Review

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Epigenetic agents in combined anticancer therapy

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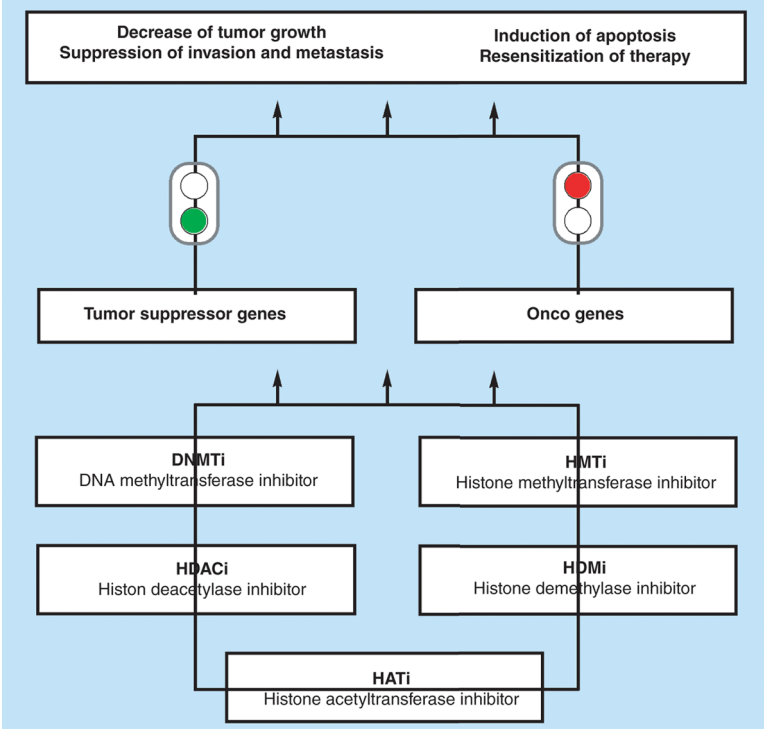
In the last decade, epigenetic drugs (such as inhibitors of DNA methyltransferases and histone deacetylases) have been intensively used for cancer treatment. Their applications have shown high anticancer effectivity and tolerable side effects. However, they are unfortunately not effective in the treatment of some types and phenotypes of cancers. Nevertheless, several studies have demonstrated that problems of drug efficacy can be overcome through the combined application of therapeutic modulates. Therefore, combined applications of epigenetic agents with chemotherapy, radiation therapy, immunotherapy, oncolytic virotherapy and hyperthermia have been presented. This review summarizes and discusses the general principles of this approach, as introduced and supported by numerous examples. In addition, predictions of the future potential applications of this methodology are included.

Graphical Abstract:



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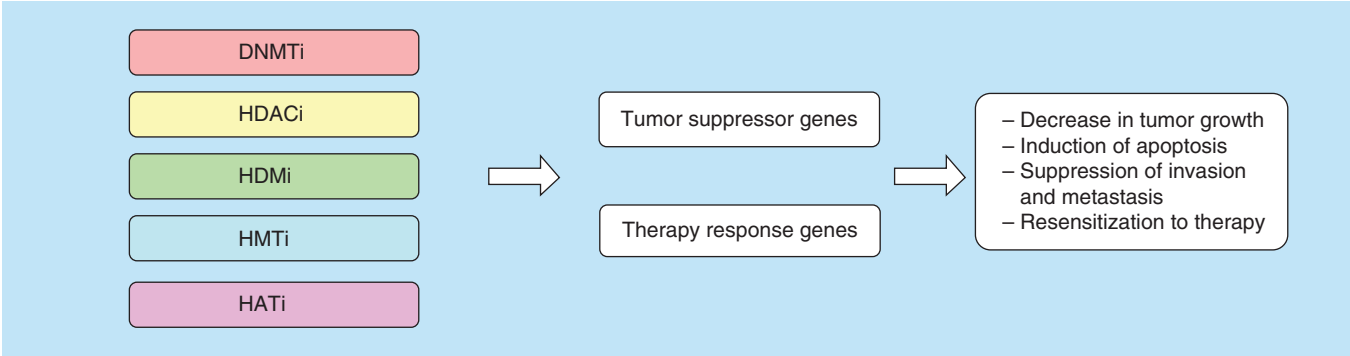

Figure 1. Principle application of epigenetic agents in combined anticancer therapy.
DNMTi: DNA methyltransferase inhibitor; HATi: Histone acetyltransferase inhibitor; HDACi: Histone deacetylase inhibitor; HDMi: Histone demethylase inhibitor.

Epigenetics is a hot topic in the biological sciences with high medicinal impact. The epigenetic control of the chromatin structure is an important mechanism for the adaptation of gene expression depending on external conditions. It is not surprising that epigenetic mechanisms, such as DNA methylation and histone post-translational modifications, are one of the most important regulation strategies in living systems. These mechanisms significantly participate in the regulation of gene expression by modifying the chromatin structure (e.g., genomic imprinting, X-chromosome inactivation, heterochromatin formation, transcriptional regulation and DNA damage repair). Changes in these mechanisms can strongly influence almost all physiological processes and have been intensely studied in the pathology of serious diseases.

Many high-impact studies have clearly demonstrated that the misregulated expression of either individual genes or complex genetic programs underlies numerous human pathologies, particularly oncologic diseases. These genes strongly influence cancer initiation and progression, as exemplified by frequent mutations in genes encoding proteins that control the epigenome, and are a necessary part of tumorigenesis. This phenomenon is typically observed for many clinically important cancer types, such as breast, colorectal, lung, ovarian, prostate, kidney, gastric, liver, brain, pancreatic and hematologic cancers [1].

This fact has led to the use and study of epigenetic therapies to transform pathological cells toward a normal state [2]. Thus, sales of epigenetic drugs have shown an increasing tendency. For example, in 2016, the global epigenetic drugs and diagnostic technologies market was valued at US$3000 million, and the expected value for 2018 is US$4000 million. However, despite promising results for their therapeutic applications, some high-impact studies have indicated that the potential of epigenetic drugs may be limited and that improving their efficiency is highly needed. One of the potential methods is the combination of these agents with other therapeutic modalities.

Therefore, combined therapies [3–11] have been intensely studied. Currently, epigenetic therapy in combination with chemotherapy, radiotherapy, immunotherapy, oncolytic virotherapy and hyperthermia has become an integral part of various cancer treatment protocols. Suitable therapeutic mixtures should suppress the disadvantages of using partial therapies, showing enhanced therapeutic benefits, lower toxicity and fewer side effects compared with monotherapies. Several studies have shown that the application of epigenetic agents can strongly positively influence the effectiveness other anticancer therapies (Figure 1 displays a high density of CpG).

Additionally, the results of clinical studies have shown that the incorporation of epigenetic agents into used therapies is patient-friendly with mild side effects. Thus, the application of epigenetic drugs is highly desirable in oncology. The individual effects of epigenetic therapy on other therapies, including the advantages of combined applications, are discussed in next chapters. The first, second and third chapters discussed a possibility and potential of DMTis, HDACis and HATis in the anticancer combination therapy, respectively. The fourth chapter is focused on the combined application of more different epigenetic agents.

