Clinical Epigenetics

REVIEW

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Epigenetic marvels: exploring the landscape of colorectal cancer treatment through cutting-edge epigenetic-based drug strategies

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Abstract

Epigenetics is currently considered the investigation of inheritable changes in gene expression that do not rely on DNA sequence alteration. Significant epigenetic procedures are involved, such as DNA methylations, histone modifications, and non-coding RNA actions. It is confirmed through several investigations that epigenetic changes are associated with the formation, development, and metastasis of various cancers, such as colorectal cancer (CRC). The difference between epigenetic changes and genetic mutations is that the former could be reversed or prevented; therefore, cancer treatment and prevention could be achieved by restoring abnormal epigenetic events within the neoplastic cells. These treatments, consequently, cause the anti-tumour effects augmentation, drug resistance reduction, and host immune response stimulation. In this article, we begin our survey by exploring basic epigenetic mechanisms to understand epigenetic tools and strategies for treating colorectal cancer in monotherapy and combination with chemotherapy or immunotherapy.

Keywords Colorectal cancer, Epigenetics, Epidrugs, Cancer treatment, DNA methylation, Histone modifications

Introduction

Colorectal cancer (CRC) poses a significant global health concern. It is estimated that 153,020 individuals will be diagnosed with CRC in the USA in 2023, with around 19,550 cases occurring in individuals under 50 years old. The decline in CRC incidence has slowed, partly due to increased cases among those under 55. Additionally, there has been a notable shift towards left-sided tumours, with rectal cancer accounting for 31% of all cases in 2019, highlighting the importance of early detection and targeted interventions. Despite advancements in treatment, CRC mortality remains a challenge, with an expected 52,550 deaths in 2023. However, there has been an overall 2% annual decline in mortality from 2011 to 2020. It is important to note that mortality rates have increased annually by 0.5% to 3% in individuals younger

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than 50 years and Native Americans younger than 65 years [1]. In summary, efforts to advance progress in CRC prevention and treatment should urgently focus on understanding the rising incidence, improving screening accessibility, and tailoring interventions to address the changing landscape of CRC diagnosis to reduce mortality and improve outcomes for affected individuals.

In most cases, surgical intervention is often necessary for complete tumour removal. Following surgery, adjuvant therapies such as chemotherapy or radiotherapy, administered before (neoadjuvant) or after (adjuvant) the procedure, can help reduce tumour size or enhance its stability. Current chemotherapy options include monotherapy with 5-fluorouracil (5-FU) and combination therapy, typically consisting of oxaliplatin (OX), capecitabine (CAP), and irinotecan (IRI) [2]. The most common combination therapy regimens are 5-FU/OX, 5-FU/IRI, CAP/OX, and CAP/IRI, which remain the mainstay approaches in first-line treatment [3]. Patients with a low risk of disease progression are typically treated with monotherapy. The choice of additive agents appears to have similar efficacy, with variations primarily observed in side effect profiles. However, this treatment approach presents several irreversible drawbacks, including systemic toxicity, suboptimal response rates, the emergence of resistance (both innate and acquired), and limited selectivity for tumour sites.

A complex interplay of factors influences CRC risk. While 5–10% of cases are attributed to genetics, 90–95% relate to lifestyle and environment [4]. Key environmental risks include tobacco, pollutants [5], radiation, certain infections (e.g., Epstein-Barr virus [6], *Helicobacter pylori* [7], *Schistosoma mekongi* [8]), and inflammatory bowel disease [9]. Family history, age over 50 [10], and living in developed countries [11] also increase risk. Furthermore, some medications, such as hormone therapies and specific chemotherapy drugs, can heighten the long-term risk of secondary cancers like CRC, particularly when combined with radiotherapy [12, 13]. In essence, CRC development is a multifaceted process requiring comprehensive risk assessment.

From the molecular aspect, CRC arises from a complex interplay of genetic and epigenetic alterations that disrupt normal cell behaviour. Genetically inherited mutations in genes like *APC*, associated with familial adenomatous polyposis, or *MLH1*, linked to Lynch syndrome, predispose individuals to CRC [14]. Environmental factors like UV radiation or chemicals in cigarette smoke can also cause DNA mutations in these critical genes. Chromosomal abnormalities, such as deletions or duplications of chromosomal regions, can further contribute to the disease [15]. Epigenetically modifications like DNA methylation can silence tumour suppressor genes such as *CDKN2A*, effectively removing a brake on cell growth [16]. Similarly, histone modifications can activate oncogenes like *KRAS*, promoting uncontrolled cell proliferation [17]. Working in concert, these genetic and epigenetic changes lead to the aberrant cell growth and tumour formation characteristic of CRC.

This review explores epigenetics' role in treating colorectal cancer (CRC) by examining innovative drug strategies that go beyond traditional therapies. We focus on key mechanisms, including DNA methylation, histone modifications, and the roles of non-coding RNAs in CRC development. Additionally, we analyse existing epigenetic drug regimens, highlighting their limitations and identifying areas for improvement. We also consider the potential of combining epigenetic drugs with chemotherapy or immunotherapy to enhance anti-tumour effects and address issues of drug resistance. Ultimately, we emphasise the importance of personalised therapies and the need for ongoing research to fully leverage the therapeutic potential of epigenetic modifications in the treatment of CRC.

Epigenetic modification mechanisms

Epigenetic alterations are pivotal in the development and progression of CRC. These modifications, which influence gene expression without changing the underlying DNA sequence, are frequently observed in CRC. A common alteration is aberrant DNA methylation, particularly the hypermethylation of promoter regions belonging to tumour suppressor genes [18]. Furthermore, changes to histones post-transcription modifications, such as altered acetylation or methylation patterns [19], can profoundly impact chromatin structure and gene expression, contributing to CRC pathogenesis. In addition to these well-established mechanisms, non-coding RNAs, including microRNAs and long non-coding RNAs, are recognised as important regulators of gene expression, with their dysregulation implicated in CRC progression [20]. The reversible nature of many epigenetic modifications makes them an attractive target for therapeutic intervention, offering the potential for novel CRC treatments either as monotherapy or in conjunction with existing therapies (Fig. 1).

DNA methylation

Numerous Aberrant DNA methylation patterns are a hallmark of many cancers and contribute significantly to tumorigenesis. In normal cells, DNA methylation, primarily occurring at CpG islands within gene promoter regions, plays a role in regulating gene expression. However, in cancer, this tightly controlled system is disrupted. A frequent observation in cancerous cells is the hypermethylation of CpG islands within the promoters

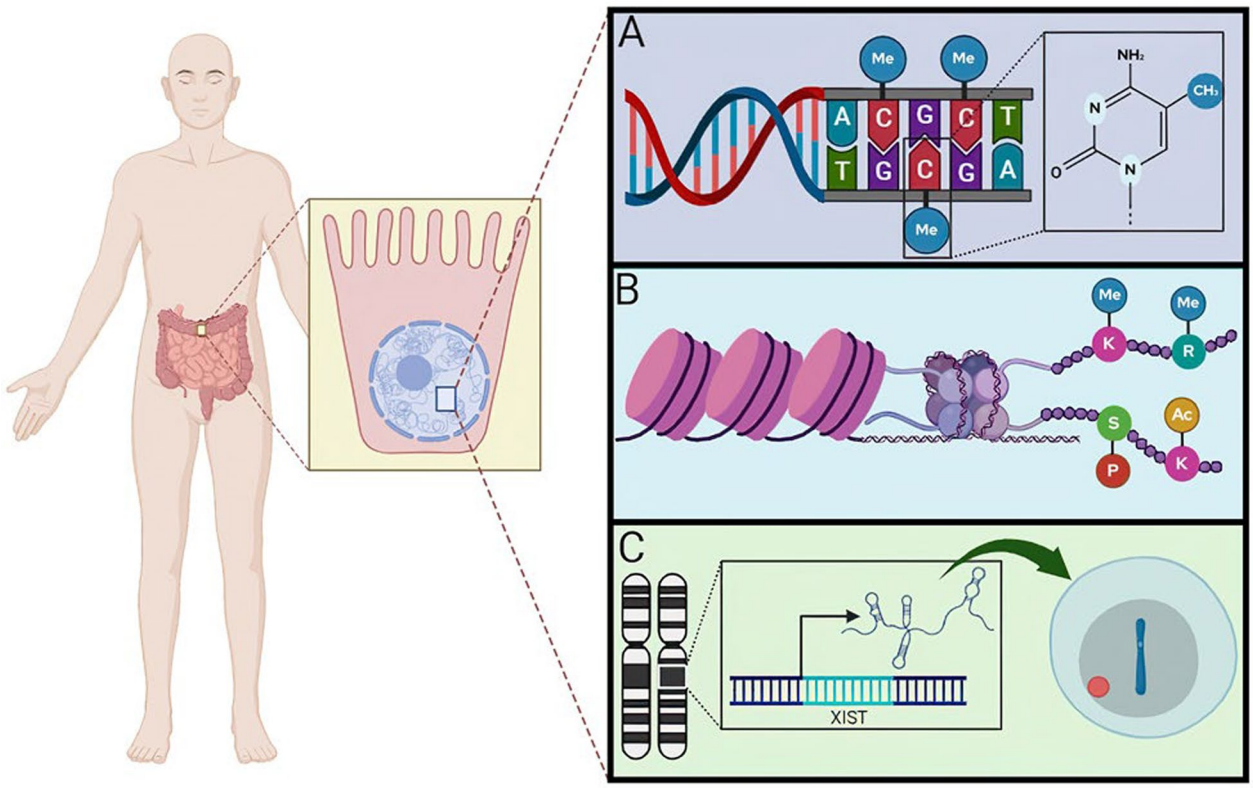

Fig. 1 Three main epigenetic mechanisms: **A** DNA methylation, in which DNA methyl transferase enzymes (DNMTs) transfer a methyl group to carbon 5 of cytosine residues **B** Histone modifications such as acetylation, methylation and phosphorylation are accomplished by their specific transferase enzymes on certain peptide residues **C** most known non-coding RNA action maybe the X chromosome inactivation which occurs in female mammals due to dosage compensation. In this process, one of the X chromosomes is randomly transcribed on the XIST gene locus; the XIST long non-coding RNA–protein complexes bind to the X chromosome in special places called 'entry sites', which plays a major role in the formation of inactivated X chromosome or 'Barr body' (the red circle). Designed by BioRender.com

of tumour suppressor genes [21]. This aberrant methylation is mediated by DNA methyltransferases (DNMTs). The addition of a methyl group from S-adenosylmethionine (SAM) to cytosine creates 5-methylcytosine (5mC), which subsequently hinders transcription factor binding and recruits methyl-binding domain proteins (MBDs) [22]. This cascade of events leads to chromatin remodelling and ultimately silences the expression of genes that would otherwise inhibit tumour growth.

