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Review Article

Potential Epigenetic Modifiers Targeting the Alteration of Methylation in Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is one of the most frequent causes of cancer-related death worldwide. Chemotherapeutic agents used in CRC treatment include oxaliplatin, irinotecan, leucovorin, Tegafur-Uracil, capecitabine, 5-fluorouracil, and monoclonal antibodies. The development of other effective drugs is urgently needed for CRC patients. As the epigenetics of CRC is increasingly understood, epigenetic modifiers (or epidrugs) targeting epigenetic mechanisms could play an important role in this process. During the past two decades, many studies have demonstrated that many specific genes are silenced by hypermethylation of their promoters in CRC, which means that the expression of these genes could be restored since epigenetic alterations are reversible. In fact, some molecules have been studied for their ability to inhibit DNA methyltransferases, and the results showed that silenced genes were reactivated. These molecules could be natural, such as curcumin, tea polyphenols, quercetin, and nanaomycin A, or synthetic, such as 5-azacytidine, decitabine, procainamide, and zebularine. On the other hand, we hypothesized in this article that ten-eleven translocation inhibitors could be another class of epigenetic modifiers since they could prevent chromosomal instability through decreasing the global hypomethylation of genomic DNA. Some studies have reported that some ten-eleven translocation inhibitors exhibit anticancer effects, which supports our hypothesis. Additionally, we have proposed combinations of these epigenetic modifiers according to different parameters.

Introduction

Colorectal cancer (CRC) is considered one of the most frequent causes of cancer-related death worldwide. In 2020, 1.9 million people were diagnosed with colorectal cancer (≈10% of all new cancer cases worldwide). This number will increase to 3.2 million new cases per year. The CRC incidence increases with age (≈80% of all new cases in individuals aged more than 55). Despite recent progress in CRC treatment, the relative five-year survival rate has decreased to ≈70%.[1] About 10% of cancer deaths are directly attributed to CRC, which is the second leading cause of cancer mortality. In 2020, the number of deaths due to CRC was estimated at 935,000 worldwide (50% occurring between ages 50 and 74) and will reach 1.6 million deaths per year by 2040 according to the WHO. Furthermore, it has been reported that CRC causes important treatment costs over time depending on the stage of disease, cancer subtype, country, and individual. On the other hand, CRC has been reported to have high mortality rates of 45%, 35% and 47.8% in Europe, the USA and worldwide, respectively, which makes it the third most frequent cancer worldwide in both sexes.[1,2]

Regarding CRC treatment, Chemotherapy can be used before surgery for some patients to shrink the tumor before it is eradicated. In the late stages, it is used to increase the life expectancy of patients. Chemotherapeutic agents may include oxaliplatin, irinotecan, leucovorin, Tegafur-Uracil capecitabine, or 5-fluorouracil. Additionally, cetuximab, panitumumab, or bevacizumab could be used as monoclonal antibodies in CRC immunotherapy.[3] The development of other effective drugs is urgently needed for CRC patients. As the epigenetics of CRC are increasingly understood, epigenetic modifiers (or epidrugs) targeting epigenetic mechanisms constitute a way to explore this process.

Keywords: CRC; Colorectal cancer; Epigenetics; Methylation; Epidrugs; DNA methyltransferase inhibitors; DNMTi; TETi.

Abbreviations: ADAMs, a disintegrin and metalloproteinase; AMPK, AMP-activated protein kinase; CIN, chromosomal instability; CRC, colorectal cancer; DNA, deoxyribonucleic acid; DNMT, DNA methyltransferases; DR, death receptor; EGCG, epigallocatechin gallate; EGF, epidermal growth factor; HDAC, histone deacetylase; IC50 with lower case, half maximal inhibitory concentration; KG, alpha ketoglutarate; LCA, laccaic acid A; LINE-1, long interspersed nuclear element; lncRNA, long non coding RNA; microRNAs, miRNAs; MMPs, matrix metalloproteinases; MSI, microsatellite instability; RNA, ribonucleic acid; SAM, S-adenosyl-L-methionine; TET, ten-eleven translocation; TNF, tumor necrosis factor; TSG, tumor suppressor genes; 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine.

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In this review, we will first elucidate epigenetic modifications involved in CRC with a focus on DNA methylation (hypermethylation and hypomethylation) and then provide a list of potential molecules (natural and synthetic) that could be used as epigenetic modifiers to target alterations in gene methylation, one of the most involved epigenetic alterations in CRC. Finally, we propose combinations of these epigenetic modifiers according to different parameters, such as their source, mechanism of action, target and chemical structure. Information given in this paper could help researchers in the development of new molecules and combinations of molecules to test as potential epigenetic modifiers in the treatment of methylation alterations in CRC and other cancers.