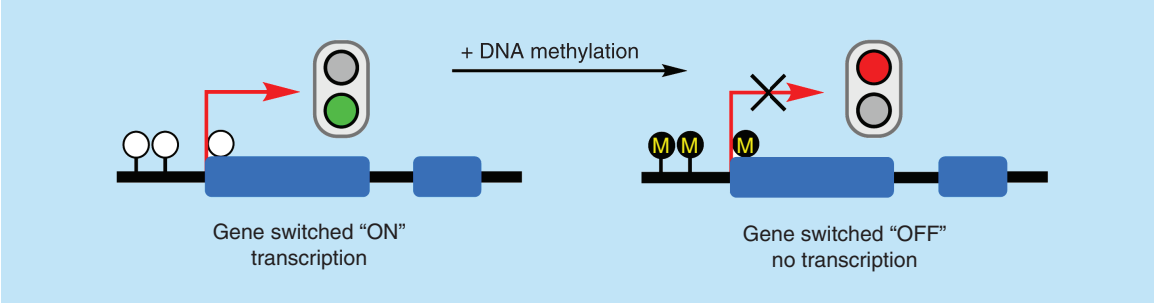

Figure 2. Regulation of gene expression through DNA methylation.

Application of demethylation agents in combined anticancer therapy

The physiological function of DNA methylation is the silencing of imprinted gene alleles and transcription from repetitive elements (e.g., retroviral genes) [12]. The most common site for the methylation of mammalian DNA is at position 5 of cytosine in CpG islands of gene promoters. This regulation site typically displays a high density of CpG dinucleotides (60–70%), ranging in size from 200 to 5000 base pairs [13]. Cancer methylation patterns are severely altered; there is both widespread genomic hypomethylation and focal gains in many normally unmethylated promoter CpG islands, and the latter are usually associated with abnormal gene silencing (CpG-island hypermethylation) (Figure 2). Nevertheless, several authors surprisingly observed in some cancer lines that the hypermethylation of the gene promoter can correlate with higher gene expression [14].

Clearly, the restoration of normal DNA methylation patterns is a well-studied method for the suppression of cancer development and the selective targeting of cancer cells. In addition, there is a correlation between the hypermethylation of cancer DNA and the difficulty in its treatment. For example, a number of high-impact studies have demonstrated that CpG methylation induces drug resistance and that this effect can be reversed by the treatment of cells or tumors with demethylating agents [15–18] such as 5-aza-2′-deoxycytidine [18,19] and zebularine [18]. However, this drug displayed slow cytostatic effects; thus, for effective cancer treatment, other therapeutic methods are needed [20].

The methylation of CpG islands is catalyzed through enzymes belonging to the DNA methyltransferase family [21,22]. Currently, there are three mammal DNA methyltransferases (DNMT1, DNMT3A and DNMT3B). DNMT1 maintains the pre-existing methylation pattern, while DNMT3a and DNMT3b process *de novo* DNA methylation.

The overexpression of DNA methyltransferases (DNMTs) was observed in several cancers with high levels of expression, often predictive of a poor prognosis [1]. These facts have naturally led to the DNA methyltransferase inhibitors (DNMTis) incorporation to the anticancer therapeutic regiments. The currently used and tested DNMTis are shown in Supplementary Table 1.

Their high potential in combination with commonly used cytostatic agents (5-flurouracil, oxaliplatin and others; Supplementary Figure 1) was demonstrated in several recent studies. For example, Flis *et al.* [23] evaluated the effectiveness of zebularine together with the application of oxaliplatin and 5-fluorouracil. The results showed high synergistic effects on the tested colorectal carcinoma cell lines. In addition, zebularine displayed minimal toxicity and side effects.

Similarly, decitabine can be used to reduce tumor immunoresistance. For example, Hu *et al.* used a combination of decitabine with the moxetumomab pasudotox immunotoxin (fused toxin with a variable fragment of the CD-22 antibody) for the treatment of immunotoxin resistance in KOPN-8 (acute lymphoblastic leukemia cell line) [24]. The application of decitabine results in the demethylation of the DPH1 promoter and a lack of immunoresistance.

Another cytostatic agent suitable for combination with decitabine is cytarabine. For example, Leonard *et al.* observed that the combination had a significant synergistic effect on an acute myeloid leukemia cell line [25]. Their co-application resulted in substantially fewer hypomethylation and hypermethylation changes than observed using these drugs alone, whereas sequential treatment (cytarabine followed by decitabine) resulted in substantially more hypomethylation and hypermethylation changes.

Table 1. The tested combination of DNA methyltransferase inhibitors with anticancer agents.

Anticancer agents	DNMTi	Model	Effect	Ref.
5-Fluorouracil	Decitabine	CC (SW480)	Strong synergistic effect	[18]
Cisplatinum	Azacytidine	OC (A2780, MCP1 and MCP3)	↑hMLH1 expression, ↓drug resistance	[19]
Moxetumomab pasudotox immunotoxin	Decitabine	ALL (KOPN-8)	↑Sensitivity (by three orders)	[24]
Cytarabine	Decitabine	Primary and primary pediatric ALL	Changes in DNA methylation ↓co-application, ↑sequential	[25]
Rapamycin	Azacytidine	CRC (HCT116), mice with HCT116	↓mTOR phosphorylation, ↓tumor volume	[27]
Quercetin and curcumin	–	PC (PC3 and LNCaP)	↑Androgen receptor expression	[31]
Oxaliplatin and 5-fluorouracil	Zebularine	CC (Colo-205)	↓Drug resistance	[32]
Oxaliplatin and 5-fluorouracil	Decitabine	CRC (SW48 and HT-29)	Synergistic effect	[33]

ALL: Acute lymphoblastic leukemia cell line; CRC: Colorectal cancer cell line; DNMTi: DNA methyltransferase inhibitor; OC: Ovarian cancer cell line; PC: Prostate cancer cell line.

This promising anticancer therapy could also be based on the combination of demethylation agents with inhibitors of the mTOR signaling pathway. These inhibitors have been studied primarily for the treatment of various cancer types, such as colorectal cancer [26]. Their application can significantly suppress cancer invasiveness and tumor growth. Nevertheless, the therapeutic effect of these agents alone is not sufficient, and therefore, these molecules are studied in combination with other anticancer agents, such as DNMTis [27–29]. For example, Zhang *et al.* successfully studied the combination of azacitidine with rapamycin (mTOR inhibitor) for the treatment of colorectal cancer [27]. Authors observed that the tested strategy displayed strong antitumor activity and a synergistic effect, particularly in the reduction of the tumor volume of colorectal carcinoma. After combined application, a five-times-smaller tumor volume was observed compared with single therapeutic applications.