The enzymes responsible for establishing and maintaining these aberrant methylation patterns are the DNMTs. While DNMT1 primarily maintains existing methylation patterns during replication, DNMT3A and DNMT3B can establish de novo methylation, contributing to the silencing of tumour suppressor genes [23]. This aberrant silencing effectively removes crucial hindrances to cell proliferation and contributes to uncontrolled tumour growth. Conversely, global hypomethylation, particularly in repetitive DNA sequences, is also observed in cancer and can contribute to genomic instability. The ten-eleven translocation (TET) family of enzymes facilitates aberrant DNA methylation reversal [24]. However, the balance between methylation and demethylation is often skewed towards hypermethylation of critical genes in cancer. Given the significant role of aberrant DNA methylation in cancer, DNMT inhibitors have emerged as promising therapeutic agents [25]. By inhibiting DNMT activity, these drugs can potentially reactivate silenced tumour suppressor genes, restoring their normal function and inhibiting cancer progression.

RNA methylation

One of the modification mechanisms carried out after the transcription is RNA methylation. There are more than 150 detected RNA modifications; moreover, N6 methyladenosine (m6A), the methylation at the 6th N of adenylate in RNA, is commonly observed in the modification processes of eukaryotic cells and impacts all RNA life cycle procedures [26]. Various types of RNA methylation, including m1A, m5C, m6Am, etc., have recently been detected. [27]; also, considerable results have been achieved as a result of m6A demethylase (FTO,

ALKBH5) discovery and sequencing technology advancement (m6A-seq, MERIP-seq) [28]. Due to the significant m6A-resulted biological functions of RNA modification, investigators tend to conduct more research and implement this procedure in different fields of study. The current study aimed to explore the functions, sequencing technology, biological functions, expression, and implementation of the mechanism mentioned above in the physiological or pathological state of RNA methylation-modified proteins and, consequently, illustrate the importance of its application in fundamental medical and clinical medicine studies.

Histone modifications

In eukaryotes, 146bps of DNA is wrapped around histone octamer proteins, which consist of two sets of H2A, H2B, H3, and H4, forming a nucleosome. The N- and C-terminal tails of these histone proteins are exposed and can be post-translationally modified [29]. Various histone modifications exist, some of which are more studied, such as acetylation, methylation, and phosphorylation.

Histone methylation is a process in which methyl groups (mono-, di-, or trimethylation) are transferred from S-adenosyl-L-methionine (SAM) to the ε-amino group of lysine or the guanidino nitrogen atom of arginine residues on histone tails. Lysine Methyltransferases (KTMs) [30] and Lysine Demethylases (KDMs) [31] are two enzyme families that catalyse the addition and removal of methyl groups on histone lysine residues, respectively. EZH2, a histone methyltransferase (KMT), and KDM6A, a histone demethylase (KDM), are frequently altered in digestive cancers [32]. Nakazawa et al. highlighted that alterations in global H3K9me2 levels are a significant epigenetic factor in colorectal tumourigenesis and carcinogenesis, influencing gene regulation in neoplastic cells through chromatin remodelling [33]. We suggest that readers explore other outstanding reviews available in the literature [34].

Histone acetylation is carried out by enzymes called histone acetyltransferases (HATs), which transfer an acetyl group from acetyl-CoA to lysine residues. HATs) catalyse the transfer of an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the ε-amino group of lysine residues on histone tails. Prominent HAT families include P300/CBP, GNAT, MYST, P160, PCAF, and TAFII230 [35]. In contrast, histone deacetylases (HDACs) remove acetyl groups, performing deacetylation. HDACs comprise a family of 18 enzymes categorised into four classes (I, II, III, and IV) based on their homology. Class I HDACs (HDACs 1, 2, 3, and 8) are localised in the nucleus. Class II HDACs are divided into two subclasses: IIa (HDACs 4, 5, 7, and 9) and IIb (HDACs 6 and 10). Class III HDACs, known as sirtuins (SIRT1-7), are found in various cellular compartments, including the nucleus (SIRT1, 6, 7), mitochondria (SIRT3, 4, 5), and cytoplasm (SIRT2). These enzymes utilise nicotinamide adenine dinucleotide (NAD +) as a cofactor for their deacetylation activity. Class IV consists of a single member, HDAC11. Class I, II, and IV HDACs require Zn^{2+} ions for catalytic activity, distinguishing them as Zn^{2+}-dependent HDACs [36]. Increased acetylation of H3K27 at oncogene promoters, like *TIMELESS*, correlates with their overexpression in CRC [37], while decreased H3K27ac and H3K9ac levels at the TP53 promoter lead to its inactivation [38].

Histone phosphorylation is one of the most common modifications in histones, occurring on certain serine residues. Histone serine phosphorylation is associated with gene expression activation in most cases. H3K9 and H3K27 share the same subsequent serine residues that can be phosphorylated; this close position modification site leads to the hypothesis that there is a crosstalk between methylation and phosphorylation that can change the affinity of some readers and writers associated with lysine residues and could regulate gene expression in a more precise and complex manner [29]. Lee et al. stated that high expression of phosphorylated H2AX in colorectal cancer tissues is linked to a more aggressive form of cancer [32].

Non-coding RNAs

Non-coding RNAs (ncRNAs) are diverse RNA molecules that do not function as templates for protein synthesis but are essential for biological processes like gene regulation and cell differentiation. They are divided into two main, including housekeeping ncRNAs, which include ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs) that are crucial for protein synthesis and ribosome biogenesis, and regulatory ncRNAs, which modulate gene expression. Regulatory ncRNAs are further classified by size: short non-coding RNAs such as microRNAs (miRNAs), small interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs) play roles in post-transcriptional regulation, while long non-coding RNAs (lncRNAs), exceeding 200 nucleotides, regulate gene expression at multiple levels, including transcription and translation [39]. ncRNAs are essential for gene regulation at various stages, including transcription, translation, and post-translational modifications. Additionally, ncRNAs are important for regulating cell differentiation and developmental processes. Some ncRNAs can also modify chromatin structure, influencing gene expression through epigenetic mechanisms [40].

The dysregulation of ncRNAs has been linked to various human diseases. In cancer, non-coding RNAs significantly contribute to tumour development and

progression. NcRNAs can act as oncogenes or tumour suppressors depending on their functions and expression levels. For instance, miR-34a suppresses tumour growth [41], while miR-138-5p can inhibit growth by targeting programmed cell death ligand 1 (PD-L1) [42]. Similarly, lncRNA NALT1 promotes cell proliferation and invasion [43], while lncRNA GAS5 suppress these processes [44].

Epigenetic tools

Epigenetic modifications are dynamically regulated through a complex interplay of enzymes that establish, interpret, and remove these marks. These enzymes can be broadly classified into writers, readers, and erasers. In the context of cancer, aberrant activity of these epigenetic regulators is frequently observed. Epigenetic writers are enzymes that catalyse the addition of chemical modifications to DNA or histone proteins. Key examples implicated in cancer include DNA methyltransferases (DNMTs), which add methyl groups to DNA, often leading to gene silencing. Other writers include histone lysine methyltransferases, protein arginine methyltransferases, and histone acetyltransferases, all modifying histone proteins, thereby influencing chromatin structure and gene expression. Epigenetic readers are proteins that contain specialised domains capable of recognising and binding to specific epigenetic marks. For example, methyl-CpG binding proteins (MBPs) recognise and bind to methylated DNA. Other readers, such as those containing Tudor domains, chromodomains, or bromodomains, recognise specific histone modifications. By binding to these marks, readers can recruit other proteins, ultimately influencing gene expression. Epigenetic erasers are enzymes that remove epigenetic modifications, effectively reversing the actions of writers. In cancer, the activity of erasers like the ten-eleven translocation (TET) family, which mediates DNA demethylation, can be dysregulated. Other important erasers include histone demethylases, such as lysine-specific demethylase (LSD1), and histone deacetylases (HDACs), which remove acetyl groups from histones.

Alterations in the expression or activity of these epigenetic regulators, through either mutations or changes in expression levels, are frequently observed in cancer and can contribute to tumourigenesis [28]. For instance, overexpression of DNMTs can lead to hypermethylation and silencing of tumour suppressor genes, while mutations in TET enzymes can impair their demethylation activity. Consequently, targeting these epigenetic tools has emerged as a promising avenue for cancer therapy. Inhibitors of writers, readers, and erasers are being developed and investigated for their potential to reverse aberrant epigenetic modifications and restore normal gene expression patterns in cancer cells. This rapidly evolving field offers a hopeful outlook for the future of cancer treatment, with the potential for more targeted and effective therapies based on manipulating the epigenetic landscape.

Epigenetic drugs (Epidrugs)

In the past few years, new treatments that affect the epigenetic mechanism have been developed by understanding the role of manipulating epigenetic changes in treating various cancers, including CRC. The proportion of epigenetic changes that cause aberrant gene expression is higher than genetic mutations, so epigenetic changes play an essential role in the formation of CRC [45].

Epidrugs are drugs that can target the epigenome. The use of epidrugs alone or combined with chemotherapy or immunotherapy shows promising results, such as increasing anti-tumour effects, overcoming the impact of drug resistance, and activating the host's immune response [46]. A large number of epigenetic modifiers are classified into different categories based on their mode of action, such as inhibitors of enzymes involved in DNA methylation (such as DNMTi), histone modification (such as HMTs, HDMs, and HDACs), and also agents that modulate miRNA expression therapeutically. Some have received FDA approval for treating diseases, including CRC, some of which have been tested preclinically or early in CRC clinical trials [47].

Their use in the early stages of CRC may be more effective because epigenetic changes are the first events in carcinogenesis, and the burden of genomic changes is lower. On the other hand, one potential application of epigenetic modifiers is in the advanced stage of CRC, although they must be combined with cytotoxic drugs. Epigenetic modifiers can sensitise CRC cells to radiotherapy, cytotoxic therapy, and immunotherapy, thereby reducing the dose of cytotoxic drugs, thus improving patient tolerance [48].

Cautious approaches to guide epigenetic inhibitors from the preclinical to the clinical level

The burgeoning field of epigenetic drug discovery has witnessed remarkable progress, fueled by an improved understanding of epigenetic mechanisms and the intricate interplay between "readers, writers, and erasers"—the key protein families orchestrating the epigenetic landscape. This deeper comprehension, coupled with encouraging preclinical findings, has spurred the development of small-molecule epigenetic inhibitors and their subsequent evaluation in clinical trials [49]. While preclinical studies have shown promise, translating these findings into robust clinical outcomes remains a significant challenge. Several factors contribute to this complexity, including the heterogeneity of epigenetic

alterations across different cancer types, the potential for off-target effects, and the need to identify predictive biomarkers to select patients most likely to benefit from epigenetic therapy.