Colorectal cancer epigenetics

Epigenetics is a heritable alteration of gene expression that does not affect the coding sequence of a gene. It has been confirmed that epigenetic alterations play a central role in cancer pathogenesis, especially in colorectal cancer.[4] During the past two decades, many studies have reported a correlation between certain CRC-specific gene expression patterns and the absence of gene mutations. For example, microsatellite instability, one of the hallmarks of the CRC molecular subgroup, is the result of a deficiency in the DNA mismatch repair system. This instability, in addition to genetic mutations in one of the mismatch repair genes, can also be the consequence of epigenetic silencing of the *MLH1* gene by hypermethylation of its promoter.[5] On the other hand, it has been demonstrated that global hypomethylation of DNA could also lead to CRC through chromosomal instability (CIN).[6]

DNA methylation in CRC

DNA hypermethylation

Compared with those in normal cells, there are hundreds of hypermethylated genes in CRC tumors, as revealed by CRC epigenome sequencing.[7] In the same study, researchers also showed that epigenetic alterations in tumors are significantly more prevalent than genetic alterations among patients.[7] Another study reported that some groups of CRC patients have greater hypermethylation of promoter regions, known as the CpG island methylator phenotype.[8] In a large study, genome methylation profiles were compared between CRC cells and normal cells using the MBD-capture protocol.[9] A total of 322,551 genomic regions (249.5 Mb of the human genome including 7 million CpG sites) were analyzed. According to the results of this study, most of the differentially methylated regions (DMRs) between CRC and normal cells were hypermethylated.[9] Furthermore, hypermethylated DMRs were more frequent in intragenic, gene-regulatory, or CpG shelf-shore island segments, as shown in Table 1.[10–32]

DNA hypomethylation

The development and progression of CRC involves a series of events. These different steps, starting with the transformation of normal colonic epithelium to an adenomatous intermediate and then to an adenocarcinoma, require multiple genetic mutations. Among these genetic events, genomic instability is now described as the main molecular driving force in CRC. In fact, CIN has been reported in 65%–70% of sporadic CRC patients.[33] There are at least three different pathways associated with genomic instability: CIN, microsatellite instability, and the CpG island methylator phenotype. The majority of CRC cases are caused by the CIN pathway. CIN is characterized by aneuploidy (imbalances in chromosome number) and loss of heterozygosity (LOH), and it can result from defects in chromosomal segregation, telomere stability, and DNA damage repair.[34]

Hypomethylation is recognized as an early molecular event leading to CIN that can cause deletion, translocation, inversion or duplication in the entire or part of the chromosome.[35] As a consequence of global DNA hypomethylation, some genes can be released from inhibition induced by the methylation of their promoter and become overexpressed, which leads to the activation of oncogenic pathways that are crucial for CRC pathogenesis. Additionally, the exploration of global DNA methylation could have potential clinical applications, such as early cancer formation by cell-free DNA (cfDNA) fraction analysis. In fact, there is a good correlation between global DNA methylation and long interspersed nuclear element (LINE-1) methylation levels since LINE-1 (mobile genetic elements) retrotransposon composes approximately 17% of the human genome.[33]

Histone modifications

Histone modifications also play a key role in carcinogenesis. In the context of CRC, histone acetylation and methylation status have been widely studied, and it has been shown that they are associated with various clinicopathological features of CRC. In fact, it has been shown that H3K9me (methylation of lysine 9 on histone 3) is more prevalent in CRC and adenomas compared to normal colonic mucosa, and H3K27ac (acetylation of lysine 27 on H3) and H4K12ac (acetylation of lysine 12 on H4) are more prevalent in CRC in comparison with normal mucosa.[36] Additionally, ChIP (chromatin immunoprecipitation technique) of circulating nucleosomes revealed decreased levels of H3K9me3 (trimethylation of lysine 9 on H3) and H4K20me3 (trimethylation of lysine 20 on H4) in individuals with CRC compared with healthy individuals.[37]

Noncoding RNAs (ncRNAs)

ncRNAs (lncRNAs (long ncRNAs) and miRNAs (microRNAs)), which are considered to act via another epigenetic mechanism, can inhibit protein expression and consequently influence many cancer-related pathways, especially at the post-transcriptional level. MicroRNAs are involved in all CRC stages (initiation, progression and metastasis).[38] For example, miR-143 prevents cell proliferation through the inhibition of the *KRAS* mRNA transcript. This mRNA was found to be frequently downregulated in CRC.[39]

