). Cell exposure to cisplatin, a DNA cross-linker, cyclophosphamide (alkylating agent), VP-16 (etoposide, a topoisomerase II inhibitor) or 5-FU, which is an antimetabolite, subsequent to pre-treatment with SAHA did not significantly change killing efficiency when compared to treatment in reverse order (killing by SAHA alone was not specifically measured in this study). In glioblastoma as well as breast cancer cell lines, a sensitization to the cytostatic agents VP-16, ellipticine (DNA intercalator), doxorubicin (DNA groove binder and intercalator) and cisplatin was observed as a consequence of pre-treatment with SAHA or TSA. These findings suggest additional cytotoxicity in cancer cells, which may be caused by increased genomic fragility subsequent to pre-treatment with HDAC inhibitors. Possible contributing effects caused by different and/or modified cell cycle regulation in tumour vs. normal cells have not been investigated. Also, conclusions should be drawn with caution, since exposure in the clonogenic survival assay was quite short for slower proliferating normal cells with only 4 h for SAHA or TSA and removal 1 h prior to exposure to the anticancer drugs, which was then followed by two more weeks in drug-free medium.

Prevention of corneal scar formation

An exposure to NaB or TSA for up to 3 days, was associated with the inhibition of myofibroblastic differen-
tiation of corneal stromal cells, repressed cell proliferation and migration of corneal fibroblasts, and the induction of cellular senescence of corneal myofibroblasts as recently published by Zhou et al. (Zhou et al. 2008). As the authors conclude, HDAC inhibition may therefore be a possible mechanism in the prevention of corneal scar formation. On the other hand, this study also underlines the wide-range and poorly understood influence of HDAC inhibition on normal cells.

Drug-induced toxicity in primary hepatocytes

A number of studies have been carried out to investigate possible effects of HDAC inhibitors on hepatocytes and hepatoma cells, because of the liver's central function in drug metabolism. Knowledge about drug-induced toxicity in primary hepatocytes by HDAC inhibitors is crucial in view of their clinical use and the development of new HDAC inhibitors. Available studies have been comprehensively reviewed by Papeleu et al., concluding disturbed tissue homeostasis by anti-apoptotic effects in primary hepatocytes due to treatment with TSA and underlining the need for future investigation of HDAC inhibitors toxicological profile in a large number of different cell types (Papeleu et al. 2005).

Reactive oxygen species

Reactive oxygen species (ROS) are an important source of endogenous DNA damage. When naturally occurring protection mechanisms, such as enzymatic and non-enzymatic ROS scavengers are compromised, damage accumulates, which then leads to apoptosis. While this is quite a desirable effect in the fight against cancer, damage caused by ROS in healthy human cells is generally feared due to its carcinogenic potential. Studies reporting an accumulation of ROS because of treatment with HDAC inhibitors have been performed almost exclusively on transformed cells (reviewed in (Eot-Houllier et al. 2009)). An international research group investigated HDAC inhibitor mediated effects in both transformed as well as normal lung fibroblasts and identified an accumulation of ROS in transformed cells treated with SAHA, but not in normal fibroblasts (WI38). Consistent with these results, apoptosis was only induced in malignant, but not in normal cells, while growth arrest occurred in both normal and transformed cells (Ungerstedt et al. 2005).

The special case of valproic acid

The capacity of valproic acid (VA) to inhibit transamination of the neurotransmitter GABA and to block voltage-gated sodium-channels and T-type calcium channels make it one of the most common drugs in the treatment of epilepsy and psychiatric disorders. Despite VA's more than 30-year-long history of clinical use, a number of effects is still cryptic and poorly investigated.

Anticonvulsant and antimigraineous effects persist for at least several days after discontinuation of VA, raising the question of possible changes at the genomic level (Lockard and Levy 1976; Rho and Sankar 1999; Rosenberg 2007; Rothrock and Mendizabal 2000). The direct comparison of gene expression levels in blood from VA vs. carbamazepine or drug-free patients revealed no less than a total of 461 genes that were either upregulated or downregulated in response to VA treatment (Tang et al. 2004).

Currently, two major principles are discussed in this context: (1) enhancement of the transcription factor AP-1 (activator protein 1) by VA and subsequent modification of gene expression (reviewed in (Rosenberg 2007)), and (2) VA mediated epigenetic modification: In 2001, the independent discovery of the HDAC-inhibiting potential of VA by Göttlicher et al. (Göttlicher et al. 2001) and Phiel et al. (Phiel et al. 2001) was a milestone in the attempt to find new explanations for the behaviour of VA.