Early clinical investigations predominantly focused on single-agent epigenetic inhibitors. However, a paradigm shift is underway, with a growing emphasis on combination therapies that leverage synergistic interactions between epigenetic agents and other treatment modalities. This shift is underpinned by meticulous preclinical studies, often spearheaded by medicinal chemists, which delve into the complexities of tumour epigenetics and provide invaluable insights for the rational design of novel therapeutic agents [50].

Beyond conventional small-molecule inhibitors, innovative approaches are emerging that hold immense promise for the future of epigenetic therapy. PROTACs (proteolysis-targeting chimaeras), for instance, represent a groundbreaking strategy that harnesses the cellular protein degradation machinery to eliminate disease-related epigenetic proteins selectively [51]. Another promising avenue involves the development of multi-targeted drugs that can simultaneously modulate multiple biochemical pathways, offering a compelling strategy for combination therapies [52].

The trajectory of epigenetic drug discovery appears poised to embrace a multifaceted approach, integrating conventional strategies with cutting-edge technologies like PROTACs and antibody–drug conjugates. While these novel modalities hold tremendous potential, it is essential to acknowledge that traditional approaches will continue to play a vital role in advancing our understanding and therapeutic exploitation of the epigenome.

Epidrugs for CRC treatment

Nowadays, the scientific community accepts that epigenetics significantly impacts the diagnosis and treatment of CRC, so the survival rate of CRC patients can be increased by focusing on the epigenetic system. The discovery of novel treatment approaches and medications involves detecting cancer-related epigenetic mechanisms that lead to the reversal of histone acetylation and DNA methylation patterns, as well as changes in non-coding RNA (Fig. 1). The epidrugs can be implemented singly or combined with other therapies. Notably, synergistic effects have been observed through combination therapy of DNMT and histone deacetylase (HDAC) inhibitors with other treatment approaches on CRC cells in vitro and in vivo [53]. The reactivation of tumour suppressor genes through the use of epigenetic drugs offers the potential to restore normal cellular functions. Observations suggest that combining conventional therapies with epigenetic treatments can yield significant benefits. A noteworthy study identified 45 drugs, primarily anticancer and antiarrhythmic agents, that synergise with DNA methylation inhibitors (DNMTi) or histone deacetylase inhibitors (HDACi) to promote the reactivation of tumour suppressor genes. For instance, the combination of the DNMTi decitabine with the antiarrhythmic drug proscillaridin A resulted in substantial changes in gene expression and downregulation of epigenetic regulators, including potential oncogenes. Conversely, 85 drugs were found to antagonise the effects of epigenetic therapy, highlighting the risk of detrimental drug interactions. These findings pave the way for innovative combination therapies and provide valuable insights for clinical practice regarding drug interactions with epigenetic treatments [54].

DNA methyltransferase inhibitors (DNMTis)

DNA methylation-mediated silencing of genes also plays a key role in CRC aetiology. Therefore, DNMTis have also been suggested for treating patients with CRC [55]. Transcriptional regulation by different epigenetic procedures such as DNA methylation/demethylation is another factor, except mutations which cause the alteration of tumour suppressor genes or oncogenes [56]. These are conserved families of cytosine methylases that can be considered promising therapeutic targets for the epigenetic treatment of cancer. DNMTis have attracted remarkable interest in modulating the improper DNA methylation pattern in a reversible route in recent years [57]. Genomic hypomethylation globally, characterised by the gradual and genome-wide depletion of 5-methyl-cytosine in cancer cells, is observed even in the early stages of CRC development and progression [58]. The process of genome hypomethylation is conducted through DNMTis, which is applied as an anticancer treatment and clarifies the role of DNA methylation in multiple procedures, such as X-chromosome inactivation or DNA imprinting [58].

DNMTis are potent anticancer therapeutics to reverse the DNA hypermethylation status of tumour suppressor genes (TSGs) [51]. DNMT inhibitors are classified into nucleoside and non-nucleoside analogues, as outlined in Fig. 2**.** Nucleoside analogues include cytidine and S-adenosyl-L-homocysteine (SAH) derivatives, while non-nucleoside inhibitors include small molecules and natural product [58, 59] (Fig. 2).

Nucleotide analogue inhibitors

The cytidine analogues are the currently most advanced drugs for epigenetic cancer therapies, which can be incorporated into the DNA or RNA backbone to replace C-5 of cytosine with N-5 and disturb the methylation, as well as form covalent bonds with DNMTs that block

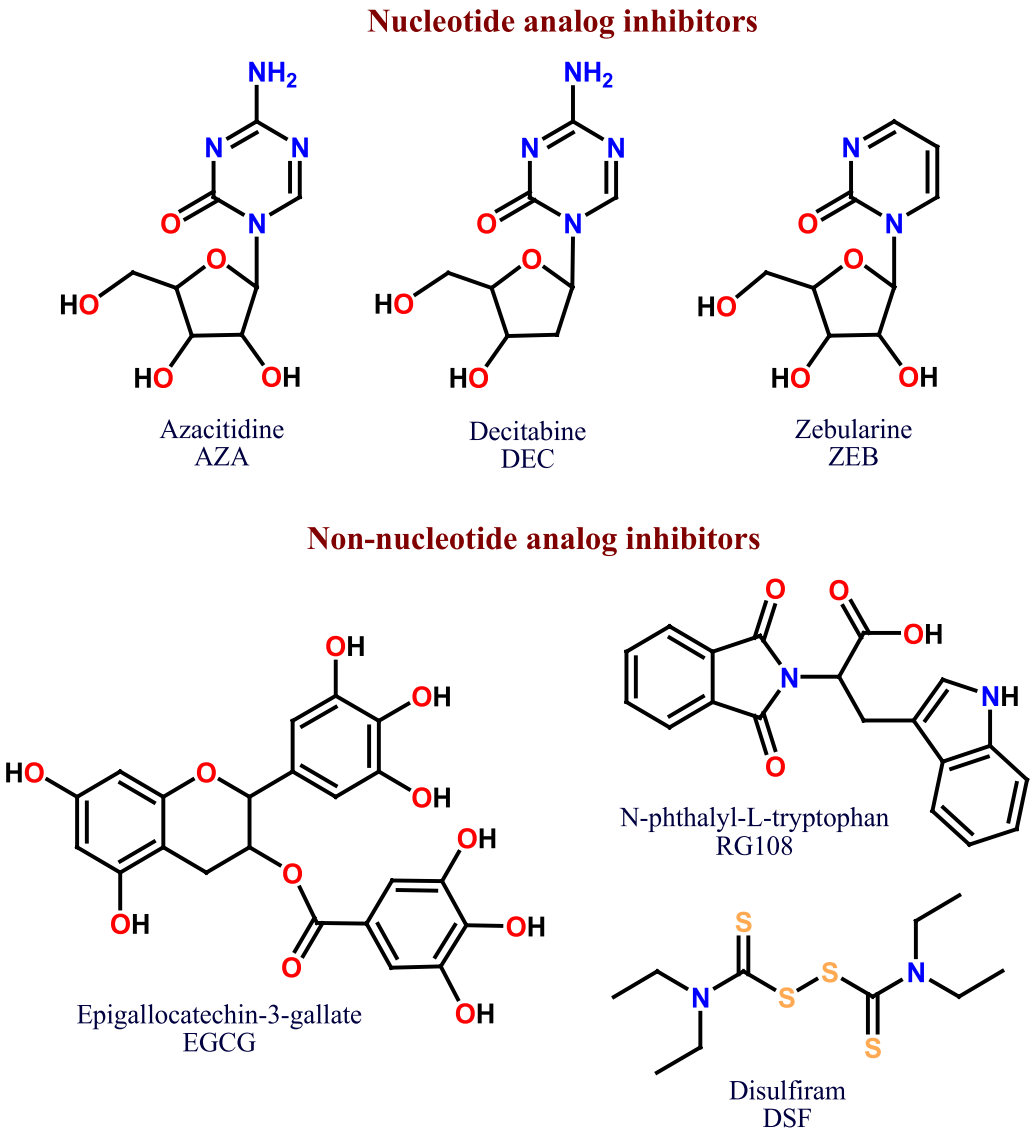

Fig. 2 Chemical structure of DNA methyltransferase inhibitors, designed by chemsketch freeware

their enzymatic activities [60]. Decitabine (DEC) and azacytidine (AZA) are classified as DNMTi drugs, and various evaluations assess their efficiencies in the treatment of multiple solid cancers, such as colorectal cancer (Fig. 2). Both are the most widely used as inhibitors of DNA methylation, which triggers demethylation, leading to consecutive reactivation of epigenetically silenced tumour suppressor genes in vitro and in vivo [61].

DEC is a deoxyribonucleoside, while AZA is a ribonucleoside. Both have the depletion activity of DNA methyltransferases with different mechanisms of action. AZA (~90%) is leading to unusual ribosome assembly and inhibiting tumour-related protein synthesis by its integration into RNA; ribonucleotide reductase can also transform AZA (~20%) to 5-aza-2'-deoxycytidine to inhibit DNA methyltransferase and lead to the re-expression of TSGs. While DEC is incorporated into DNA, high-dose DEC inhibits DNA synthesis and cross-linking by cytotoxicity, and low-dose DEC inhibits DNA methyltransferase and reactive silent tumour suppressor genes [62].

DEC, with trade name Dacogen (DAC), engages the DNMTs by binding to it irreversibly through a covalent bond and inhibiting the methylation of a daughter strand during the replication [63]. DAC remodels the tumour microenvironment to improve the effect of PD-L1 immunotherapy due to directly enhancing tumour programmed cell death-1 (PD-L1) expression

and eliciting stronger anticancer immune responses, which can accomplish potential clinical benefits to CRC patients [64]. The main side effects of DEC are neutropenia, nausea, and fatigue in solid tumours [65]. In CRC, DEC has been used alone or in combination with other drugs. Low expression of NALP1 (NLRP1 encodes NACHT, LRR, FIIND, CARD domain, and PYD domains-containing protein 1) is associated with survival and tumour metastasis in colon cancer. DEC treatment increases the expression of NALP1, suppressing colon cancer growth [66]. It has also been used in combination with other drugs like gefitinib and oxaliplatin as the effective treatment approach for CRC [67, 68].

Azacitidine, a 5-azacitidine analogue marketed as Vidaza, has a structure similar to that of decitabine. It acts as a DNMT inhibitor by modifying the carbon 5 of the pyrimidine ring [69]. AZA works in a dose-dependent manner. Low doses, it causes DNA hypomethylation by inhibiting DNMT through covalent binding. Conversely, at high doses, it functions as a cytotoxic agent by incorporating into DNA and RNA of abnormal cells, leading to cell death [45, 69]. In dose-limiting toxicities, AZA's general side effects include thrombocytopenia, febrile neutropenia, and fever in advanced malignancies [70].