LncRNAs have attracted increasing interest as markers of CRC over the past few years. Many lncRNAs related specifically to colon cancer have been identified in different databases (NONCODE database, PubMed, etc.). Most of these lncRNAs are upregulated, and it seems that they function as miRNA sponges. HOX transcript antisense intergenic RNA (HOTAIR), a lncRNA that has been analyzed in both serum and tissue, was shown to be upregulated in the early stages of CRC development.[40] Colon cancer-associated transcript 1 (CCAT1) is another CRC-related lncRNA that is reported to be upregulated in both cancer cells and blood.[41] In addition, it has been demonstrated that the expression of the lncRNA growth arrest specific 5 (GAS5) is downregulated in human colon cancer cells compared to normal tissues.[42]

Signaling pathways

In CRC, as well as in all other cancers, alterations in several signaling pathways, such as the mitogen-activated protein kinase (MAPK)

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Table 1. Genes silenced by hypermethylation in CRC classified according to their function

Gene (Protein)	Function	Target	Effect of gene silencing	Reference
APC (Adenomatous polyposis coli)	Wnt signaling pathway inhibition	Wnt signaling pathway inhibition	Increased Wnt/β-catenin signaling	10
SFRP2 (Secreted frizzled-related protein 2)	Wnt antagonist		Increased Wnt/β-catenin signaling	11
SFRP1 (Secreted frizzled-related protein 1)	Wnt antagonist		Increased Wnt/β-catenin signaling	11
MGMT (O-6-methylguanine-DNA methyltransferase)	Repair of alkylation DNA damage	DNA repair	Increased G→A mutation frequency	12
MLH1 (MutL homolog 1)	DNA Mismatch repair		Microsatellite instability	13
HLTF (Helicase-like transcription factor)	dsDNA translocase, fork remodeling activity, ubiquitin ligase		Impaired DNA repair	14
RUNX3 (Runt-related transcription factor 3)	Transcription factor	DNA Transcription	Decreased TGF-β/BMP signaling	15
ID4 (Inhibitor of DNA binding 4)	Transcription factor		?	16
IRF8 (Interferon regulatory factor 8)	Transcription factor		Interferon signaling	17
ESR1 (Estrogen receptor 1)	Ligand-activated transcription factor		Loss of estrogen receptor signaling	18
CDH1 (E-Cadherin)	Calcium dependent cell-cell adhesion glycoprotein	Adhesion	Loss of cell adhesion, possible increased Wnt/β-catenin signaling	19
CDH13 (Cadherin 13)	Selective cell recognition and adhesion, anti-apoptotic		Increased PI3K/Akt/mTOR signaling, MAPK signaling	20
THBS1/TSP1 (Thrombospondin 1)	Cell-to-cell and cell-to-matrix adhesive glycoprotein		Decreased TGF-β1 signaling	21
CRABP1 (Retinol-binding protein 1)	Carrier protein for transport of retinol, promotes apoptosis	Transport	?	22
SLC5A8 (Sodium solute symporter family 5 member 8)	Sodium and short-chain fatty acid transporter, suppress colony formation		?	23
DAPK (Death associated protein kinase)	Induction of cell death	Apoptosis	Interferon gamma signaling, TNF alpha signaling, Fas/APO1 signaling	24
RASSF1A (Ras association domain family 1 (isoform A))	Negative RAS effector, pro-apoptotic, microtubule stabilization		Increased RAS/RAF/MAP kinase signaling, death receptor-dependent apoptosis	25
CDKN2A (P14, ARF) (Protein 14)	Inhibits E3 ubiquitin ligase	Cell cycle regulation	Decreased p53 stabilization and activation	26
CDKN2A/p16 (Cyclin-dependent kinase inhibitor 2A)	Regulates cell cycle G1 progression		Increased cell proliferation	27
SEPT9 (Septin 9)	GTPase, formation of filaments		Impaired Cytokinesis and loss of cell cycle control	28
SEPT9 (Septin 9)	GTPase, formation of filaments	Invasion and Migration	Impaired Cytokinesis and loss of cell cycle control	29
CXCL12 (Chemokine)	(C-X-C motif) ligand 12 Alpha chemokine		Increased tumor cell metastases	30
VIM (Vimentin)	Stabilizing cytoskeleton		Increased tumor cell metastases	31
TIMP3 (Tissue inhibitor of metalloproteinase 3)	Inhibition of MMPs and ADAMs	Angiogenesis	Increased EGF receptor signaling, TNF alpha signaling	32

ADAMs, A Disintegrin And Metalloproteinase; CRC, colorectal cancer; EGF, Epidermal Growth Factor; MMPs, Matrix Metalloproteinases; TNF, Tumor Necrosis Factor.