On the other hand, some anticancer agents, such as methylated DNA-binding protein 2 antisense nucleotide, could be used to improve DNMTi's therapeutic effectivity. This protein regulator collaborates with DNMTs in gene silencing and is necessary for the expression of some oncogenes (*uPA*, *FABP7*). Cheishvili *et al.* studied the combination of decitabine with antisense oligonucleotides using a mouse model with breast cancer [30]. These authors observed that antisense oligonucleotides had a highly positive effect on DNMTi application, such as oncogene repression. In addition, antisense nucleotides also suppressed some side effects from decitabine application (e.g., induced invasiveness).

An easily applicable method of DNMT inhibition could be the combined application of bioactive food components. The potential of this method for the treatment of prostate cancer was demonstrated by Sharma *et al.* [31]. One method of treatment is androgen deprivation therapy with anti-androgens. Nevertheless, an important problem due to its wide application is resistant cell lines with epigenetic repression of the targeted androgen receptor. However, these authors showed that the combined application of phytochemicals (e.g., quercetin and curcumin) strongly suppressed DNMT activity and restored the expression of androgen receptors.

The above facts strongly suggest that the incorporation of DNMTi can significantly enhance existing therapies, as shown in Table 1.

Inhibition of histone deacetylases in combined anticancer therapy

Histone covalent post-translational modifications (acetylation, methylation, phosphorylation, ubiquitylation, etc.) are the key mechanisms for the control of chromatin structure. One of the most studied and pharmaceutically utilized mechanisms is the deacetylation of histone lysine groups [34]. In general, histone deacetylation leads to gene repression (Figure 3).

This process is catalyzed through the specific enzyme family of HDACs. Mammals express 18 types of HDACs, which are divided into four groups according to homology and cellular localization. Class I (HDACs 1, 2 and 3), Class IIA (HDACs 4, 5, 7 and 9), Class IIB (HDACs 6 and 10) and Class IV (HDAC11) HDACs are zinc dependent. Class III HDACs, including sirtuins 1–7, are not zinc dependent. HDACs not only specialize in histone deacetylation but also control the acetylation levels of other important proteins (e.g., β-tubulin [35] or DMMT1 [36]). It has been indicated that therapeutic control of their activity by histone deacetylase inhibitors (HDACis) can be a prospective therapeutic method.

Most used and studied HDACis (e.g., vorinostat, belinostat, panobinostat and others) were designed as targets of zinc HDACs and therefore displayed a nonsignificant effect on sirtuins. Their structure is shown in Supplementary

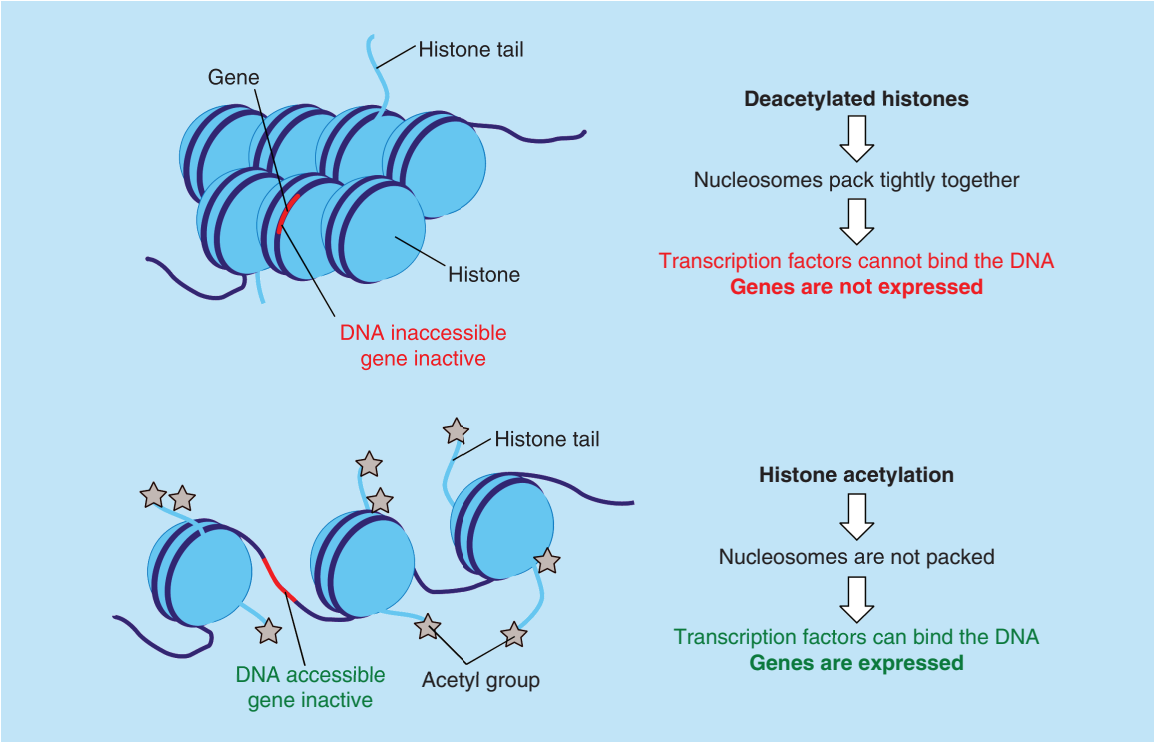

Figure 3. Regulation of gene expression by histone acetylation.

Table 2. They are clinically well-tolerated, potent, antiproliferative agents with relatively reduced effects on normal tissues [37]. However, their therapeutic potential may be properly evaluated only in combination with classical therapies used and studied for cancer treatment (chemotherapy, radiotherapy, immunotherapy, virotherapy and hyperthermia).

Application of HDACi in combination with cytostatic agents

HDACi application with commonly used cytostatics (paclitaxel doxorubicin and others, [Supplementary Figure 2]) can significantly improve therapeutic effectivity. The positive influence of HDACi on cytostatic effects likely reflects a combination of several mechanisms, such as the augmentation of the apoptotic signal and factors (e.g., caspase activation) [38–40], reduced expression of tumorigenic and proangiogenic factors (e.g., VEGF and HIF-1α) [41,42] and the repression of DNA repair systems [40].

An important benefit of HDACi application is the reduction of drug resistance, particularly for DNA-targeting drugs [43–49]. The removal of acetyl groups from lysine residues in the N-terminal tails of histones causes chromatin condensation and transcriptional repression. Therefore, the application of HDACs leads to a more open chromatin structure, thereby increasing the accessibility of DNA-damaging chemotherapeutic agents. Consistent with this effect on the DNA accesibility, one of the most well-studied HDACi combinations is with cisplatin drugs [38,42,50–56]. A number of recent works clearly demonstrate great potential of this approach in cancer treatment, particularly in the case of tumor chemoresistance [42,47,48,51,54,55].