Necdin (NDN) is a member of the melanoma-associated antigen (MAGE) protein family; NDN affects tumour cell proliferation by inhibiting the expression of LRP6, which is a key factor in the activation of the Wnt signalling pathway in CRC [71]. Reduced expression of NDN is linked to poor differentiation, advanced TNM stage, and poor CRC prognosis. Administration of azacytidine results in hypomethylation of the NDN promoter. Therefore, increased expression of NDN causes it to bind to the LRP6 promoter, leading to reduced transcription and inhibition of the Wnt signalling pathway in CRC [72]. Decitabine, a DNA demethylation agent, increased NDN expression in the CRC cell line and decreased the Wnt signalling pathway. Thus, the hypermethylation of the NDN promoter leads to NDN gene silencing [61].

Zebularine (ZEB), a 1-beta-D-ribofuranosyl-2(1H)-pyrimidinone, is a cytosine analogue similar to AZA. It inhibits DNA methylation by being incorporated into DNA, making it an attractive option for suppressing rapidly dividing cancer cells [73]. ZEB forms a covalent bond with DNMTs when acting as a substrate DNA and becomes entrapped in the complex. In the case of CRC, ZEB has been shown to induce p53-dependent ER stress and autophagy while inhibiting tumorigenesis and stemness [74]. Zebularine also operates at the mRNA level, increasing the expression of let-7b, a tumour suppressor microRNA that suppresses the invasion activity of CRC cells [75]. These findings suggest that ZEB may inhibit invasion activity by upregulating the intracellular expression of let-7b in highly invasive CRC cells [75].

Although nucleotide analogue inhibitors are effective in treating solid tumours, the formation of irreversible covalent adducts with DNA that is caused by DNA mutagenesis and conclusively a potential cause of tumour recurrence is from their long-term side effects [76]. On the other hand, the side effect caused by DNMT inhibitors can limit their application in cancer treatment [77].

Non-nucleotide analogue inhibitors

This class of DNMTis are small molecules that bind to the CpG-rich sequences or bind to the catalytic site of DNMTs, preventing the binding of DNMTs to the target sequences [78]. These epigenetic drugs, with a minor inhibitory effect on various aggressive tumour cells, present weaker anticancer activity than cytosine analogue inhibitors. Non-nucleoside DNMTis do not incorporate into DNA and thus might exhibit less cytotoxicity than cytidine analogues. Among them can mention to hydralazine, epigallocatechin-3-gallate (EGCG), N-phthalyl-L-tryptophan (RG108), a 20-base pair antisense oligonucleotide (MG98), and disulfiram (DSF) [79–81]. DSF, a bis-diethylthiocarbamoyl disulfide, is an irreversible inhibitor of aldehyde dehydrogenase (ALDH), which is responsible for ethanol metabolism and is applied as an Antabuse. DSF/Cu exerts anti-colorectal cancer by enhancing the molecule expression of cell immunogenic cancer cell death (ICD). DSF can be considered a safe and effective candidate for CRC prevention and therapy [82].

Histone deacetylase inhibitors (HDACis)

HDACis are epigenetic compounds recently considered for their promising anti-tumour activity. Histone acetylation/deacetylation is another epigenetic process for transcriptional regulation that can lead to alterations in tumour suppressor genes or oncogene [83]. Typically, there is a proper balance of histone acetyl-transferases (HATs) and Histone deacetylases (HDACs) that mediate histone acetylation and deacetylation; however, this balance can be disrupted by various diseases such as cancer [84].

HDAC inhibitors are categorised clinically based on their chemical structure into hydroxamic acids, cyclic peptides, short-chain fatty acids, and benzamides [85]. Hydroxamic acids include vorinostat (VRS), resminostat (RES), belinostat (BEL), panobinostat (PAN), trichostatin A (TSA), givinostat (ITF2357), and quisinostat (QST) [86]. Cyclic peptides are a group of potent HDAC inhibitors; romidepsin, a cyclic tetrapeptide, is known as this group's most potent HDAC inhibitor [87]. Valproic acid, a branched short-chain fatty acid, causes the specific degradation of HDAC2 [91, 92].

Benzamides like mocetinostat and entinostat are generally effective against class I HDACs [88]. Some HDACis, which can indifferently inhibit all HDAC isoforms, are called pan-inhibitors, such as VRS and PAN. Others can specifically inhibit certain HDACs and are considered HDAC class- or isoform-selective inhibitors (for instance, entinostat and rocilinostat) [89] (Fig. 3).

Several clinical trials have demonstrated numerous side effects of HDACis, including diarrhoea, fatigue, taste disturbances, weight loss, hematologic toxicity, disordered clotting, electrolyte changes, cardiac arrhythmias, and myelosuppression [90–92]. Before initiating treatment, the selectivity of HDAC inhibitors should be carefully deliberated to achieve the expected therapeutic effects with low toxicity on malignant tumours, including CRC.

Hydroxamic acids HDACis

VRS, also known as suberoylanilide hydroxamic acid (SAHA), is an orally bioavailable broad-spectrum HDAC inhibitor that commonly targets HDAC class I and II without inhibiting HDAC class III enzymes [93]. SAHA, a second-generation HDAC inhibitor, was approved by the US FDA for the treatment of cutaneous T cell lymphoma (CTCL) [94]. As mentioned above, the activity of HDAC is known to be involved in CRC progression, making it a potential target for CRC treatment. SAHA can disrupt the interaction between FLIP, an apoptosis inhibitor, by blocking caspase 8 activation and the DNA repair protein Ku70, which regulates FLIP protein stability. SAHA induces apoptosis in CRC by enhancing the acetylation of Ku70, ultimately disrupting the FLIP/Ku70 complex and triggering FLIP polyubiquitination and degradation by the proteasome [95].

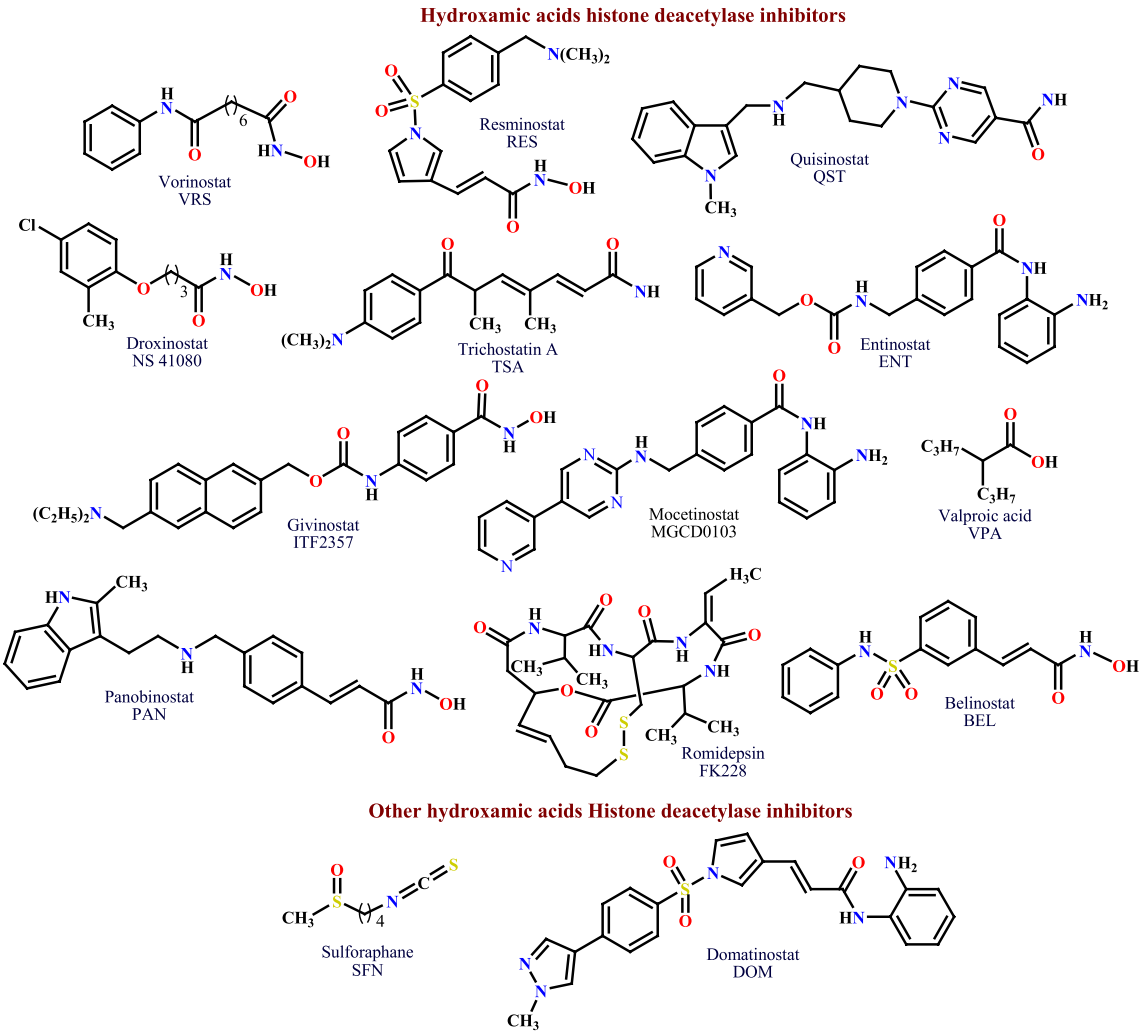

Fig. 3 Chemical structure of histone deacetylase inhibitors, designed by chemsketch freeware

RES is a novel HDACi that inhibits classes I, IIb, and IV of HDACs. Resminostat reduces the levels of cyclin cdk4, cdc25a, D1, and pRb and, by upregulating p21, also prevents cell proliferation and stops the cell cycle in G0/G1. RES can be effective in treating CRC by affecting the AKT signalling pathway and inhibiting proliferation, migration and apoptosis [45]. The anticancer activity of RES on advanced CRC is still being investigated in clinical trials.

BEL is a pan-HDAC inhibitor comprising a sulphonamide-based hydroxamate structure that inhibits class I, II, and IV HDAC isoforms with nanomolar potency [96, 97]. Importantly, it has tolerable side effects with infrequent toxicity when compared to other HDACis. Combination therapy BEL with fluorouracil showed an inhibitory effect on the growth of colon cancer cells in vivo and in vitro [98]. Co-administration of 5-FU with HDACIs exerts a synergistic effect in various types of cancer, especially colorectal cancer [99]. BEL can increase the expression of the tumour suppressor gene TGFβRII in colon cancer and repress the expression of survivin, a cancer-associated gene, through the TGFβ/protein kinase A (PKA) pathway and leads to cancer cell death and reduced metastasis [100].