In addition, a weaker HDAC-inhibiting effect was described for constitutional isomers and metabolites of VA (Eyal et al. 2005). Also, it was demonstrated in vitro that carbamazepine and its metabolite carbamazepine-10,11-epoxide as well as the common antiepileptic agents topiramate and the main metabolite of levetiracetam exert HDAC-inhibiting activities (Beutler et al. 2005; Eyal et al. 2004). While carbamazepine was initially reported to lack an HDAC inhibitory activity (Eyal et al. 2004), contradicting results were soon published after this report, demonstrating the hyperacetylation of histone proteins in response to exposure to carbamazepine despite its poor solubility (Beutler et al. 2005).

Histone H3 and to a lesser extent histone H4 hyperacetylation was shown in vivo in isolated PBMCs from patients with bipolar disorders and/or schizophrenia who had received 4 weeks of treatment with VA (Sharma et al. 2006). Persisting effects after discontinuation of the drug (Lockard and Levy 1976; Rho and Sankar 1999; Rothrock and Mendizabal 2000) may indeed be ascribed to persistent hyperacetylation, but since affected cells carry the potential to restore ‘original’ acetylation pattern rapidly after drug removal, effects attributed to HDAC inhibition are in fact expected to disappear after a maximum of a few days.

In view of the relatively long clinical use of VA when compared with other HDAC inhibitors—its toxicological profile is of special interest (Bokelmann and Mahlknecht 2008). The most common side-effects comprise weight gain, dyspepsia and hepatic disturbances. Nausea, vomiting, indigestion are also frequently observed as well as sedation,
fatigue and blood dyscrasia, which may occur due to VAs influence on HDAC activity, gene expression and genomic stability of rapidly proliferating cells (Qiao et al. 2006; Rosenberg 2007). The teratogenic side-effects of VA, which are known since long, have also been attributed to the inhibition of HDACs (Göttlicher et al. 2001; Phiel et al. 2001; Rosenberg 2007), the exact underlying epigenetic mechanism remains however to be further elucidated (Ornoy 2009).

Long-term safety of VA

The controversial data on genomic instability due to treatment with HDAC inhibitors raise the question of long-term safety of VA. General genomic instability anticipates an increase in the rate of malignant transformations, but fortunately, until today no increase in the frequency of solid or haematological neoplasias has been reported in association with the administration of VA.

To date, the anti-carcinogenic or chemopreventive effects that have been ascribed to the anti-proliferative and differentiating effects of HDAC inhibition have not been demonstrated in humans in vivo (Hallas et al. 2009; Singh et al. 2005). If on the other hand VA is able to inhibit DSB repair to a certain degree not only in tumour cells, as demonstrated (Camphausen et al. 2005; Chinnaiyan et al. 2008; Harikrishnan et al. 2008), but in normal cells as well (Purrucker et al. 2010), one should expect noticeable side-effects in patients who are being exposed to therapeutic irradiation. We recently did not find a significant decrease of DSB repair subsequent to exposure of VA in cultured human fibroblasts. Nevertheless, our observation of an increase in radiosensitivity resulting in decreased clonogenic survival underlines the biological importance of a possible slight decrease in DSB repair in human normal cells (Purrucker et al. 2010).

At present, retrospective data are only available from an abstract published at the 50th ASTRO annual meeting in 2008 indicating a favourable outcome in patients with high-grade glioma who underwent VA therapy during radiotherapy, without suffering additional severe side-effects (Barker et al. 2008). Also an active clinical trial is currently being conducted (NCI-06-C-0112), which combines radiotherapy with HDAC inhibition. Patients with glioblastoma multiforme are treated with VA and/or the classic cytotoxic agent temozolomide in addition to a fractionated radiotherapy of up to 60 Gy (Camphausen and Tofilon 2007). Since this is the first prospective study which combines radiotherapy with HDAC inhibition, results are expected with special interest.