For example, the influence of vorinostat application on etoposide/cisplatin chemotherapy using a mouse model with non-small-cell lung carcinoma was studied by Pan *et al.* [57]. As expected, triple combination engendered a significant reduction in cell viability and high apoptotic cell death. In addition, vorinostat combined with cisplatin enhanced cell growth inhibition, induced apoptosis, and promoted cell cycle arrest, and vice versa; the application of the above cytostatics had a positive influence on vorinostat's effect, and the acetylation levels of histone H3 were higher in combination treatments than in vorinostat treatment alone [57]. This study also showed that application of etoposide enables a significant reduction of the cisplatin dosage. Furthermore, an *in vivo* study of the combination of vorinostat and cisplatin (one-fifth the volume of a nontreated tumor) showed significant inhibition of tumor growth in xenograft nude mice with the combined treatment compared with treatment with cisplatin (one-half

tumor volume) or vorinostat (without change) alone. However, the positive influence of HDACis on their toxicity (e.g., etoposide) may be directed toward sensitive types of normal cells, such as neurons. The combination of etoposide and vorinostat results in more double-stranded breaks of chromosomal DNA and the activation of pro-apoptotic factors such as p53 [58].

Another anticancer drug that can be used for the improvement of vorinostat effectivity is arsenic trioxide. The application of vorinostat for leukemia treatment can be complicated by subpopulations of lymphoma cells with drug resistance. This fact leads to its combined application with suitable anticancer drugs such as arsenic trioxide (used for the clinical treatment of hematologic malignancies). Its anticancer effect is coupled with the stimulation of oxidative stress and the suppression of DNMT1 expression, including resistant cell lines [59–61]. Therefore, Li *et al.* studied its combination with vorinostat for the apoptosis of cells [62]. As expected, the tested combination showed a significant improvement of apoptosis in comparison to the single drugs alone. Their combined application also led to increased levels of acetylation of histones H3 and H4.

Intensively studied application of clinically used HDACis (e.g., vorinostat) [37], or new developed agents (N-hydroxy-7-(2-naphthylthio) heptanomide) have been treatment of highly malignant oncological diseases such as anaplastic thyroid cancers [63]. These malignancies displayed high invasiveness, extensive necrosis and poor prognoses. Patients bearing this cancer still have a median survival of 5 months and less than 20% survival for 1 year [64]. Nevertheless, Baldan *et al.* found that vorinostat's combination with PJ34 (inhibitor of poly(ADP-ribose) polymerase-l) led to a significant increase of cytotoxicity [65]. In addition, the tested combination increased the expression of thyroid-stimulating hormone receptors associated with the inhibition of metastasis and apoptosis of thyroid carcinoma cells [66]. It has been confirmed that this method can be useful for the treatment of this highly malignant cancer.

The discussed and cited works clearly demonstrate the high potential of HDACi's combination with existing cytostatics for anticancer treatment, as shown in Table 2. We expect that this combination could enhance the effectivity of cytostatics and suppress drug resistance and the induction of apoptosis, among others.

The application of HDACi in combination with biological treatments

Biological therapy is the application of living organisms or agents obtained or derived from living organisms. For combined anticancer therapy, immunotherapies and oncolytic virotherapy have been studied.

One oncologic disease studied for the application of immunotherapies is metastatic melanoma. It was observed that this tumor type can be occasionally sensitive to immunotherapy, with durable response rates ranging from 5 to 15% [73,74]. Overcoming this tumor resistance is complicated because this phenomenon is initiated and controlled by several factors and processes, including suboptimal immune system activation, tumor-derived local immunosuppressive factors, immune-suppressive cells, decreases in tumor antigen and intrinsic tumor antiapoptotic mechanisms in cancer cells [75]. However, HDACi can induce immune potentiation by increasing the antitumor activity of IL-2 [76]. Vo *et al.* observed that the application of dacinostat had a highly positive effect on pmel-1 splenocytes (white blood cell lines isolated from the spleen) [77]. These authors observed that dacinostat application led to the reduced viability of melanoma cells and stimulates antitumor activity of lymphocytes by increased presentation of tumor-associated antigens. Another benefit of its application was suppression of immunosuppressive endogenous lymphocytes.

Oncolytic virotherapy was successfully studied for the selective targeting of cancer cells [78] and the stimulation of immune resistance. More than 12 different oncolytic viruses are currently undergoing Phase I–III clinical trials against various types of cancer [79]. Nevertheless, in some cases, the tumor environment can suppress viral susceptibility and permissivity and attenuate apoptotic tumor death and intrinsic/extrinsic antiviral immune responses. However, the inhibition of HDAC activity can effectively repress these phenomena [80–84]. In the field, Otsuki *et al.* successfully tested valproic acid with the combination of herpes simplex virus in glioma cell lines [85]. These authors observed that its application significantly improved the effectivity of subsequent virotherapy, such as the repression of the antiviral response, and improved viral propagation, even in the presence of interferons.

Similarly, Han *et al.* observed that the combined application of the vorinostat had a synergistic effect on the killing HeLa cells and tumor destruction [86]. On the molecular level, this effect was related to the HDACi suppression of NF-κB (oncogenic survival factors) and the virus induction of apoptotic proteins (e.g., caspase 8 and 9). However, Hotti *et al.* observed that the application of valproic acid suppressed adenovirus replication and spread in prostate cancer cell lines [87]. Additionally, Shulak *et al.* observed vorinostat suppression of virus replications in prostate tumor [88] Nevertheless, Kim *et al.* found that this phenomenon could be coupled with the increased expression

Table 2. The studied combination of histone deacetylase inhibitor with anticancer agents.

Anticancer agents	HDACi	Model	Effect	Ref.
Paclitaxel	Valproic acid	TC (CAL-62 and ARO)	↑β-tubulin acetylation	[35]
Cisplatin	Trichostatin A	LC (A549)	↑caspase 8	[38]
Cisplatin	YCW1	NSCLC (H1435, H1299, H460, A549, 4T1 and C1–5), LC (IMR90) nude mice with A549	↓Drug resistance, ↑caspase 3	[40]
Cisplatin	Vorinostat	NSCLC (Calu 6) and BC (NCI-H23)	↑cAMP-dependent transcription factor ATF-3, synergistic effect	[44]
Cisplatin	Curcumin	CC (SiHa and SiHa R)	↓HDAC 1 and 2 expression, ↑acetylation of 53, ↓drug resistance	[47]
Cisplatin	Belinostat	NSCLC (H460, H460R and A549, H406R, H1299) HEK (HEK293) and transfected HEK293	↓ABCC2 (membrane drug-efflux pumps) expression, ↑drug accumulation, ↓*ERCC1* (DNA repair gene)	[48]
Cisplatin	Panobinostat	MPM (MSTO-211H) MCL (Met-5A)	↑Apoptotic gene (*FOXO3A* and *CASP9*), ↑cytoselectivity	[50]
Cisplatin	Vorinostat	OSCC (HSC-3), LC (A549) and BC (MCF-7)	↑Oxidative endoplasmic reticulum stress, ↑caspase 4	[51]
Cisplatin	Valproic acid	LC (A549)	↑Caspase 3	[52]
Cisplatin	Sodium butyrate	OSCC (HSC-3)	↑Caspase 3	[54]
Cisplatin and pemetrexed	Valproic acid	MCL (M14K, M38K and ZL34) and their sarcomatoid subtypes	↑Oxidative stress, ↑histone 3 acetylation	[56]
Cisplatinum and etoposide	Vorinostat	SCLC (H209 and H146), Nunu mice with H209 cells	↑Caspase-3, ↑acetylation of histone 3 and α-tubulin, ↓tumor growth	[57]
Arsenic trioxide	Vorinostat	ML (K562)	↑Histone H3 acetylation and 4, ↓apoptosis	[62]
PJ34	Vorinostat	ATC-derived cell line (SW1736)	↑TSHR expression	[65]
Cisplatin	ST3595	NSCLC (H460, A549, H460/Pt and A549/Pt) mice with H460 and H460/Pt	↓IL-8, ↓vascular endothelial growth factor signaling pathway	[67]
Cisplatin	Vorinostat, or trichostatin A	CCA (KKU-100 and KKU-M214)	↑p21, ↑p53, ↓CDK4, ↓Bcl-2	[68]
Mitomycin C, or cisplatin	Valproic acid	TCC (T24, BIU87 and 5637)	↓Survivin (apoptosis inhibitors), ↑histone H3 acetylation	[69]
5-Fluorouracil	SBHA, or entinostat	CRC (SW48, HT-29 and Colo-205)	↓Cyclin A1, ↑p53, ↑p21 expression, triggering mitochondrial dysfunction ↓ pro-caspase 3 and 8	[70]
Cisplatin	Scriptaid	LC (A549 and H460)	↓Hypoxia, ↓drug resistance	[71]
Sorafenib, or bortezomib	Entinostat	ChC (EGI-1 and TFK-1)	↑Caspase-3, ↑Bax, ↑p21(Waf/CIP1), ↓Bcl-2	[72]