PAN, a nonselective HDACi, is another member of the hydroxamic acid class which is effective against all the classes of HDACs, including class I, II, and IV, and causes cell cycle arrest and apoptosis, leading to it being an antineoplastic drug [101]. PAN affects both histone (H3 and H4) and non-histone proteins (HIF-1α, β-catenin, α-tubulin, chaperons (HSP90), estrogen receptor (ERα), androgen receptor (AR), DNA repair proteins (Ku70), and…) leading to transformations in some transcription factors including NF-κB, E2F, p53, and c-Myc [102, 103]. In CRC, the tumour suppressor gene death-associated protein kinase (DAPK) was activated by PAN [104]. DAPK plays a key role in the induction of autophagy and apoptosis.

TSA, a pan-HDAC inhibitor that inhibits HDACs in a non-competitive and reversible way and is structurally related to SAHA, could induce suppressors of cytokine signalling genes (SOCS1 and SOCS3) expression by inducing histone modifications and consequently inhibit JAK2/STAT3 signalling in CRC cells [105]. TSA could also induce G2/M cell cycle arrest and Bax-dependent apoptosis in wild-type and mutant p53 colorectal cancer cell lines by both p53-dependent and -independent mechanisms [106]. In addition, TSA induced cell death by arresting the cell cycle in the G2/M phase, which was dependent on the production of mitochondria-mediated ROS derived from reduced mitochondrial respiratory chain activity [106]. A study showed the potent inhibitory activity of VRS and TSA against HDACs by their chelation with the zinc atom within the catalytic pocket of HDAC8 was related to the hydroxamic acid moiety of drugs with [107].

QST, an orally and potent pan-HDACi with a hydroxamate structure, has broad activity in solid tumour models [108, 109]. This drug could reduce the migration rate of colon cancer cells (> 50%) and prevent metastasis and spread of cancer in the long term by chromatin compression and subsequent suppressed effective genes in the expression of essential proteins for cell migration. [108]. QST inhibits class I and II HDACs and also presents continuous H3 acetylation, and inhibits tumour progression in CRC in preclinical and clinical studies [109, 110].

Droxinostat (NS 41080, inhibitor of HDAC3, 6, and 8) potently increased the acetylation of Ku70 by inhibiting a key enzyme of HDAC6 and caused rapid FLIP protein downregulation. The Ku70/FLIP interaction disruption subsequently led to FLIP degradation by the ubiquitin–proteasome system and induction of caspase 8-dependant apoptosis in droxinostat-induced apoptosis [111]. Based on these results, NS 41080 could be introduced as an efficient post-transcriptional suppressor of FLIP expression in CRC. On the other hand, cancer cells are very sensitive to oxidative stress [112]. Anticancer activity of NS 41080 on CRC was also mediated by the induction of oxidative stress and contributed to cellular apoptosis [113].

Romidepsin (FK228), a selective inhibitor of HDACs 1 and 2, inhibits proliferation, induces G0/G1 cell cycle arrest and increases apoptosis in various solid tumour cells [114–116]. FK228 upregulated costimulatory molecules (PD-L1) in colon cancer cells and suppressed cellular immune functions, including the decreased ratio of Th1/Th2 cells and the percentage of IFN-γ + CD8 + T cells in the peripheral blood and the tumour microenvironment [116].

Givinostat is a hydroxamate HDAC class I and class II inhibitor. Its anti-tumour effect in colon cancer cells that are characterised by oncogenic BRAF mutations has been confirmed [117]. Furthermore, the combination of givinostat with other epigenetic drugs, including DNMTis, indicated that this drug amplifies the effects of both general and selective DNMTis on colon cancer cells [118].

Other HDACis

Sulforaphane (SFN), a phytochemical compound known in some green leafy vegetables, is found to be effective in preventing and treating various cancers, including colon cancer [119]. SFN (1-isothiocyanate-4-(methylsulfonyl) butane) is known to act on the epigenetic regulation of gene expression by suppressing HDACs [120, 121]. Treatment of colon cancer xenograft mice with SFN (10 μmol/

mice) presented its activity in suppressing the cancer cell growth through increased histone acetylation [121].

SFN treatment showed decreasing cell density, significantly inhibiting cell viability and inducing apoptosis in CRC cells. SFN significantly down-regulated oncogenic miR-21, HDAC and hTERT mRNA, protein and enzymatic levels in CRC cells. Indeed, HDACis may play a vital role in regulating microRNAs (miRs) and human telomerase reverse transcriptase (hTERT). SNF can delay and/or prevent CRC.

SFN treatment also decreased cell density, remarkably inhibited cell viability, and induced apoptosis in CRC cells [122].SFN also down-regulated oncogenic microRNA (miRNA-21), HDAC, and telomerase reverse transcriptase (hTERT) mRNA, proteins, and enzymatic levels in CRC cells [122]. In this way, SNF can delay and/or prevent CRC. hTERT plays a vital role in cancer progression using to promote invasion and migration of CRC through hTERT-β-catenin/TCF-4-CCL2 signalling pathway [123]. Notably, miRNA-21 regulates hTERT expression and plays a key role in the development and progression of CRC [124].

Domatinostat (DOM) is a selective inhibitor of HDACs, HDAC1, 2, and 3, which are applied for the treatment of various types of cancer and probably play important roles in regulating improper cancer signalling [125, 126]. DOM potently inhibits survival, proliferation, and cell cycle progression and also activates apoptosis in CRC cells [127, 128]. DOM is very effective against HDACs because colon epithelial cells with low HDAC1/2 expression were least affected by DOM treatment. Fortunately, monotherapy of HDAC inhibitors is broadly used against solid tumours despite their modest efficacy for sufficient tolerance and efficient clinical functions [129]. Multiple clinical trials are underway to evaluate their therapeutic potential in co-combination with other anticancer agents.

Other epigenetic drugs for CRC treatment

The best epigenetic drug classes in CRC therapy are HDACis and DNMTis. Although recently, some new epigenetic therapeutic targets have been applied for the treatment of CRC, such as antagomirs, bromodomain (BRD), and extra-terminal domain (BET) protein family [130–133]. Antagomirs inhibit a specific miRNA and change its function. For instance, antagomirs can target miR-21, downregulating tumour suppressor genes in CRC. In 2014, song and coworkers designed an antagomir complementary to the miR-21 sequence. This antagomir was specifically engineered to inhibit angiogenesis and proliferation in CRC effectively [131]. On the other hand, Nedaeinia et al. confirmed the therapeutic potential of LNA-anti-miR-21 in CRC for targeting miR-21 expression [134]. Locked nucleic acid (LNA), a modified RNA nucleotide, can also target miRNAs. These nucleic-acid-based approaches for gene silencing are stable, safe, and non-toxic [120].

BET proteins, a protein family known to be overexpressed in multiple tumour types, can regulate various cellular functions and play a key role in oncogene expression. BET, the best-characterised class of acetylation readers, can promote the transcription of target genes by binding to acetylation motifs present in histones and accumulating on hyper-acetylated chromatin regions. The potential of BET proteins as targets for anticancer strategies among CRC, similar to HDAC proteins, is a source of inspiration and motivation in the fight against CRC. BET inhibitors can suppress the effect of these promoters and affect their transcriptional activity. BRD4, one of the BET proteins, is extremely expressed in colorectal cancer tissue samples, and BRD4 inhibitors led to reduced CRC proliferation [132].

Natural epigenetic products in CRC treatment

Natural products have yet to be important in treating various diseases worldwide. Epigallocatechin-3-gallate (EGCG), a major polyphenol component in green tea, reversibly inhibits DNMT, resulting in the reactivation of various key genes, including P16, hMLH1, and RA, in different cancer cell lines, including colon [135] (Fig. 4). EGCG is an epigenetic modulator for cancer chemoprevention and treatment effects on many kinds of tumours [136]. Previously, topoisomerase I (TOPI) inhibitory effect of EGCG had also been reported in CRC [137].

Psammaplin A (PSA) is a group of natural products isolated from marine sponges and is capable of inhibiting both DNMTs and HDACs with minimum cytotoxicity [138, 139]. However, Godert and colleagues reported that PSA was not a potent DNMT inhibitor in vivo because it did not cause any changes in the level of genomic DNA methylation in treated human CRC cells [140]. Recent studies reported antiproliferative activities of PSA against several human cancer cell lines, especially colon cancer cell lines [141, 142]. Furthermore, the anticancer activity of PSA was related to other enzyme inhibition, such as aminopeptidase, topoisomerase II, farnesyl protein transferase, leucine aminopeptidase, and DNA polymerase α-primase that are at least responsible in tumour cell proliferation, intracellular signal transduction, invasion, and angiogenesis [141].

Overview of co-combination therapies and preclinical / clinical studies on epigenetic drugs in CRC and metastatic CRC

To a certain extent, dynamic and reversible epigenetic abnormalities can lead to the continuous evolution of cancer cells. These reversible changes in epigenetic signs

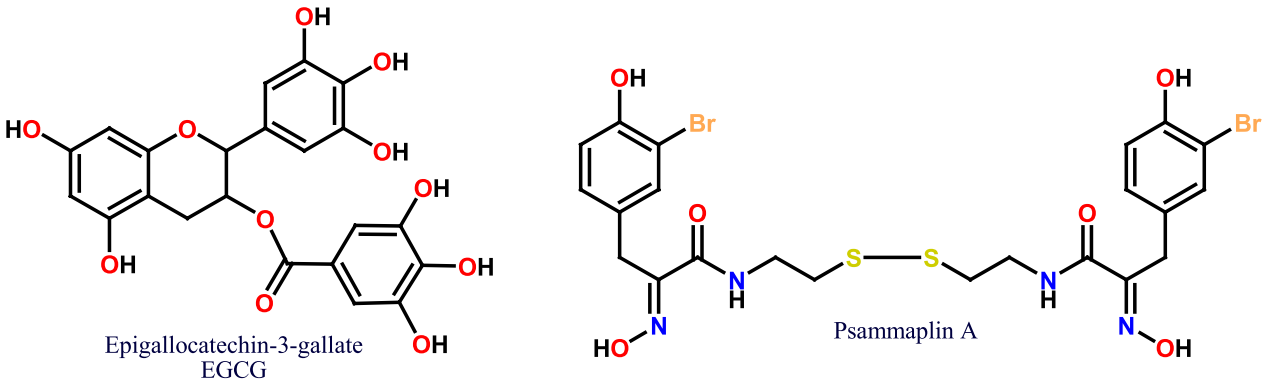

Fig. 4 Chemical structure of natural epigenetic products, designed by chemsketch freeware

on DNA, histone, and non-histone proteins, as well as the functional efficacies of these alterations, are implicated in primary and acquired resistance to various anticancer medications by modulation of tumour cells or their microenvironment [143, 144]. As tumours progress, epigenetic changes and genetic mutations increase. For this reason, if epigenetic modifiers are used alone in the final stages of CRC, they will be inefficient. It is also likely to require long-term treatment because cell reprogramming takes time and is probably not sustainable after stopping treatment [90]. (Table 1).