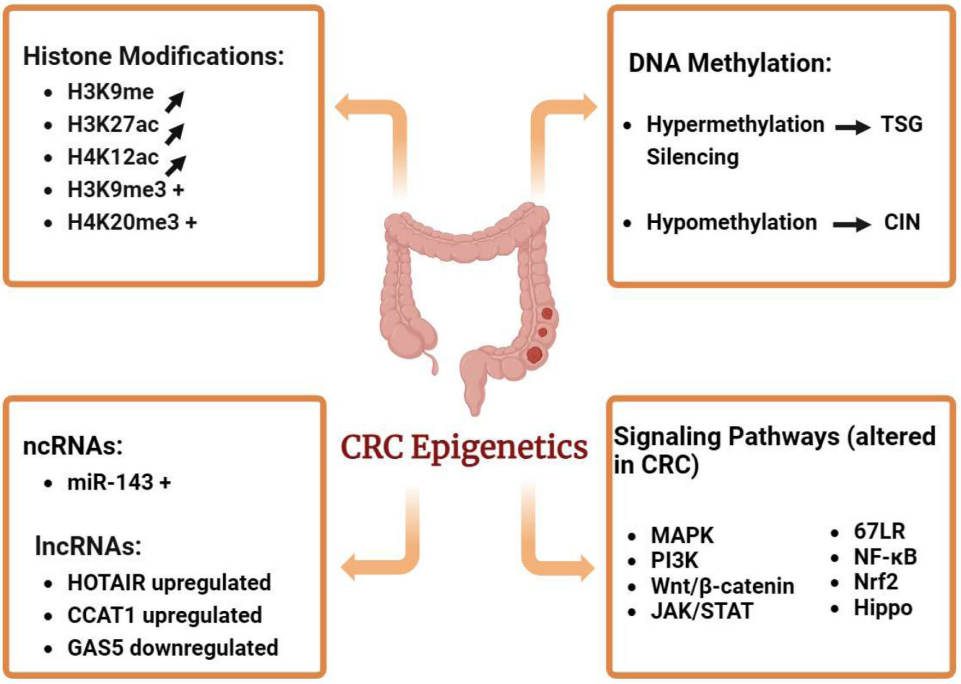

Fig. 1. Some epigenetic alterations detected in CRC. Epigenetic alterations leading to CRC include DNA methylation (hypermethylation of TSG promotors and global hypomethylation that leads to CIN), histone modifications, and ncRNA (miRNA and lncRNA). ↗ (increased), + (presence), → (leads to). CRC, coloractal cancer; TSG, tumor suppressor gene, CIN, chromosomal instability; miRNA, microRNA; ncRNA, non coding RNA; lncRNA, long non coding RNA.

pathway, PI3K pathway, and Wnt/β-catenin pathway, can lead to the onset and development of cancer.[43] Crosstalk between these pathways can also promote colon cancer invasion and increase resistance to drugs. Other pathways were reported to be involved in CRC, such as the Janus-activated kinase/signal transducers and activators of transcription pathway, 67 kDa laminin receptor pathway, nuclear factor-kappa B pathway, nuclear factor-erythroid 2-related factor pathway, and Hippo pathway. The Hippo pathway was shown to be responsible for cell proliferation, differentiation, apoptosis, and tumorigenesis,[44] and its interaction with the Wnt/β-catenin pathway is crucial for colorectal cancer development (Fig. 1).[45]

Inhibitors targeting molecular signal transduction have modest efficacy in nonhematologic malignancies because of the complexity of the genome in solid tumors. Thus, molecules designed to target abnormal DNA methylation, particularly in CRC, might be a more efficacious anticancer treatment strategy. As epigenetic modifications are reversible, the molecules that can target the enzymes involved in these mechanisms may be good drug candidates to fix these alterations and subsequently restore normal gene expression. These molecules are referred to as epidrugs or epigenetic modifiers. In fact, many recent studies have reported that certain molecules are able to inhibit enzymes like DNMT (DNA methyltransferase) and HDAC (histone deacetylase), which are responsible for DNA methylation and histone deacetylation, respectively.[46]

Epigenetic modifiers as potential epidrugs for CRC treatment

As we have mentioned before, many studies have confirmed that epigenetic mechanisms, such as DNA methylation, histone modifications and ncRNAs, are strongly involved in many processes of cellular physiology and development, and the alteration of these mechanisms could, under certain conditions, lead to carcinogenesis through alteration of the expression of oncogenes or tumor suppressor genes. Thus, since epigenetic modifications are reversible, molecules able to restore normal gene expression by fixing epigenetic alterations could be good candidates for use as agents in the treatment of cancer, either alone or in combination with conventional treatment. These molecules, called epigenetic modifiers or epidrugs, can target DNA methylation, histone modifications or ncRNAs. In this article, we will focus on molecules that target DNA methylation in CRC, namely, DNMT inhibitors (as hypomethylating or demethylating agents) and ten-eleven translocation (TET) inhibitors (as potential hypomethylating agents).