ATC: Anaplastic thyroid carcinoma; BC: Breast cancer; CC: Cervical cancer; CCA: Cholangiocarcinoma; CRC: Colorectal cancer; HDACi: Histone deacetylase inhibitor; HEK: Human embryonic kidney cells; LC: Lung carcinoma cell line; MCL: Mesothelioma cell line; ML: Myelogenous leukemia; MPM: Malignant pleural mesothelioma; NSCLC: Non-small-cell lung cancer; OSCC: Oral squamous cell carcinoma; SCLC: Small-cell lung cancer; TC: Thyroid cancer; TCC: Transitional cell carcinoma; TSHR: Thyroid stimulating hormone receptor.

of cell cycle-related proteins, such as p21 [89]. More importantly, they also presented a possible solution to this problem based on the sequential application of antitumor agents. It was confirmed that the addition of vorinostat prior to adenovirus CAR led to the significant synergistic effect.

Another positive influence of HDACi application on oncolytic virotherapy can be the overexpression of their cellular receptors. For example, Watanabe *et al.* showed that valproic acid or a small dosage of FR901228 (1 ng/ml) significantly improved the expression of coxsackie-adenovirus receptor and thereby the toxicity of adenoviruses such as OBP-301 for lung cancer cell lines [90,91]. In addition, this effect was observed even for lines with a low level of expression of virus receptors (e.g., A149 and H358). Because these viruses displayed high selectivity for cancer cells against normal ones [78], this approach may have a significant impact on anticancer treatment. We expect a profoundly positive impact of HDACi on the clinical implementation of oncolytic virotherapy.

The application of HDACi in combination with physical stimuli

Some anticancer therapeutic methods (hyperthermia and radiotherapy) that can be applied with HDACi are based on cellular stress from heating or radiation. HDACi can sensitize cancer cells for these therapies by blocking protective mechanisms or inducing apoptotic factors.

For example, Narita *et al.* observed the *in vitro* and *in vivo* induction of HDAC3 activity by hyperthermia in hypoxic cells [92]. Because the suppression of this phenomenon can strongly enhance hyperthermia efficiency,

HDACi could sensitize cancer cells to prospective agents. In addition, data have shown that this strategy could be gentle for potential patients. It is most likely caused by the low possibility of hypoxia for normal nontransformed cells against cancer cells. Another benefit of combined hyperthermia applications with HDACi was demonstrated by Hassan *et al.* [93]. They examined an HDACi combination (nicotinamide and trichostatin A) for the enhancement of hyperthermia in PC-10 cells (lung cancer line). This combination with hyperthermia reduced cell resistance and the activation of apoptotic pathways, most likely reflecting the deacetylation of Ku70. Antiapoptotic factors (Bcl-2 and Bcl-xL) prevent proapoptotic proteins, such as Bax, from translocating into mitochondria in cells without stress. Under hyperthermia application, the Bcl-2 level is decreased, and some Bax is liberated. In addition, more Bax is overexpressed by hyperthermia. However, activation is blocked because deacylated Ku70 binds liberated Bax. Nevertheless, SirT-1 inhibited by HDACis and acetylated Ku70 cannot suppress the activation of the apoptotic pathway. Therefore, targeting Ku70 and/or its acetylation during hyperthermia may be a promising therapeutic method for cancer treatment.

The effectivity of the HDACi abexinostat for radiosensation was tested by Gressette *et al.* using a nunu mouse model with induced nasopharyngeal carcinoma [94]. A possible explanation for the synergistic cytotoxic effects can be based on the silencing of the RAD51 protein (a key effector for the repair of DNA double-stranded breaks by homologous recombination). Its deactivation is one prospective mechanism of cell sensitization to chemotherapy or radiotherapy.

Similarly, Wu *et al.* observed that vorinostat application significantly enhanced the radiosensitivity to pancreatic carcinoma (Panc-1) cells [95]. In agreement with the above results, this effect was coupled with the repression of important survival factors such as Ku70, Ku86, RAD51 and RAD54.

Application of histone acyltransferase inhibitor in combination with cytostatic agents

Another therapeutic method for the regulation of histone acetylation could be based on the inhibition of histone acyltransferases (HATs). Nuclear HATs (type A) can also modulate the acetylation level of other nuclear proteins, such as transcription factors (e.g., Myc proto-oncogene protein, p53 and NF-kB). Second group HATs (type B) are cytoplasmic enzymes, which act in the posttranslational modification of histone acetylation. Their influence on carcinogenesis is still ambiguous. Even within the same type of cancer, HATs can play tumor suppressive and supportive roles [96]. Nevertheless, the finding of histone hyperacetylation in hepatocellular carcinoma and the correlation of the acetylation of lysine on histone H3 (H3K18) with prostate cancer recurrence indicate the high potential of histone acyltransferase inhibitor (HATi) for cancer treatment [97,98]. More importantly, the number of high-impact studies strongly implies HATi applicability for the treatment of melanoma, leukemia, breast, prostate and colon cancer, among others [96]. The structures of known inhibitors are shown in Supplementary Table 3.

Especially in combination with other therapeutic regimens, HATi could be very promising agents for the treatment of dangerous oncological diseases. For example, the high potential of this strategy for the treatment of thyroid cancers was demonstrated by Kim *et al.* [99]. The authors showed that triptolide's combination with BIIB021 (inhibitor of heat shock protein 90) displayed a strongly cytotoxic effect on the viability of thyroid cancer cell lines. This effect was most likely caused by the inhibition of p3K/AKt/mTOR and NF-kB signaling pathways and the activation of apoptotic factors such as caspase 3 and p53.