The potential of combining epigenetic drugs and chemotherapeutic drugs or immunotherapy significantly enhances therapeutic effects and reduces drug resistance (Tables 2 and 3). Mechanically, epidrugs like DNMTis and HDACis can increase chromatin accessibility to chemotherapeutic agents through chromatin decomposition, amplifying their anticancer effects [145]. Among the benefits of such co-combinations, the results of specific combinations stand out, offering a ray of hope in the fight against solid tumours. Epigenetic drugs have shown promising synergistic effects with other anticancer therapies, increasing antitumour effects, sensitivity to chemotherapy agents, and inducing apoptosis in solid tumours. These results inspire confidence in the potential of combined therapies for cancer treatment [90]. Co-combination of vorinostat with panobinostat against cancerous cells in colon adenocarcinoma-induced immunogenic cell death (ICD) [146]. The combination of sodium butyrate (SB), an HDAC inhibitor, and 5-AZA-2'-deoxycytidine (5-AZA-DC), a demethylating agent, along with radiation, showed lower survival in 5-aza-DC or SB than radiation alone in colon cancer cell line [147]. Indeed, a

Table 1 Epidrug classifications based on their mechanisms

Targeting epigenetic modification	Drugs	Signalling pathway in colorectal	Ref
DNMTis	Disulfiram	Blocking the enzyme acetaldehyde dehydrogenase, targeting drug efflux pumps, induction of production of ROSs, activating the JNK and p38 MAPK signalling pathways, inhibition of NFκB and the proteasome activity	[193]
	Zebularine	Downregulation of GRP78 and p62 and upregulates a pro-apoptotic CHOP	[74]
	Decitabine	PD-1 blockade	[194]
	Azacitidine	At low doses, azacytidine targets CRC-initiating cells by inducing viral mimicry via the MDA5/MAVS/IRF7 pathway	[72]
	Zebularine	Induces p53-dependent ER stress and autophagy. Increases the expression level of let-7b, which functions as tumour suppressor microRNA	[195]
HDACIs	Sulforaphane	Blocks Wnt/β-catenin signalling	[196]
	Vorinostat	Autophagy modulation	[197]
	Resminostat	Amplification of AKT signalling	[198]
	Belinostat	Induction of apoptosis	[199]
	Panobinostat	Arresting the G1, G2/M cell cycle, activation of tumour suppressor gene death-associated protein kinase (DAPK), induction of autophagy, apoptosis and cell death	[200, 201]

HDACIs: Histone Deacetylases Inhibitors; DNMTis: DNA Methyltransferases Inhibitor

Table 2 Epigenetic drugs in phases of clinical trials

Epigenetic class drug	Drug	Phases of Clinical Trials	Outcome	Toxicity	Ref
HDACIs	Vorinostat	Phase I	Once-daily MTD vorinostat 600 did not significantly alter the PK of vorinostat	nausea, anaemia, fatigue, diarrhoea, weight loss, and elevated creatinine	[202]
	Panobinostat	phases I–II	Co-administration of panobinostat with CYP3A inhibitors is feasible	Diarrhea, vomiting, nausea, hypophosphatemia, myalgia, fatigue, and anorexia	[203]
	Carbamazepine	phase II	Administration of carbamazepine did not reduce oxaliplatin-induced neuropathy	Nausea, dizziness, memory disorders, problems, headaches, vision problems	[204]
	Resveratrol	Phase I	It is worth investigating further clinical studies to replace non-steroidal anti-inflammatory agents and selective COX inhibitors	No side effects	[205]
	Bevacizumab	Phase I	Stability (more than 3 months) was seen in four patients	Grade 3 fatigue, grade 3 myalgia, and elevated ALT	[206]
	Romidepsin	Phase II	Administration of 13 mg/m^2 on days 1, 8 and 15 in cycle 28 days was not successful	Thrombocytopenia, changes in ECG, fatigue, weight loss, nausea, vomiting, anorexia, fever, and weakness	[207]
	Entinostat	Phase I	Half-life of 39 to 80 h. MTD was 10 mg/m^2	Anorexia, fatigue, nausea, and vomiting	[208]
	Epsipeptide	Phase II	Epsipeptide 13 mg/m^2 administration is ineffective in CRC patients with previous chemotherapy	Thrombocytopenia, electrocardiographic, fatigue, nausea and vomiting, weakness, anorexia, fever, and weight loss	[209]
DNMTi	5-Azacitidine	phase II	An increase in the regulation of AZA immune gene set	–	[210]

combination of 5-aza-DC and SB could enhance radiosensitivity in the RKO cell line.

DSF, in combination with 5-fluorouracil (5-FU), is used as the significant chemotherapeutic component for CRC [148]. It imparts chemosensitisation, significantly enhances the apoptotic effect, and synergistically potentiates the toxic effect of 5-FU on CRC cell lines [45].

CRC cell lines treated with PAN confirmed the alteration of genes responsible for the process of angiogenesis, mitosis, DNA replication, and apoptosis [149]. Co-administration of PAN with lapatinib (LAP, EGFR/HER2 kinase inhibitor) showed synergistic effect and inhibited the proliferation and colony formation in all CRC cell lines tested with varying expression and KRAS/BRAF/PIK3CA mutations [150]. Caspase-8 activation, increased DNA double-strand breaks, and PARP cleavage, combined with downregulation of transcriptional targets, including IRAK1, NF-κB1, and CCND1, led to rapid apoptosis induction by the combination drug PAN-LAP.

Combining FK228 with an anti-PD-L1 antibody can amplify the anti-tumour effects and provide a more potent treatment for colon cancer. This finding underscores the potential of our research to contribute to developing novel and more effective cancer treatments. [116].Reversing the influence of FK228 on immune cells affected the considerable anti-tumour effect of this co-combination. Romidepsin also inhibited the catalytic activity of HDAC8 by perturbing its coordination with the zinc atom [151].

VRS was the first of the class of HDAC inhibitors to be FDA-approved and evaluated via phase clinical trials in advanced and chemotherapy-resistant CRC, especially in combination with 5-FU [152]. VRS showed a synergistic effect with 5-FU and oxaliplatin in inducing apoptosis in CRC cells; however, this synergy was abolished in cells overexpressing FLIP(L) [141]. VRS, in combination with decitabine, could also increase the sensitivity of Fas ligand (FasL)-induced apoptosis and CTL immunotherapy by promoting CD8 + T cells in colon cancer cells [153, 154]. The combination of EGCG with irinotecan not only had a stronger inhibitory effect on tumour cells than irinotecan or EGCG alone but also prevented tumour cell migration and invasion in CRC cells [155].

In the combination of the tolerated dose of 5-AZA (75 mg/m^2) with valproic acid, stable disease was observed in patients with colorectal cancer (median = 6 months) [147], a significant decrease in global DNA methylation was also observed. EGCG and irinotecan (anti-tumour agent with low solubility and high toxicity) synergistically inhibited the migration, invasion, and proliferation of colorectal cancer cells [156]. EGCG alone did not cause DNA damage, but in co-combination with irinotecan, it could induce S or G2 phase arrest by inhibiting TOPI to cause more extensive

Table 3 Co-combination of anticancer drugs with epigenetic drugs in clinical phase studies

Co-combination of anticancer drug with epigenetic drug	anticancer class drug/ Epigenetic class drug	Phases of Clinical Trials	Outcome	Toxicity	Ref
Erlotinib/5-AZAcitidine	Tyrosine kinase inhibitor/DNMT1 inhibitor	Phase I in solid tumour	1 Patient with mCRC had PFS of 2 months	–	[161]
Valproic acid/ Bevacizumab		Phase I	Being safe for up to 6 months and creating a stable condition of the disease	Proteinuria, increased blood pressure and mental problems	[158]
Capecitabine/ Oxaliplatin /5-AZAcitidine	Thymidylate synthase inhibitor / Thymidylate synthase inhibitor / DNMT1inhibitor	Phase I/II in mCRC	–	No dose-limiting toxicities were observed for 26 patients	[211, 212]
Carboplatin/Decitabine	Alkylating agents /DNMT1 inhibitor	Preclinical and Phase I in solid tumours, including mCRC	7 Patients with mCRC who experienced PD	The main toxicity is related to myelosuppression. Dose-limiting toxicities include grade 4 long-term neutropenia, sepsis, grade 3 anorexia, and fatigue	[213]
Gefitinib/Decitabine	Tyrosine kinase inhibitor /DNMT1 inhibitor	Preclinical and Phase I in solid tumours, including mCRC	was synergistic at inducing apoptosis in colon cancer cells	Minimal toxicity to NCM460 cells	[67]
Capecitabine/ Azacitidine/Oxaliplatin	Inhibitor of DNA synthesis /DNMT1 inhibitor/	Phase I/II in mCRC	26 Patients (SD in 17 patients, median duration of 4.5 months)	No dose-limiting toxicities were observed	[214]
Panitumumab/Decitabine	Monoclonal antibody /DNMT1 inhibitor	Preclinical and Phase I in solid tumours, including mCRC	20 Patients (two had a partial response). 10 patients had stable disease (3 of them longer than 16 weeks)	Grade 1–2 (rash and hypomagnesemia) /neutropenia /neutropenic fever	[157]
Tetra-hydrouridine(THU)/5-fluoro-2-deoxycytidine	Cytidine deaminase /DNMT1 inhibitor	Phase I in solid tumours, including mCRC	35% of Patients treated with different types of solid tumours had SD	–	[161]
Irinotecan/Guadecitabine:DNMTs	Topoisomerase I inhibitor /DNMT1 inhibitor	Ongoing Phase II in mCRC	At a median follow-up of 20 months, the median OS for the 22 patients in the study is 10.7 months	Neutropenic fever / biliary drain infection / colonic obstruction / severe dehydration / Most common toxicities were neutropenia and leukopenia	[215]
Irinotecan/Disulfiram/ copper	Topoisomerase I inhibitor /DNMT1 inhibitor	Phase II in mCRC	–	severe fatigue/ headache/ cerebral confusions	[193]
Regorafenib and Hydroxy Chloroquine/ Entinostat	Kinase Inhibitor /antimalarials / HDAC inhibitors	Phase I/II in mCRC	Ongoing	Ongoing	[161]
Radiation therapy w/wo Capecitabine/Valproic Acid	Fluoropyrimidine carbamate/ anticonvulsants	Phase I /II (preoperative setting in rectal cancer)	Ongoing	Ongoing	[161]
Hydroxy Chloroquine/ Vorinostat	Antimalarials/ HDACs	Phase I/II in mCRC	Ongoing	Ongoing	[161]
Capecitabine/CI-994	Anti-metabolite /HDAC inhibitors	Phase I in advanced solid tumours, including mCRC	24 Patients (1 patient had PR and 1 had SD)	The main dose-limiting toxicity is thrombocytopenia	[216]
13-cis retinoic acid/Entinostat	Agonist at retinoic acid receptors (RARs) and retinoic X receptors (RXRs) /HDAC inhibitors	Phase I in advanced solid tumours, including mCRC	Only one patient with mCRC who experienced PD	haematological and Skin toxicities	[217]