Demethylating (hypomethylating) molecules: DNA methyltransferase inhibitors (DNMTis)

DNA methyltransferases

DNA methylation is the most studied epigenetic mechanism, and it could be considered the most important. In fact, DNA methylation is involved in many physiological mechanisms, such as cellular differentiation, parental imprinting, and X-chromosome inactivation. DNA methylation is catalyzed by enzymes called DNA methyltransferases (DNMTs). These enzymes lead to the formation of 5-methylcytosine (5mC) by transferring a methyl group from S-adenosyl-L-methionine to cytosine at CpG sites in the gene promoter. In humans, *de novo* DNA methylation is ensured by DNMT3, while DNA methylation is maintained by DNMT1 (a multidomain protein consisting of 1,616 amino acids).

Currently, there is no doubt that deregulation of DNA methylation is associated with diseases, especially cancers. In fact, this epigenetic modification, when it occurs on oncogenes or tumor suppressor genes, could lead to carcinogenesis.[47] Additionally, be-

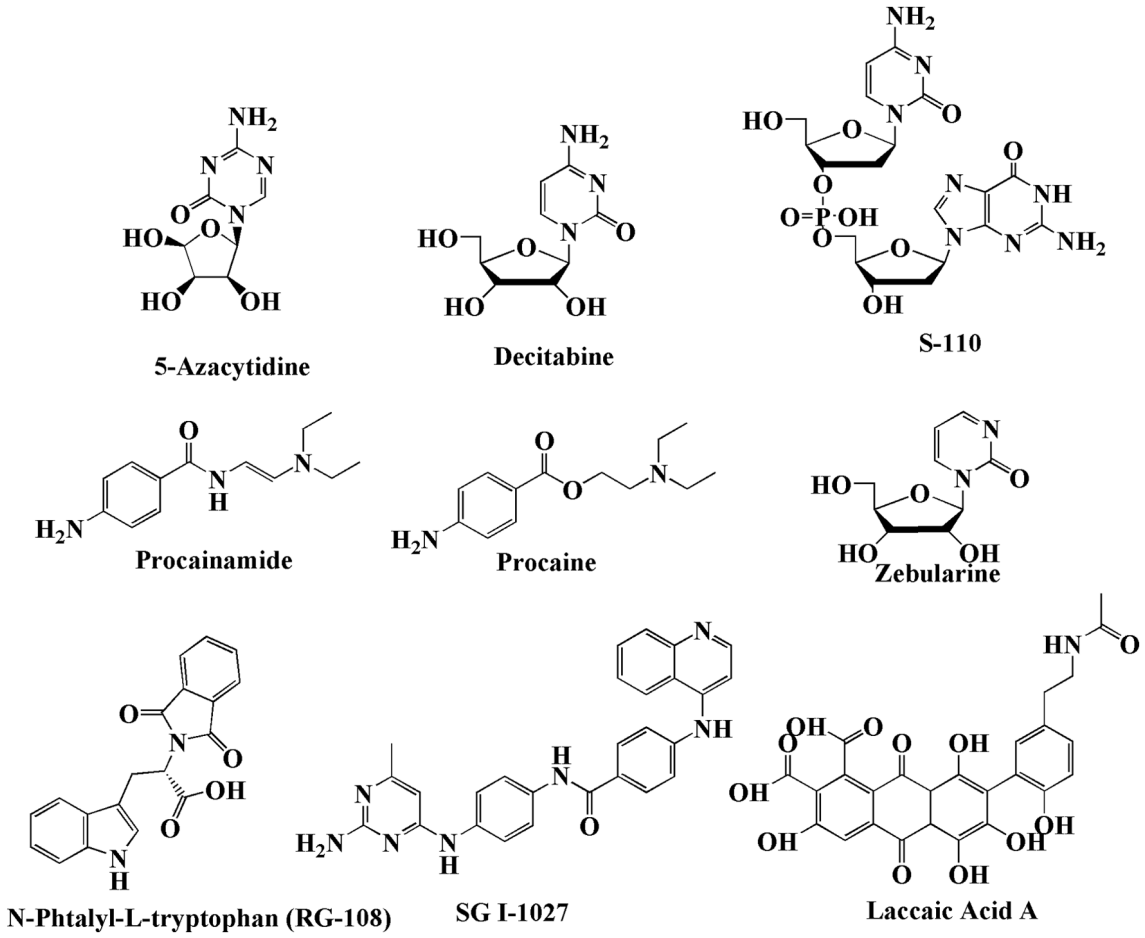

Fig. 2. Synthetic demethylating (hypomethylating) molecules.