Despite mentioned risks in long-term-therapy concepts, VA could assist to improve outcome in less-toxic therapy regimes, especially in elderly patients. In patients with MDS or de novo/secondary acute myeloid leukaemia not eligible for intensive chemotherapy, VA in addition to all-trans-retinoic acid was well tolerated and resulted in an overall response rate of 27% (Bellos and Mahlknecht 2008).
Clinical relevance
We are witnessing steadily growing fields of studies demonstrating anticancer effects of epigenetic modifying drugs while at the same time reports on effects in non-deranged normal cells are single blades of grass. This might be simply caused by the fact that experiments with slow proliferating primary human cells generally seem to be by far more time-consuming and complicated. In addition, a strong publication bias, excluding studies with ‘negative’ (or less significant) results may also contribute to this. The effects of drug-induced epigenetic modifications furthermore seem to be far more heterogeneous and complex than it was initially expected (Fig. 1).
Global hypomethylation which leads to undercondensation of chromosomes is shown to be responsible for chromosomal breakage and could lead to transformation of normal cells (Cimini et al. 1996; Guttenbach and Schmid 1994; Satoh et al. 2004; Schmid et al. 1984; Viegas-Péquignot and Dutrillaux 1976). HDAC inhibitors may not only introduce reversible or irreversible growth arrest in healthy human cells (Qiu et al. 2000; Saunders et al. 1999), but may also be responsible for apoptosis in certain constellations (Qiu et al. 1999; Zhou et al. 2008).

Radiosensitization is not limited to diseased cells, but also occur in healthy tissue cells (Purrucker et al. 2010; Stoilov et al. 2000). In the case of VA, new (prospective) studies have to clarify uncertainties of its long-term safety and provide a full toxicity profile, contributing to a more exact epigenetic image of VAs behaviour especially in the central nervous system.

Future generations of HDAC inhibitors will have to be HDAC specific, allowing a more targeted influence on cell functions. Until then, in the absence of comprehensive toxicological long-term data, physicians should be aware of possible side-effects of epigenetic modifications. Patients will have to be informed and educated explicitly on potential carcinogenic side effects, as it is already common procedure in the administration of classic cytotoxic chemotherapy. Given the enormous potential of epigenetics, scientists and clinicians should work as close together as possible to provide the best outcome for patients—in view of effective therapies as well as best possible risk reduction.

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We apologize to all authors whose work was not discussed or cited in this manuscript. Since this review summarizes results based on experiments with healthy human cells, studies either using only malignant or diseased cells or cell lines whose origin was not human have not always been considered.

Conflict of interest 
The authors declare that they have no conflicts of interest.

References
Allis CD, Berger SL, Cote J, Dent S, Jenuwien T, Kouzarides T, Pillus L, Reinberg D, Shi Y, Shiekhattar R, Shilatifard A, Workman J, Zhang Y (2007) New nomenclature for chromatin-modifying enzymes. Cell 131:633–636
Banuelos CA, Banath JP, MacPhail SH, Zhao J, Reitsema T, Olive PL (2007) Radiosensitization by the histone deacetylase inhibitor PCI-24781. Clin Cancer Res 13:6816–6826
Barker C, Camphausen K, Tofilon P, Chan T (2008) Outcome of patients undergoing radiotherapy for high grade glioma while taking the histone deacetylase inhibitor, valproic acid. Int J Radiat Oncol Biol Phys 72:S214–S214
Bellos F, Mahlknecht U (2008) Valproic acid and all-trans retinoic acid: meta-analysis of a palliative treatment regimen in AML and MDS patients. Onkologie 31:629–633
Beutler AS, Li S, Nicol R, Walsh MJ (2005) Carbamazepine is an inhibitor of histone deacetylases. Life Sci 76:3107–3115
Bokelmann I, Mahlknecht U (2008) Valproic acid sensitizes chronic lymphocytic leukemia cells to apoptosis and restores the balance between pro- and antiapoptotic proteins. Mol Med 14:20–27
Breuninger DJ, Hall EJ (2007) Computed tomography—an increasing source of radiation exposure. N Engl J Med 357:2277–2284
Brinkmann H, Dahler AL, Popa C, Serewko MM, Parsons PG, Gabrielli BG, Burgess AJ, Saunders NA (2001) Histone hyperacetylation induced by histone deacetylase inhibitors is not sufficient to cause growth inhibition in human dermal fibroblasts. J Biol Chem 276:22491–22499
Camphausen K, Tofilon P (2007) Inhibition of histone deacetylation: a strategy for tumor radiosensitization. J Clin Oncol 25:4051–4056
Camphausen K, Cerna D, Scott T, Sproull M, Burgan W, Cerra M, Fine H, Tofilon P (2005) Enhancement of in vitro and in vivo tumor cell radiosensitivity by valproic acid. Int J Cancer 114:380–386
Chinnaiyan P, Cerna D, Burgan W, Beam K, Williams ES, Camphausen K, Tofilon P (2008) Postradiation sensitization of the histone deacetylase inhibitor valproic acid. Clin Cancer Res 14:5410–5415
Cimini D, Tanzarella C, Degrassi F (1996) Effects of 5-azacytidine on the centromeric region of human fibroblasts studied by CREST staining and in situ hybridization on cytokinesis-blocked cells. Cytogeten Cell Genet 72:219–224
Dah-Yousef N, Lagouge M, Froelich S, Koehl C, Schoonjans K, Auwerx J (2007) Sirtuins: the ‘magnificent seven’, function, metabolism and longevity. Ann Med 39:335–345
Entin-Meer M, Rephaeli A, Yang X, Nudelman A, VandenBerg SR, Gabrielli BG, Burgess AJ, Saunders NA (1999) Histone hyperacetylation induced by histone deacetylase inhibitors is not sufficient to cause growth inhibition in human dermal fibroblasts. J Biol Chem 276:22491–22499
Fauth E, Scherthan H, Zankl H (1998) Frequencies of occurrence of all human chromosomes in micronuclei from normal and 5-azacytidine-treated lymphocytes as revealed by chromosome painting. Mutagenesis 13:235–241
Geigl JB, Obenauf AC, Schwarzbauer T, Speicher MR (2008) Defining ‘chromosomal instability’. Trends Genet 24:64–69
Goll MG, Kirpekar F, Maggert KA, Yoder JA, Hsieh C-L, Zhang X, Golic KG, Jacobsen SE, Bestor TH (2006) Methylation of rRNA Asp by the DNA methyltransferase homolog Dnmt2. Science 311:395–398