This suggests that the incorporation of HATis in anticancer therapy, especially in combination regimens, could be a promising method for clinical oncology.

The combination of various types of epigenetic agents in anticancer therapy

A number of recent studies demonstrated that multiple hypermethylated genes can be effectively reactivated in combination with DNMT1 and HDAC inhibition [100]. It was indicated that DNMT1 and HDAC can cooperate for the silencing of gene expression in cancer cells. This can be explained by the effect of methyl CpG-binding proteins (as shown in Figure 4).

MBD (Methyl CpG-binding proteins) interact with methyl-CpGs in DNA and frequently associate with some HDACs [101], implicating their important role in the control of gene expression and potential gene targeting in tumor treatment. For example, their depletion by antisense oligonucleotides resulted in the silencing of these genes and the inhibition of the invasiveness and metastasis of breast, prostate and liver cancer cell lines [102]. Previous studies have revealed another therapeutically important link between HDAC and DNMT [95,103]. For example, HDACi application can lead to DNMT inhibition [36]. A possible model of this process can be based on DNNT

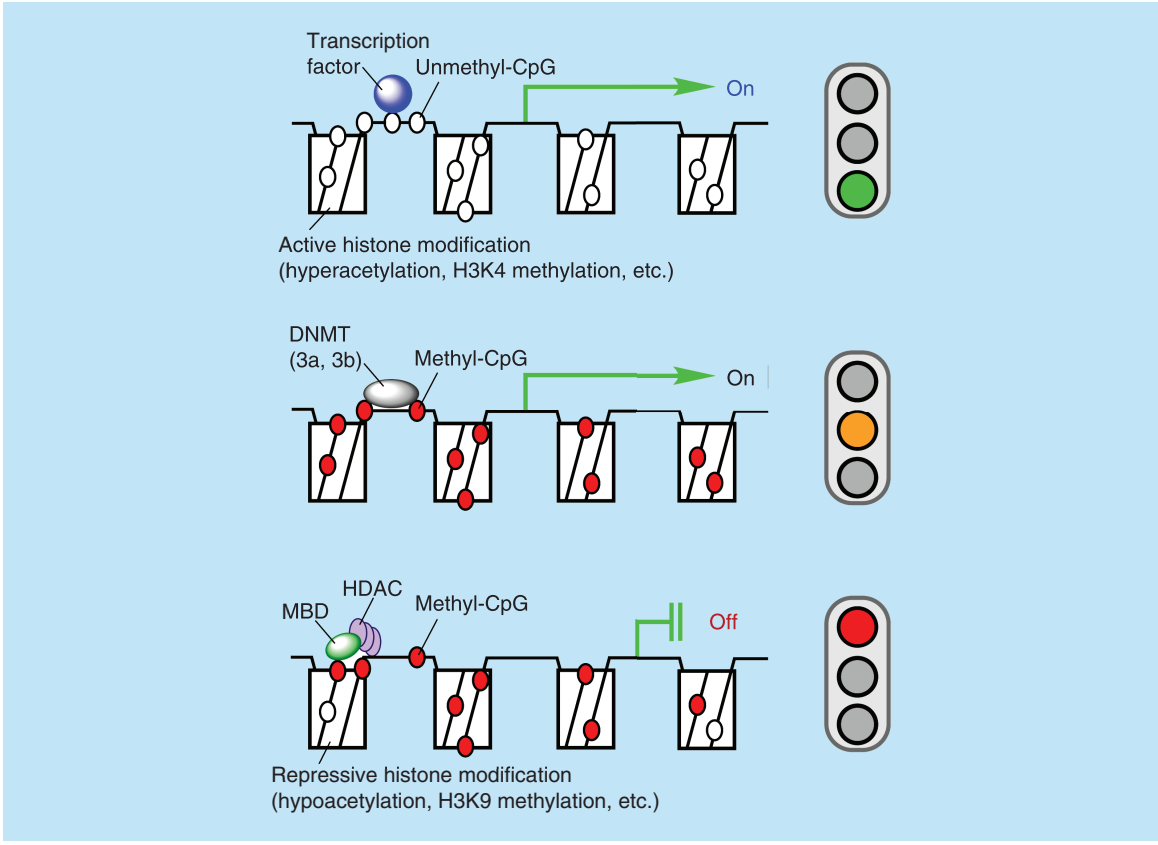

Figure 4. Principle of gene suppression via methyl CpG-binding proteins.
DNMT: DNA methyltransferase; HDAC: Histone deacetylase.

ubiquitination and accelerated degradation [103] or DMMT hyperacetylation, resulting in a disturbance of DNMT1 binding to other nuclear components [36].

These facts led to the development of new anticancer therapies based on the combined modulation of DNA and histone epigenetic factors, mainly using HDACis and DNMTis [49,104–109]. In addition, Souza *et al.* found that the combination of trichostatin A and decitabine revealed changes in melanocyte morphology and gene expression, which could indicate epigenetic flexibility in normal melanocytes [110].

Another important benefit of the combination of HDACi and DNMTi may be the sensitization of cancer cells to other therapies (e.g., chemo and immunotherapy). For example, Steele *et al.* showed that the combined application of decitabine and belinostat is more effective than decitabine alone at reactivating the silenced tumor suppressor hMLH1 and re-sensitizing resistant ovarian cancer cells to cisplatin [111]. Similarly, Cacan *et al.* observed that the combination of decitabine and trichostatin A is significantly more effective in the suppression of ovarian cancer chemoresistance than the application of these agents alone [100]. The above results clearly demonstrate the high usability of this approach as adjuvant therapy to overcome cancer chemoresistance.

Similarly, Dubovsky *et al.* studied the combination of decitabine and LAQ824 for the treatment of chronic lymphocytic leukemia [112]. These authors confirmed that this method effectively restored the immunogenicity of tested cell lines as well as primary cells from patients. This combination led to significant changes in the expression pattern of all tested cell lines, for example, a strong reduction in immunosuppressive factors such as IL-10.

Nevertheless, for some tumor types, such as glioma, obtaining suitable epigenetic agents can be complicated. For example, the application of decitabine, valproic acid or their combinations can induce tumor differentiation (e.g., induction of CD133 in human glioma [113]). However, $CD133^+$ tumor cells can initiate neurospheres, which exhibit self-renewal, differentiation and proliferation, resembling those of normal neuroblastoma cells [114]. Moreover, other phenotypical characteristics of tumor stem cells, such as enhanced chemoresistance and radioresistance, resulting in the regulation of tumor progression and recurrence, are coupled with its expression [115].