cause of alterations in DNA methylation, cancer cells can escape apoptosis and resist chemotherapy.[48]

Recently, some studies reported that certain drugs approved for other indications could be good candidates for use as DNA methylation inhibitors. Among these drugs, hydralazine, procaine,[49] procainamide,[50] and certain antibiotics, such as mithramycin A.[51] Other natural and synthetic molecules could also be good candidates for DNMT inhibitors.[52] Recently, researchers have been working on the rational development of small-molecule non-nucleoside inhibitors.[53] This family of molecules is steadily growing and comprises a large variety of different chemical scaffolds like polyphenolic compounds such as epigallocatechin-3-gallate (EGCG) or compounds with acidic functions such as caffeic acid or methylenedisalicylic acid.[54–56]

Synthetic molecules

5-Azacytidine

5-Azacytidine, shown in the Figure 2, is a cytidine analog modified at position 5 of the pyrimidine ring (with nitrogen instead of carbon). Its incorporation into DNA disrupts the interaction between DNA and DNMTs (1 and 3). Consequently, the enzyme remains covalently bound to DNA, and its function is inactivated.[57] Furthermore, because of the inhibition of cytosine methylation, there is a passive loss of methylation in daughter cells after replication. Azacitidine, known to reactivate the expression of some tumor suppressor genes (TSGs), is approved by the US FDA for the treatment of myelodysplastic syndrome.[58] This molecule restores the normal growth and differentiation of cells via the demethylation of TSG.[59]

A clinical study reported that treatment with 5-azacytidine in combination with entinostat (an HDAC inhibitor) restored TSG and inhibited the growth of CRC cell lines. The same study showed that the reversal of hypermethylation was observed in a subset of patients and correlated with improved performance status.[60]

Decitabine (and its derivative S-110)

Decitabineis a nucleoside analog of cytidine obtained by substitution of a carbon by a nitrogen at position 5 of the pyrimidine ring of deoxyribose (Fig. 2). Like 5-azacytidine, decitabine acts by incorporating into RNA and/or DNA during the S phase of the cell cycle. Decitabine is more specific and less toxic than azacitidine, but both are good DNMT inhibitors even at low concentrations.[61] In addition, decitabine alone or in combination with other chemotherapeutic agents, such as oxaliplatin, inhibited CRC cell proliferation.[62]

Procainamide

Procainamide, shown in the Figure 2, is a drug used to treat a variety of atrial and ventricular dysrhythmias. Investigations have

shown that this molecule is a specific inhibitor of DNMT1. It is a competitive non-nucleoside inhibitor that interacts with the binding pocket of the enzyme, which increases the affinity of DNMT1 for hemimethylated DNA and S-adenosyl-L-methionine. This inhibition of methylation leads to a reduction in the global 5mC content in the cell and decreases gene-specific hypermethylation at promoter CpG islands, which could lead to the reactivation of TSG inhibited by hypermethylation in cancer cells.[63]

Shih *et al*. showed that the *IL27RA* gene was downregulated in a group of rats with lung injury induced by LPS and upregulated in the LPS+procainamide group. Thus, the demethylation of the *IL27RA* promoter by procainamide restored the activation of this gene, which is known to have an anti-inflammatory effect in several models of inflammation.[64] Another study reported that excessive methylation of antioxidant gene promoters increases oxidative stress in many diseases. The involvement of DNMT inhibitors in decreasing reactive oxygen species production has been observed in many diseases, such as osteoarthritis chondrocytes and lung adenocarcinoma cells.[65] Moreover, Chih-Chin has shown that hypermethylation induced by microbial infection is associated with increased inflammation and oxidative stress.[66] These results showed that procainamide may be a good candidate for use as a therapeutic agent in diseases caused by oxidative stress, like colorectal cancer (often associated with an alteration of the microbiota), because of its involvement in DNA demethylation, suppression of superoxide production and neutrophil infiltration.[66] Gao *et al*. reported that Procainamide inhibits the Wnt (Wingless type) pathway, which is involved in carcinogenesis. Aberrant promoter methylation of Wnt inhibitory factor-1 (WIF-1) is a crucial mechanism of epigenetic silencing in human cancers, especially in colorectal cancer, where this hypermethylation targets the *APC* gene (adenomatous polyposis coli), which functions as an inhibitor of the Wnt signaling pathway.[67]