Göttlicher M, Minucci S, Zhu P, Krämer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pellicci PG, Heinzel T (2001) Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 20:6969–6978

Guttenbach M, Schmid M (1994) Exclusion of specific human chromosomes into micronuclei by 5-azacytidine treatment of lymphocyte cultures. Exp Cell Res 211:127–132

Haaf T (1995) The effects of 5-azacytidine and 5-aza-deoxycytidine on chromosome structure and function: implications for methylation-associated cellular processes. Pharmacol Ther 65:19–46

Hallas J, Friesi S, Bjerrum L, Stavring H, Nørnerud SF, Heyerdahl T, Grønbaek K, Andersen M (2009) Cancer risk in long-term users of valproate: a population-based case-control study. Cancer Epidemiol Biomark Prev 18:1714–1719

Harikrishnan KN, Baragianis TC, Chow MZ, El-Osta A (2008) Effect of valproic acid on radiation-induced DNA damage in euchromatic and heterochromatic compartments. Cell Cycle 7:468–476

Heilig CE, Loffler H, Mahlknecht U, Janssen JW, Ho AD, Jauch M, Marks PA (1998) A class of hybrid polar inducers of transformed cells and reduces DNA double-strand break repair capacity. Mol Cell Biol 14:895–902

Issa JP (2004) CpG island methylator phenotype in cancer. Nat Rev Cancer 4:988–993

Issa JP, Kantarjian HM (2009) Targeting DNA methylation. Clin Cancer Res 15:3938–3946

Jackson-Grusby L, Laird PW, Magge SN, Moeller BJ, Jaenisch R (1997) Heterochromatin compartments. Cell Cycle 7:468–476

Kiziltepe T, Hideshima T, Catley L, Raje N, Yasui H, Shiraishi N, Harikrishnan KN, Karagiannis TC, Chow MZ, El-Osta A (2008) Effect of valproic acid on radiation-induced DNA damage in euchromatic and heterochromatic compartments. Cell Cycle 7:468–476

Kim MS, Blake M, Baek JH, Kohlhagen G, Pommier Y, Carrier F (2003) Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res 63:7291–7300

Kiziltepe T, Hideshima T, Catley L, Raje N, Yasui H, Shiraishi N, Okawa Y, Ikeda H, Vallet S, Pozzi S, Ishitsuka K, Ocio EM, Chauhan D, Anderson KC (2007) 5-Azacytidine, a DNA methyltransferase inhibitor, induces ATR-mediated DNA double-strand break responses, apoptosis, and synergistic cytotoxicity with doxorubicin and bortezomib against multiple myeloma cells. Mol Cancer Ther 6:1718–1727