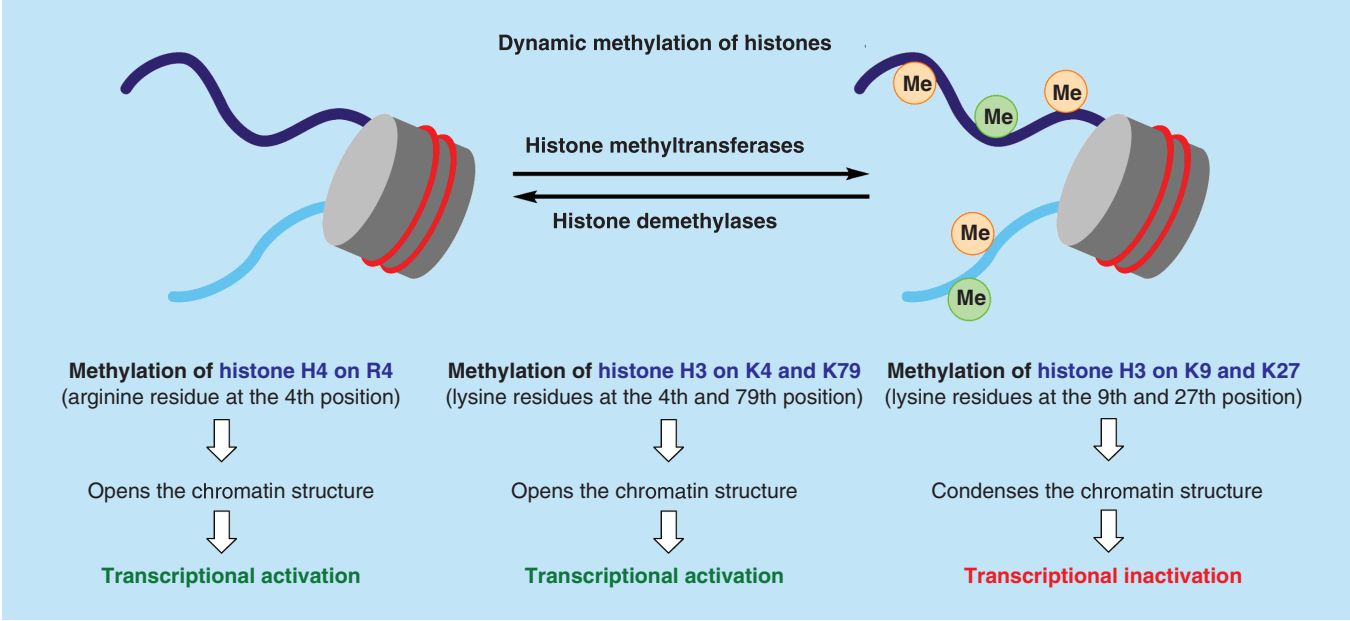

Figure 5. Regulation of gene expression by histone methylation.

A tested therapeutic strategy for targeting histone methylation in combination with other epigenetic agents

A prospective strategy for the influence of gene expression can also be the modulation of proteins, particularly histone methylation. Unlike histone acetylation, methylation/demethylation can lead to activation and even the deactivation of expression, depending on the position of the methyl groups (Figure 5).

For the regulation of the histone methylation pattern, histone methylase and demethylase inhibitors (HDMis and HMTi), such as tranylcypromine, statins, 3-deazaneplanocin A or other drugs (e.g., antifolates and inhibitors of adenosine transporters), can be used (Supplementary Figure 3).

Combined modulation of histone methylation and acetylation pattern can be effective method for the treatment this appearance of cancer drug resistance. For example, Takashina *et al.* found that combinations of vorinostat and 3-deazaneplanocin A synergistically suppressed the proliferation of resistant cancer cell lines against kinase inhibitors, including EGFR-mutant cells *in vitro* and in the mouse model [116]. The observed effect was associated with decreased histone methylation (H3K27Me3) and increased histone acetylation.

An interesting strategy based on targeting the methionine cycle through the inhibition of adenosine transporters and folate metabolism for the combined targeting of epigenetic mechanisms was presented by Montenegro *et al.* [117]. Methionine is an essential amino acid for many human cancer cell lines and primary tumors [118]. Folate is a resource for the synthesis of N5-methyl-tetrahydrofolate, a cofactor of methionine synthase. Adenosine is a product of the methionine cycle, and its transport from cells can be an important mechanism for cancer cell survival. In folate-deficient cells (including cells treated with antifolates), adenosine accumulation can lead to suppressed homocysteine methylation. Homocysteine methylation subsequently induces the synthesis of S-adenosylhomocysteine (inhibitor of cellular methyltransferases). In agreement with the above, the authors observed that the application of dipyridamole (inhibitor of specific adenosine transporters) with of 3-O-(3,4,5-trimethoxybenzoyl)-(2)-catechin (tyrosinase-processed antifolate prodrug) had a strong synergistic effect on the cytotoxicity of breast cancer lines [117]. This effect was coupled with the reactivation of the expression of tumor suppressor genes such as *RASSF1A* and the inhibition of protein methylation (e.g., E2F1, p53).

In summary, we can conclude that combinations of more epigenetic agents can more significantly and effectively suppress cancer phenotypes via the reversion of the 'normal' epigenome (as shown in Table 3).

Conclusion

Epigenetic agents are promising tools in cancer therapy. This review presents and discusses various methods for the combined application of epigenetic drugs, primarily focusing on inhibitors of DNA methyltransferases and

Table 3. Tested combinations of epigenetic agents for anticancer treatment.

Epigenetic agents		Model	Effect	Ref.
Trichostatin A	Azacytidine	OC (A2780 and A2780-AD)	↑G-protein signaling, ↓cisplatin resistance	[100]
Entinostat	Azacytidine	LC (Calu6 lung), Rowett nude rats with Calu6	↓Tumor volume, ↑DNA methylation	[104]
SAHA, MS-275 and FK228	Azacytidine	ESCC (OE21, Kyse-270, Kyse-410), and EAC (OE33, SK-GT-4), NNOE (Het-1A)	↑Cytotoxicity ↑DNA damage, ↑apoptosis, ↓cell proliferation, ↑metastatic selectivity	[105]
Resveratrol	Pterostilbene	BC (HCC1806 and MDA-MB-157), BEC (MCF10A)	↓SIRTI, ↓DNMT 1, 3a and 3b, ↑cancer selectivity	[108]
Sodium phenylbutyrate	Azacytidine	A/J mouse with inducted lung cancer	↓Number of pulmonary lesions	[107]
MS-275	Azacytidine	AML (MV4–11, MV4–11 TP53 and R248W)	↓Proliferation, ↓p21waf1, ↑caspase 3	[109]
Trichostatin	Azacitidine	MM (4C11$^+$), M(4C) NMM (4C11$^-$), Human primary melanocytes C57Bl/6 mice with 4C11$^+$ and 4C11$^-$	↑HSPB1 (molecular chaperone), ↑blim1 (assembler and stabilization of actin-filaments), ↓SERPINE1 (serine proteinase inhibitor) in 4C11$^+$	[110]
Belinostat	2-Deoxy-5′ azacytidine	OC (OVCAR-3 and MDAH-2774)	↑Caspase 3 and 7, ↑p27, strong synergistic effect	[111]
LAQ824	Decitabine	CLL (MEC1, MEC2, WaC3), primary CLL	↓IL-4, 6 and 10, ↑CCL–T-cell interaction,	[112]
Vorinostat	3-Deazaneplanocin A	NSCLC (H1299, H1975, A549 and PC-3), BALB/cAJcl-nu/nu mice with H1975	↓Cell proliferation, ↓epidermal growth factor signaling, ↓histone H3 lysine 27 trimethylation, ↑histone acetylation	[116]
Dipyridamole	Catechin	BC (MDA-MB-231)	↓*E2F1* and *p53* methylation, ↑tumor suppressor genes	[117]
Valproic acid	Decitabine	N (UKF-NB-3 and UKF-NB-4)	↑CD133 receptors expression	[119]
Valproic acid, or CI-994	2-Deoxy-5′ azacytidine	SCLC (H69, H82, H1417, H2171 (ATCC) and U1906)	Sensation to tumor necrosis factor-related apoptosis-inducing ligand ↑caspase 8, ↑Bax (apoptotic factor)	[120]
Tranylcypromine	Decitabine	MDA-MB-231 with knocked down LSD2	↑Expression of progesterone, oestrogen and retinoid acid receptors, ↑tumor suppressor genes	[121]
Deazaneplanocin A	5-Aza-deoxycytidine	CML (EG-01, and K562) MDS (MDS-L), AML (KG1 HL-60, THP-1, OCI/AML3)	↓Histone methylation (H2K27Me2 and H3K27Me3)	[122]
MC1568	Simvastatin	CC (DLD1, SW620, HCT116, HT29, LoVo and colo320)	↑p27KIP1	[123]
Decitabine	Trichostatin A	NSCLC (A549 and H460), mice with H460 cells	↓Cisplatin resistance, ↑tumor suppressor (miR-512 and miR-373)	[124]