Procaine

Procaine, shown in the Figure 2, a local anesthetic drug, is a non-nucleoside inhibitor. Its DNMT inhibitor effect was first reported in breast cancer cells, where it induced global DNA demethylation and restored the activation of certain TSGs.[68] Moreover, it has been demonstrated that procaine, at high concentrations, decreases the proliferation of different cancer cells.[69] Procaine acts by specifically binding to sequences rich in CpG islands, which inhibits the DNMT-DNA interaction.[70]

Procaine inhibits the proliferation and migration of CRC cells. Chang *et al*. reported that, when tested on HCT116 cells, procaine significantly inhibited cell viability, increased apoptosis, and decreased the expression level of Ras homolog family member (RhoA) in a dose-dependent manner ($p < 0.05$). In fact, this drug increased the proportion of HCT116 cells in the G1 phase and downregulated cyclin D1 and cyclin E expression. In this study, it was demonstrated that procaine inhibits the proliferation and migration of CRC cells through inactivation of the ERK/MAPK/FAK pathways via the regulation of *RhoA*.[71]

Zebularine

Zebularine, shown in the Figure 2, is a nucleoside analog. Its DNMT inhibitor effect is specific since it forms a close covalent complex between DNMT and zebularine-substituted DNA, which prevents methyl group transfer and subsequently inhibits DNA methylation.[72] In human squamous carcinoma cell lines, zebularine has been shown to reduce viability and DNA synthesis through cell cycle arrest at the G2/M phase and through apoptosis.[73]

The effect of zebularine on colorectal cancer was investigated by Yang *et al*., who reported that zebularine has cytotoxic effects on cancer cell cultures, tumor xenografts and a mouse model of colitis-associated CRC. This effect is achieved through the stabilization of *p53* via the ribosomal protein S7 (RPS7)/MDM2 pathway and DNA damage.[74]

N-Phthalyl-L-tryptophan (RG-108)

N-Phthalyl-L-tryptophan is a non-nucleoside DNA methyltransferases inhibitor (IC_{50} = 115 nM) that blocks the active site of DNMTs (Fig. 2). It has been shown that N-phthalyl-L-tryptophan induces the demethylation and reactivation of TSG. In fact, incubation of NALM6 and HCT116 (human colon carcinoma) cell lines with low concentrations of this molecule results in significant DNA demethylation without any detectable toxicity.[75]

SGI-1027

SGI-1027, shown in the Figure 2, is a quinoline-based compound. It is a nonnucleoside inhibitor of DNMT3B, DNMT3A, and DNMT1 with IC_{50} values of 7.5 µM, 8 µM, and 12.5 µM, respectively (with poly-dI-dC as the substrate). Jharna Datta *et al*. demonstrated that SGI-1027 inhibits these DNMTs by competing with S-adenosylmethionine in the methylation reaction. Different cancer cells were treated with SGI-1027, and the results showed that there was selective degradation of DNMT1 with minimal or no effect on DNMT3A or DNMT3B. Moreover, prolonged treatment of RKO (colorectal cancer cells) with SGI-1027 led to demethylation and reactivation of the silenced TSG *P16*, *MLH1*, and *TIMP3*. Additionally, the same study reported the involvement of the proteasomal pathway in the mechanism of action of this molecule. In addition, no significant toxicity has been detected in a rat hepatoma (H4IIE) cell line.[76]

Laccaic acid A

Laccaic acids or laccainic acids, shown in the Figure 2, are a group of five anthraquinone derivatives. Laccaic acid A (LCA) is a tetrahydroxyanthraquinone (3,5,6,8-tetrahydroxy-9,10-anthraquinone) substituted by two carboxy groups at positions 1 and 2. It has been reported that LCA is a DNMT1 inhibitor with K_i = 310 nM and IC_{50} = 650 nM. In a study aiming to evaluate the anti-colorectal cancer activity of the combination (LCA+ 5-FU), the results demonstrated that LCA is a highly DNA-competitive inhibitor of DNMT1. According to *in vitro* methylation assays, LCA competes with DNA substrates and alters the expression of methylated genes in the MCF-7 cell line.[77] In another study, it was shown that LCA combined with phenethyl isothiocyanate has a strong synergistic effect on CRC. Additionally, LCA inhibited human colon carcinoma HT29 cell growth with an IC50 value of 6.08 µM after 72 h of treatment and induced cell apoptosis and cell cycle arrest at the sub-G1 phase.[78]