Lieber MR (2008) The mechanism of human nonhomologous DNA end joining. J Biol Chem 283:1–5

Lockard JS, Levy RH (1976) Valproic acid: reversibly acting drug? Epilepsia 17:477–479

MacPhail SH, Banath JP, Yu TY, Chu EH, Lambur H, Olive PL (2003) Expression of phosphorylated histone H2A.X in cultured cell lines following exposure to X-rays. Int J Radiat Biol 79:351–358

Mahlknecht U, Hoelzer D (2000) Histone acetylation modifiers in the pathogenesis of malignant disease. Mol Med 6:623–644

Mai S, Ho AD, Mahlknecht U (2004) Role of histone deacetylase inhibitors in the treatment of cancer (Review). Int J Oncol 25:1509–1519

Morales-Ramirez P, Villarrino-Kelly T, Cruz-Vallejo VL (2008) Mechanisms of DNA breaks induction in vivo by 5-azacytidine: paths of micronucleus induction by azac. J applied toxicology JAT 28:254–259

Munshi A, Kurland JF, Nishikawa T, Tanaka T, Hobbs ML, Tucker SL, Ismail S, Stevens C, Meyn RE (2005) Histone deacetylase inhibitors radiosensitize human melanoma cells by suppressing DNA repair activity. Clin Cancer Res 11:4912–4922

Ornay A (2009) Valproic acid in pregnancy: how much are we endangering the embryo and fetus? Reprod Toxicol 28:1–10

Pandita TK, Richardson C (2009) Chromatin remodeling finds its place in the DNA double-strand break response. Nucleic Acids Res 37:1363–1377

Papeleu P, Vanhaecke T, Elaut G, Vinken M, Henkens T, Snyskers S, Rogiers V (2005) Differential effects of histone deacetylase inhibitors in tumor and normal cells-what is the toxicological relevance? Crit Rev Toxicol 35:363–378

Parsons PG, Hansen C, Fairlie DP, West ML, Danoy PA, Stumm RA, Dunn IS, Pedley J, Abllet EM (1997) Tumor selectivity and transcriptional activation by azelaic bis hydroxyacid in human melanocytic cells. Biochem Pharmacol 53:1719–1724

Priel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J Biol Chem 276:36734–36741

Plummer R, Vidal L, Griffin M, Lesley M, de Bono J, Coulthard S, Sludden J, Siu LL, Chen EX, Oza AM, Reid GK, McLeod AR, Besterman JM, Lee C, Judson I, Calvert H, Boddy AV (2009) Phase I study of MG98, an oligonucleotide antisense inhibitor of human DNA methyltransferase 1, given as a 7-day infusion in patients with advanced solid tumors. Clin Cancer Res 15:3177–3183

Purrrucker JC, Fricke A, Ong MF, Rübe C, Rübe CE, Mahlknecht U (2010) HDAC inhibition radiosensitizes human normal tissue cells and reduces DNA double-strand break repair capacity. Oncol Rep 23:263–269

Qiao L, Schack J, Shao J (2006) Suppression of adiponectin gene expression by histone deacetylase inhibitor valproic acid. Endocrinology 147:865–874

Qiu L, Kelso MJ, Hansen C, West ML, Fairlie DP, Parsons PG (1999) Anti-tumour activity in vitro and in vivo of selective differentiating agents containing hydroxamate. Br J Cancer 80:1252–1258

Qu L, Burgess A, Fairlie DP, Leonard H, Parsons PG, Gabrielli BG (2000) Histone deacetylase inhibitors trigger a G2 checkpoint in normal cells that is defective in tumor cells. Mol Biol Cell 11:2069–2083

Rasheed WK, Johnstone RW, Prince HM (2007) Histone deacetylase inhibitors in cancer therapy, Expert Opin Investig Drugs 16:659–678

Rho JM, Sankar R (1999) The pharmacologic basis of antiepileptic drug action. Epilepsia 40:1471–1483

Richon VM, Emiliani S, Verdin E, Webb Y, Breslow R, Riikkind RA, Marks PA (1998) A class of hybrid polar inducers of transformed cell differentiation inhibitors histone deacetylases. Proc Natl Acad Sci USA 95:3003–3007