AML: Acute myelogenous leukemia; BC: Breast cancer; BEC: Breast epithelial cells; CLL: Chronic lymphocytic leukemia; CML: Chronic myelogenous leukemia cell lines; CRC: Colorectal cancer; EAC: Oesophageal adenocarcinoma; ESC: Oesophageal squamous carcinoma; LC: Lung carcinoma; MDS: Myelodysplastic syndrome; MM: Metastatic melanoma; N: Neuroblastoma; NNOE: Non-neoplastic oesophageal epithelial cells; NSCLC: Non-small-cell lung cancer; OC: Ovarian cancer; SCLC: Small-cell lung carcinomas.

histone deacetylases. The number of examples and cited studies presented here demonstrate the potential efficacy of this therapeutic strategy in the field of modern anticancer treatment. These studies show that the application of epigenetic drugs can positively influence existing therapies, such as the sensitization of cancer cells by HDACis and DNMTis.

Several recent studies have also indicated that the application of epigenetic agents can reverse the disease process and return cancer cells back to their normal state. In addition, other clinical studies confirmed that the applications of epigenetic agents are limited and clinically manageable.

Future perspective

We expect that the incorporation of epigenetic agents into combined anticancer therapy would be a useful treatment for a significant majority of cancer patients, with a high impact on the efficiency of anticancer therapy (prolonging the average survival time, suppressing metastatic ability and cancer recurrence, etc.). Achieving this important goal would be difficult without obtaining more detailed information about the expression and regulation of epigenetic enzymes.

This improvement demonstrated the high potential of epigenetic agents, particularly those used in the multitherapeutic mode, for cancer treatment. Nevertheless, the full incorporation of the promising strategies and approaches discussed above is still often limited by the incomplete knowledge of epigenome behavior and regulation, and their effective use is unlikely without suitable diagnostic methods. However, rapid developments of advanced bioanalytical methods for epigenetic analysis promise potential solutions to these problems [125,126]. In addition,

their incorporation in clinical practice in combination with a wide selection of epigenetic agents could represent a strategy for the personalization of cancer therapy. This could enable the therapeutic modulation of activities of other types of epigenetic-active enzymes that participate in disease mechanisms.

In the case of DNA, an interesting enzyme target might be TET proteins, especially TET 1. TET protein 1 is an oxidase that oxidizes a cytosine methyl group to hydroxymethyl. The higher activity of TET protein 1 and the hydroxymethylcytosine level was found in leukaemic diseases such as acute myeloid leukemia [127]. Its targeting by iron chelators (structure moiety of potential inhibitors, confirmed *in vitro*) [128,129] in combination with other therapies is a prospective therapeutic strategy. The application of iron chelators for combined anticancer therapy has been extensively studied [130]. The cited studies showed high potential for this strategy for cancer treatment.

For the control of histone pattern targeting, histone demethylases and histone methyltransferases are prospective strategies. Nevertheless, predicting this achievement is not easy, as these enzymes can suppress or even activate gene expression depending on the position of methyl groups. However, the potential for the modulation of histone methylation was clearly demonstrated in several high-impact studies [121,123].

An interesting strategy could be the application of epigenetic agents for the prevention of oncological diseases. Epigenetic changes can be observed in early cancer states or even prior to the start of tumorigenesis. Some authors say that lifestyle adjustment, including meal modification (e.g., the incorporation of epigenetic active agents), can significantly decrease cancer incidence in the population [5,131]. However, in this field, it is much easier to be optimistic rather than critically evaluate the obtained data. Nevertheless, we predicted that precise knowledge concerning the cancer epigenome and the personation of therapy and prevention would lead to epigenetically active food supplements for cancer patients and persons with a higher risk of cancer.

In the future, we also expect that epigenetic agents for cancer treatment will be used in veterinary medicine. Studies discussing the application of epigenetic drugs or diagnostic methods for the animal treatments have been published [132,133]. We may assume the relatively rapid transfer of methodology from humans to veterinary medicine, as industries targeting animals such as pets are increasing. In agriculture, affordable epigenetic drugs and diagnostic methods improve the health and performance of livestock that is, the 'personalization' of fodder mixtures.

Thus, we propose that the combination of epigenetic agents with classical anticancer modalities will be used more often in the near future for oncologic therapeutic methodologies, and we hope that this trend will lead to the introduction of more efficient and safer anticancer regimens.

Executive summary

Introduction

- Epigenetic mechanism such as histones and DNA modification play a key role in the control of gene expression. Their dysregulation initiates and supports of many serious pathological states such as oncological diseases.

Combined application of DMNT, HDAC and HAT

- Epigenetic agents such as DMTis, HDACis and HATis displayed significant anticancer effectivity. Their application can be used for the improvement of the effectiveness of other anticancer regiments (e.g., reduction of drug and immunoresistance, activation tumor suppressor genes and silence of oncogenes).

Combined application of various epigenetic drugs

- Due to the complexity of epigenetic mechanisms, applications of more various epigenetic agents, for example combination of DNMTis and HDACis can be a perspective cancer treatment.

Conclusion & future perspective

- To date, the number of combinations of epigenetic agents with anticancer therapeutic regiments was designed and tested. Both *in vitro* and *in vivo* experiments found promising anticancer therapeutic regimens for the future clinical studies and clinical practice.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/fmc-2017-0203

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