Table 3 (continued)

Co-combination of anticancer drug with epigenetic drug	anticancer class drug/ Epigenetic class drug	Phases of Clinical Trials	Outcome	Toxicity	Ref
Sorafenib/Entinostat	Tyrosine kinase inhibitors /HDAC inhibitors	Phase I in advanced solid tumours, including mCRC	5 Out of 10 patients with mCRC had SD	grade 3–4 toxicities were muscle weakness (13%), skin rash (10%), fatigue (6%), diarrhoea (6%), and hand-foot syndrome (3%)	[218]
Bevacizumab/Panobinostat	Angiogenesis inhibitors /HDAC inhibitors	Phase I in advanced solid tumours, including mCRC	9 Patients with mCRC, 3 had SD	–	[219]
Lapatinib/Panobinostat	GFR/HER2 kinase inhibitor/HDAC inhibitors	Colorectal Cancer Models	–	–	[150]
5-FU/Phenylbutyrate	Inhibition of thymidylate synthase / HDAC inhibitors	Phase I in mCRC	9 patients with mCRC (4 patients evaluable, 3 SD lasting 12, 25 and 54 weeks, respectively)	Fatigue, confusion, hearing loss, triglyceridemia, and hyperuricema	[220]
Modified FOLFOX6 or Bortezomib/ Vorinostat	Proteasome inhibitors/HDAC inhibitors	PhaseI/II in solid tumours, including mCRC	23% of 21 patients had SD	Thrombocytopenia, gastrointestinal toxicities, neutropenia, and fatigue increased at higher doses	[221]
Pazopanib/ Vorinostat	VEGFR-PDGFR inhibitor /HDAC inhibitors	PhaseI/II in solid tumours, including mCRC	Long PFS and OS in patients, especially in patients with mutation TP53	-	[222, 223]
FOLFOX/ Vorinostat	HDAC inhibitors	PhaseI in solid tumours, including mCRC	–	Thrombocytopenia, neutropenia, GIT toxicities, Fatigue	[221]
Doxorubicin/ Vorinostat	Anthracycline group of chemotherapeutic agents (Antineoplastics)/ HDAC inhibitors	PhaseI/II in solid tumours, including mCRC	–	Venous thromboembolism and haematological	[224]
Hydroxychloroquine or Regorafenib/ Vorinostat: HDAC,HDAC1,HDAC3	/Multi-targeting kinase inhibitor / HDAC inhibitors	PhaseI/II in solid tumours, including mCRC	Median PFS (1.8 months) and OS (5.2 months)	grade 3 fatigue/ rapid weight loss	[225]
Oxaliplatin/ACY-1215:HDAC6	Alkylating agent /HDAC inhibitors	Preclinical in CRC cells and mouse xenografts	Improve overall survival	It had profound side effects, including fatigue, nausea, vomiting, diarrhoea, thrombocytopenia, and neutropenia	[222]
Oxaliplatin/ 5-FU Irinotecan/ CG2:HDAC	Alkylating agent/ alkylating agent/ DNA topoisomerase I inhibitor / HDAC inhibitors	Preclinical in CRC cells and mouse xenografts	CG2, in combination with irinotecan, shows promising anti-tumour effects both in vitro and in vivo	In vitro, results showed increased cytotoxicity when CG2 was combined with oxaliplatin	[223]
Bortezomib /Vorinostat	Proteasome Inhibitors /HDAC inhibitors	Phase I	Potential clinical efficacy of the combination therapy with primary concern of late-cycle toxicities that precluded long-term administration	Thrombocytopenia, increased alanine transaminase (ALT) and fatigue	[225]
Bevacizumab /Valproic Acid	Kinase Inhibitors/ HDAC inhibitors	Phase I	–	Grade 3 altered mental status/ Grade 3 proteinuria/Grade 3 hypertension	[158]

Table 3 (continued)

Co-combination of anticancer drug with epigenetic drug	anticancer class drug/ Epigenetic class drug	Phases of Clinical Trials	Outcome	Toxicity	Ref
5-fluorouracil (5-FU) /vorinostat	Inhibition of thymidylate synthase / HDAC inhibitors	phase I/II clinical trials in (mCRC)	10 Metastatic colorectal cancer patients who failed to all standard therapeutic options	Grade 3 and 4 toxicities were fatigue, mucositis, and thrombocytopenia	[226]

DNMT: DNA methyltransferase; mCRC: metastatic colorectal cancer; mPFS: median progression-free survival; OS: overall survival; PD: progression disease; SD: stable disease; PFS: progression-free survival

DNA damage. EGCG also elevated apoptosis synergistically by promoting autophagy with irinotecan.

In a study conducted by Azad et al. for combined treatment in CRC patients, they showed that the combined therapy of a demethylating agent such as [5] and an HDACi agent such as entinostat caused more DNA demethylation in people who had a higher than average (progression-free survival) PFS. In another study conducted by Garrido-Laguna et al. on mCRC cancer phase patients with KRAS mutations, they showed that the combination of decitabine and panitumumab is well tolerated. In this study, by prescribing decitabine 45 mg/m^2 on days 1 and 15 of the 28-day cycle along with the administration of panitumumab 6 mg/kg on days 8 and 22 of the 28-day cycle for 20 patients, the results indicate (10% PR(Partial response), 55% SD (Stable disease) 3 patients had SD lasting > 16 weeks) [157]. A preclinical study conducted by Yun-feng et al. showed that the combination of gefitinib and decitabine was effective in inhibiting cell proliferation, migration, and induction of apoptosis by reducing the expression of p-AKT, p-mTOR, and p-S6 and by inhibiting Bcl2. Indeed, it confers a pro-apoptotic phenotype to CRC cell lines.

The combination of lapatinib and Panobinostat can prevent proliferation and colony formation in all CRC cell lines. Panobinostat activates death-associated protein kinase (DAPK), which induces apoptosis and autophagy. By inhibiting EGFR/HER 2, lapatinib causes double-strand breaks and increases apoptosis, which causes PARP cleavage and reduction of transcriptional targets, such as IRAK 1, NF-κB1, and CCND1. This happens under the influence of caspase eight activations, and ultimately, MAPK and PI3K/AKT pathway signalling is reduced [150]. Wheler et al. showed that the combined treatment of valproic acid with a dose of 5.3 mg/kg and 11 mg/kg of bevacizumab causes 6-month disease stability in patients with advanced malignancies, including CRC [158].

Epigenetic drugs and cancer stem cells (CSCs)

CSCs are the main causes of cancers that lead to conventional treatment deficiency. They contain several pathways and molecular mechanisms that cause stemness and resistance against chemotherapy; however, they could be controlled through specific epigenetics-based agents. Table 1 provides a number of these agents applied in various therapeutic phases. It is found that transformations of particular miRNA expression can cause EMT, CSC phenotype, and resistance against chemotherapy. It is noteworthy that novel cancer treatment approaches are provided through the elimination of the resistance of CSC to chemotherapy. Due to the epigenetic nature of some tumour progressive aberrant signals, particular inhibitors promote the treatment efficacy. Finally, using epigenetic modifiers decreases CSC chemotherapy resistance by targeting their stemness-like traits.

Epigenetic manipulation to improve endogenous anti-tumour response

It has been found that epigenetic changes affecting tumour immunogenesis and immune cells, including lymphocytes and macrophages involved in anti-tumour responses, form the favourable tumour microenvironment (TME) conducive to tumour growth. Cancer cells escape from the immune system using different mechanisms; these mechanisms include loss of antigen processing and presentation machinery (APM), downregulation of tumour-associated antigens (TAAs), and expression of a tumour-promoting balance in costimulatory and co-inhibitory molecules. A new field of research has been formed in the field of cancer immunotherapy, which can affect the function of immune cells in the environment using molecules that inhibit epigenetic changes and thus play an important role in cancer treatment [159, 160].

Combination of epidrugs and vaccine for cancer treatment

Today, combination therapies emerging after epigenetic therapy include immune checkpoint blockade, vaccines, and other immunotherapeutic agents [161]. Immunotherapy encompasses diverse strategies, including monoclonal antibodies, checkpoint inhibitors, cancer vaccines, and cell-based therapies. Specific long non-coding RNAs (LncRNAs), such as Lnc-TIM-3 and LncSNHG1, play crucial roles in sustaining the expansion of exhausted T cells. Combining these LncRNAs with cell-mediated therapies like dendritic cell vaccines and adoptive T cell therapy aims to enhance treatment effectiveness.

Cancer immunotherapy can be divided into passive and active approaches. Passive immunotherapy involves administering monoclonal antibodies (mAbs), cytokines, and ex vivo "educated" immune cells. Active immunotherapy includes anticancer vaccines (such as peptide, dendritic cell-based, and allogeneic whole cell vaccines), immune checkpoint inhibitors, and oncolytic viruses. Researchers continue to explore innovative methods to boost anticancer immune responses further [162, 163].

Peptide vaccines can elicit effective anti-tumour T cell responses. Anticancer vaccines typically contain immunogenic epitopes derived from tumour-specific antigens (TSAs) or tumour-associated antigens (TAAs). However, initial clinical trials using TSA- or TAA-derived peptide vaccines as monotherapy demonstrated limited effectiveness due to the narrow range of immune responses induced in vivo and the constraints of MHC restriction. Subsequent approaches included single and multiple

peptide vaccines tailored to patients expressing specific MHC alleles, but their efficacy remained modest. The latest generation of anticancer peptide vaccines involves multi-peptide cocktails, synthetic long peptides, or hybrid peptides that incorporate both cytotoxic T lymphocyte (CTL) and helper T (Th) cell epitopes. These advanced vaccines are administered alongside other therapies and are employed in treating various cancer types, including colorectal, breast and prostate cancers [164].

Specifically, vaccines containing targeted epitopes have undergone phase I-III clinical trials for breast cancer and phase II trials in patients with metastatic castrate-resistant prostate cancer. It is important to note that, based on current knowledge, these specific epitope-based vaccines have been evaluated in these clinical settings [164, 165]. Also, recent studies have proved a synergistic anti-tumour activity in mouse MC38 and CT26 colorectal tumour models with concurrent, but not sequential, CTLA-4 and PD-1 blockade (Ipilimumab and Nivolumab) [166].