Rosenberg G (2007) The mechanisms of action of valproate in neuropsychiatric disorders: can we see the forest for the trees? Cell Mol Life Sci 64:2090–2103

Rothrock JF, Mendizabal JE (2000) An analysis of the “carry-over effect” following successful short-term treatment of transformed migraine with divalproex sodium. Headache 40:17–19

Sato T, Yamamoto K, Miura KF, Sofuni T (2004) Region-specific chromatin decondensation and micronucleus formation induced by 5-azacytidine in human TIG-7 cells. Cytogenet Genome Res 104:289–294

Saunders N, Dicker A, Popa C, Jones S, Dahler A (1999) Histone deacetylase inhibitors as potential anti-skin cancer agents. Cancer Res 59:399–404

Schaefer M, Hagemann S, Hanna K, Lyko F (2009) Azacytidine inhibits RNA methylation at DNMT2 target sites in human cancer cell lines. Cancer Res 69:8127–8132

Schmid M, Haaf T, Grunert D (1984) 5-Azacytidine-induced undercondensations in human chromosomes. Hum Genet 67:257–263

Sedelnikova OA, Rogakou EP, Panyutin IG, Bonner WM (2002) Quantitative detection of (125)Iu-induced DNA double-strand breaks with gamma-H2AX antibody. Radiat Res 158:486–492

Sharma RP, Rosen C, Kartan S, Guidotti A, Costa E, Grayson DR, Chase K (2006) Valproic acid and chromatin remodeling in schizophrenia and...
bipolar disorder: preliminary results from a clinical population. Schizophr Res 88:227–231
Singh G, Driever PH, Sander JW (2005) Cancer risk in people with epilepsy: the role of antiepileptic drugs. Brain 128:7–17
Sorm F, Piskala A, Čihák A, Veselý J (1964) 5-Azacytidine, a new, highly effective cancerostatic. Experientia 20:202–203
Stoilov L, Darroudi F, Meschini R, van der Schans G, Mullenders LH, Natarajan AT (2000) Inhibition of repair of X-ray-induced DNA double-strand breaks in human lymphocytes exposed to sodium butyrate. Int J Radiat Biol 76:1485–1491
Stresemann C, Brueckner B, Musch T, Stopper H, Lyko F (2006) Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. Cancer Res 66:2794–2800
Tang Y, Glauser TA, Gilbert DL, Hershey AD, Privitera MD, Ficker DM, Szaflarski JP, Sharp FR (2004) Valproic acid blood genomic expression patterns in children with epilepsy—a pilot study. Acta Neurol Scand 109:159–168
Ungerstedt JS, Sowa Y, Xu WS, Shao Y, Dokmanovic M, Perez G, Ngo L, Holmgren A, Jiang X, Marks PA (2005) Role of thioredoxin in the response of normal and transformed cells to histone deacetylase inhibitors. Proc Natl Acad Sci USA 102:673–678
Viegas-Péquignot E, Dutrillaux B (1976) Segmentation of human chromosomes induced by 5-ACR (5-azacytidine). Hum Genet 34:247–254
Villar-Garea A, Esteller M (2004) Histone deacetylase inhibitors: understanding a new wave of anticancer agents. Int J Cancer 112:171–178
Winquist E, Knox J, Ayoub J-P, Wood L, Wainman N, Reid GK, Pearce L, Shah A, Eisenhauer E (2006) Phase II trial of DNA methyltransferase 1 inhibition with the antisense oligonucleotide MG98 in patients with metastatic renal carcinoma: a National Cancer Institute of Canada Clinical Trials Group investigational new drug study. Invest New Drugs 24:159–167
Witt O, Deubzer HE, Milde T, Oehme I (2009) HDAC family: what are the cancer relevant targets? Cancer Lett 277:8–21
Xu Y, Voelter-Mahlknecht S, Mahlknecht U (2005) The histone deacetylase inhibitor suberoylanilide hydroxamic acid down-regulates expression levels of Bcr-abl, c-Myc and HDAC3 in chronic myeloid leukemia cell lines. Int J Mol Med 15:169–172
Zhou Q, Wang Y, Yang L, Wang Y, Chen P, Wang Y, Dong X, Xie L (2008) Histone deacetylase inhibitors blocked activation and caused senescence of corneal stromal cells. Mol Vis 14:2556–2565