Dendritic cells

Dendritic cells (DCs) are a type of professional antigen-presenting cells (APCs) that release IL-12 by absorbing antigens, so they play a major role in starting the immune response [167]. Special AT-rich sequence-binding protein-1 (SATB1) is necessary for generating DCs. SATB1 uses chromatin remodelling complexes and binds these complexes to the AT-rich sequence, causing HAT or HDACs to be applied to gene promoter regions. Finally, changing the position of nucleosomes leads to histone modification, which affects the amount of gene transcription.

A large increase in SATB1 increases the secretion of cytokine IL-6. IL-6 is effective in converting anti-tumour DCs into pro-tumour DCs. SATB1 also increases the immunosuppressive factor Galectin 1. Because of this, SATB1 is overexpressed in a large number of tumours, including breast, lung, pancreatic, colorectal, liver, bladder, prostate, and ovarian cancer. Kruppel-like factor 4 (KLF4) modulates IL-6 production at the post-translational level by histone acetylation. KLF4 reduction has been seen in many cancers, including the oesophagus, lung, colon, colon, and prostate. Finally, understanding the mechanism and the role of epigenetics on the activity of DCs makes it possible to create an effective anti-tumour response with changes [159].

Empowering the potential of CAR-T cell immunotherapies by epigenetic reprogramming

Cancer stem cells (CSCs), a small fraction of malignancies, exhibit unique characteristics such as self-replication, tumourigenesis, and resistance to therapy. These CSCs manipulate immunological pathways to escape immune surveillance. Specifically, they express tumour-associated antigens (TAAs), secrete cytokines and anti-apoptotic molecules, and upregulate survival signalling pathways like STAT3 or PI3K/AKT. These immunomodulatory features allow CSCs to evade immune detection, contributing to tumour growth and treatment challenges [168]. A crucial feature of CSCs is their ability to evade immune system responses. Consequently, selective targeting of CSCs has become a critical focus in cancer research [169].

Abnormal epigenetic reprogramming contributes to the emergence and persistence of CSCs. Specific epigenetic alterations, such as changes in DNA methylation, histone-modifying enzymes, chromatin remodelers, and long non-coding RNAs (LncRNAs), play a pivotal role in initiating and sustaining the CSC compartment, ultimately driving tumourigenesis [170].

Chimeric antigen receptor (CAR)-T cell therapy, a highly promising treatment, has significant potential for addressing both haematological disorders and solid tumours. This approach involves modifying cancer patient T lymphocytes ex vivo to express a CAR specifically targeting tumour-associated antigens (TAAs), followed by reinfusion into the patients [171]. Combining CAR-T (chimeric antigen receptor T cell) therapy with epigenetic compounds has emerged as a promising strategy for pursuing more effective cancer therapies. This approach specifically targets a small subset of stem-like cells known as CSCs. These CSCs possess unique epitopes and epigenetic alterations distinguishing them from other cancer and normal cells. By harnessing CAR-T immunotherapy alongside epigenetic probes, we aim to overcome treatment barriers and achieve a more precise and personalised medicine approach for patients with specific CSC alterations [172].

Epidrugs targeting CSCs in combination with CAR-T therapy is more efficient. Several aberrant epigenetic alterations have been linked to the initiation and maintenance of CSCs in several cancers [173, 174]. Epigenetic reprogramming has been used to modulate the differentiation state and to promote the memory phenotypes of CAR-T, to improve CAR-T infiltration and persistence, and finally, as an alternative strategy to avoid their exhaustion [172].

Epigenetic reprogramming resulting from epidrug treatment can have multiple effects on both CSC subpopulations and CAR-T cells. These effects include the Impairment of CSC self-renewal and stemness capabilities, leading to inhibition of CSC initiation; upregulation of tumour-associated antigens (TAAs) specific to CSCs, enabling CAR-T cells to target CSCs selectively; and enhancement of T cell-intrinsic properties through

modifications in histones, DNA, and miRNAs, promoting a memory phenotype and reversing T cell exhaustion [175]. Recent research has demonstrated that miR-153 and miR-448 effectively inhibit the expression of Indoleamine 2,3-dioxygenase 1 (IDO1) in colorectal xenograft models. Additionally, miR-153 overexpression in cancer cells enhances CAR T cell killing capacity in vitro and suppresses tumour growth in a murine colorectal cancer xenograft model by downregulating IDO1 expression [177, 178].

Roadblocks in epigenetics treatments

Despite the benefits of epidrugs, their efficacy has various limitations. DNMT inhibitors lead to slow remission but, in most cases, do not eradicate cancer [178]. Their cytotoxicity and side effects, such as fatigue, nausea, increased infection susceptibility, and bone marrow suppression, are related to the lack of specificity of DNMT inhibitors and their indiscriminate demethylation [179]. One significant limitation of DNMT inhibitors is their cost, which may restrict access for many patients. The resistance mechanisms to these drugs are still a mystery, although possible re-methylation of DNA regions could be a crucial reason for resistance.

It is correct that precise targeting of histone deacetylases by HDACIs slow cancer growth, but these drugs have notable limitations. Off-target toxicity due to the influence of both histone and non-histone proteins is one of the most critical limitations [180]. Resistance to HDAC inhibitors in cancer cells may involve both "intrinsic" and "acquired" mechanisms. Intrinsic resistance to HDACIs in cancer cells is raised by abnormal expression and modifications of signalling molecules [181].

Furthermore, the complex molecular interactions with the targets of epidrugs, coupled with tumour heterogeneity and the wrapped nature of epigenetic regulation, have complicated their application [182]. In conclusion, not all cancers have equal responses to epidrugs therapies, and their delivery to target tissue has encountered challenges [183]. Therefore, future research must urgently focus on improving treatment with epidrugs through dose adjustment, resistance management, and, most importantly, targeted delivery. This targeted delivery is crucial in ensuring the efficacy of epidrugs in cancer treatment.

Discussion

Epigenetic modifications significantly influence CRC drug resistance through changes like tumour suppressor gene hypermethylation (e.g., MLH1) and oncogene hypomethylation (e.g., KRAS), affecting gene expression and drug interactions. However, challenges exist in clinical applications.

A primary challenge is the need for further research into epigenetic compounds. For instance, MG98 effectively downregulates DNMT1 and exhibits antiproliferative properties in vitro but has not shown considerable clinical success. Similarly, entinostat had promising preclinical results yet did not achieve its primary phase III trial endpoint for CRC. This 'translational gap' highlights the need for novel epidrugs with enhanced specificity and efficacy. Furthermore, the complexity of epigenetic regulation may lead to off-target effects, necessitating a deeper understanding of the epigenetic landscape for selective therapies. Common epidrugs like VPA risk causing adverse epigenetic changes and have a limited therapeutic window. Despite potential acquired resistance, combination therapies appear promising, integrating epidrugs with immunotherapy, targeted therapy, or chemotherapy to enhance effects. Another strategy includes combining HDAC inhibitors with chemotherapy to resensitise resistant tumours.

Studying the tumour microenvironment's impact on the cancer epigenome, such as immune and stromal cells, can shed light on heterogeneity and resistance. For instance, tumour hypoxia can modify epigenetics and exacerbate drug resistance, guiding the development of combination therapies targeting both tumour and microenvironment, such as pairing epidrugs with immune modulators. Identifying biomarkers for predicting response to epidrugs and resistance likelihood is vital for personalising treatment, and analysing epigenetic modifications, gene expression, or circulating tumour DNA to find suitable candidates for epidrug therapies.

Understanding the relationship between epigenetic modifications and CRC drug resistance is crucial for developing effective therapeutic strategies. By addressing these challenges, notably the limitations of preclinical models and robust patient stratification, and exploring innovative areas like next-generation epidrugs and combination therapies, we can leverage the potential of epigenetic therapies against CRC.

Conclusion and future perspectives

A wide range of genetic changes-independent dynamic alterations resulting from epigenetics lead to tumourigenesis formation and progression through TME reengineering. 'TME' refers to the tumour microenvironment, which includes immune and stromal cells. These alterations also adjust the on/off statuses of oncogenes and TSGs. Hereditary and reversibility are the main traits of epigenetic modifications that make them appropriate targets in cancer treatment. Nowadays, epidrugs are efficiently implemented for patients with various types of cancers singly or combined with other anticancer agents. However, there are

deficits associated with the mentioned drugs, including increased demands for appropriate personalised therapies resulting from the heterogeneity and plasticity traits of human cancer. Based on personal variations, standard cancer treatments are associated with limited prognosis. Moreover, both genome and epigenome maps of a determined cell population of patients were investigated using high throughput epigenome mapping technologies to evaluate drug sensitivity and screening. Therefore, therapy optimization of CRC associated with increased efficacy and decreased off-target effects could be achieved for all patients.

Abbreviations

5-caC	5- Carboxylcytosine
5-fC	5- Formylcytosine
5-hmC	5- Hydroxymethylcytosine
5mC	5- Methylcytosine
AKT	RAC-alpha serine/threonine-protein kinase
AML	Acute myeloid leukemia
APCs	Professional antigen-presenting cells
AR	Androgen receptor
BCR-ABL	Breakpoint cluster region gene- Abelson gene
CGIs	CpG islands
c-RAF	Proto-oncogene serine/Threonine-protein kinase
CSCs	Cancer stem cells
CTCL	Cutaneous T cell lymphoma
DNA	Deoxyribonucleic acid
DNMTIs	DNA methyltransferase inhibitors
DNMTs	DNA methyltransferases
EZH2	Enhancer of Zeste 2
HATs	Histone acetyl-transferases
HDAC	Histone deacetylase
HDACIs	Histone deacetylase inhibitors
HSP	Heat shock protein
ICD	Immunogenic cell death
ICIs	Immune checkpoint inhibitors
KLF4	Kruppel-like factor 4
m6A	N6 methyladenine
MBDs	Methyl-binding domain proteins
MDK	Midkine
MDS	Myelodysplastic syndrome
MEST	Mesoderm Specific Transcript
MHC-I	Major histocompatibility class-I
miRNAs	MicroRNA
MLH1	MutL homolog 1
NAD^+	Nicotinamide adenine dinucleotide
OS	Overall survival
PD-L1	Programmed death-ligand 1
piRNAs	Piwi-interacting RNAs
PR	Partial response
PROTACs	Proteolysis-targeting chimeras
PSA	Prostate-Specific Antigen
RNA	Ribonucleic acid
RUNX3	Runt-related transcription factor 3
SAHA	Suberanilohydroxamic acid = Vorinostat
SAM	S-adenosylmethionine
SD	Stable disease
SATB1	Special AT-rich sequence-binding protein-1
siRNAs	Small interfering RNAs
SIRT	Sirtuin
TET	Ten-eleven translocation methylcytosine dioxygenases
TFs	Transcriptional factors
TME	Tumour microenvironment
tMPRSS2	Transmembrane Serine Protease 2
TNBC	Triple-negative breast cancer

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