MG-98

MG-98 is referred to as the second-generation DNMT inhibitor. It is a 20-mer antisense compound with a phosphorothioate backbone. MG-98 is a highly specific inhibitor of DNMT1 mRNA translation in humans. It acts by binding to the 3′ untranslated region of DNMT1 mRNA. *In vitro* studies have reported that this oligonucleotide restores the expression of the cyclin-dependent kinase inhibitor *p16* (α-CDKN2A) through suppression of DNMT1 expression.[79] In preclinical studies and clinical phase I/II trials, it has been demonstrated that MG-98 can lead to the reactivation of silenced tumor suppressor genes safely and effectively. Thirty-two patients with gastric, colonic, ovarian, breast, renal, lung cancer,

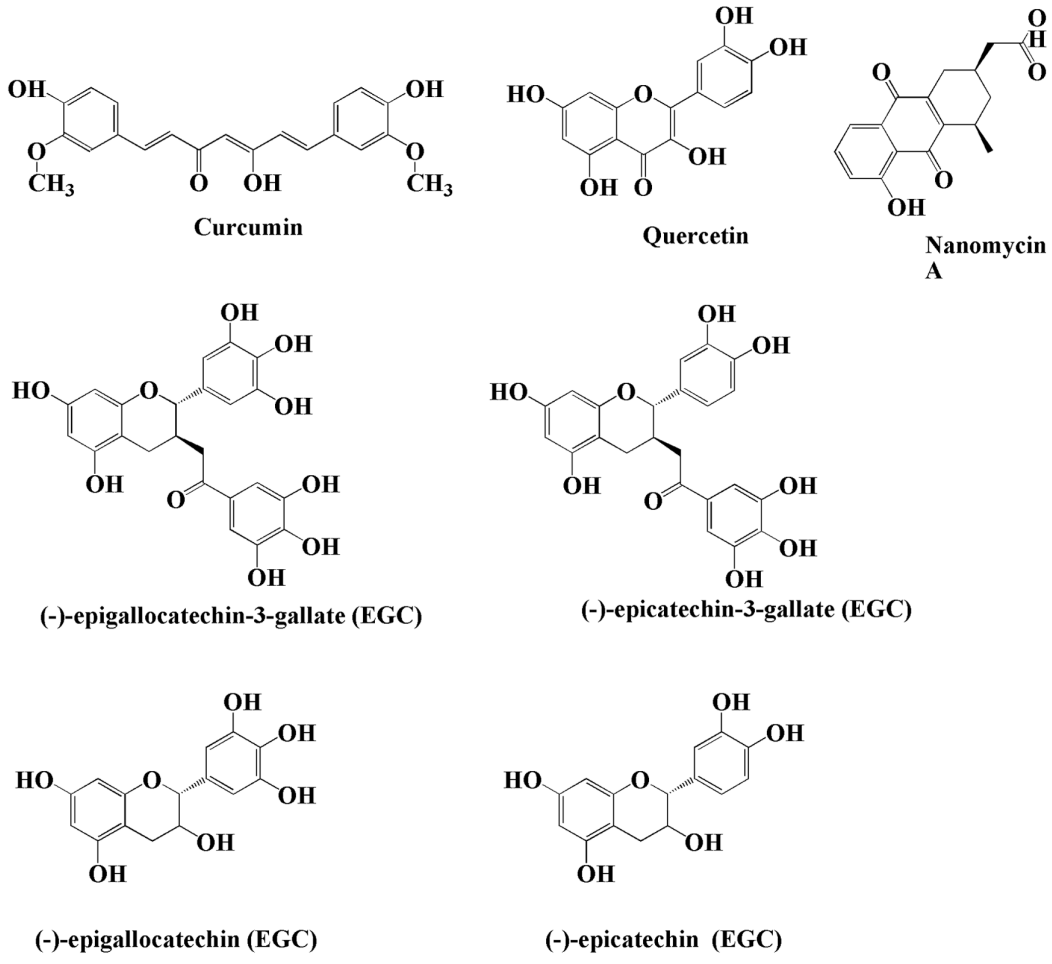

Fig. 3. Natural demethylating (hypomethylating) molecules.

and melanoma were treated with MG-98 administered as a 7-day continuous infusion every 14 days, and DNMT1 activity in PBMCs was monitored during two cycles of therapy. DNMT1 inhibition was reported in 26 of 32 patients, and MG-98 was well tolerated, with early evidence of a clinical effect.[80]

Natural molecules

Curcumin

Curcumin, shown in the Figure 3, a plant-derived polyphenol, has been shown to inhibit DNMT activity in various cancer cell lines, including colorectal cancer cells, through the regulation of multiple signaling pathways. These pathways include cell proliferation pathway (cyclin D1, c-myc), cell survival pathway (Bcl-2, Bcl-xL, cFLIP, XIAP, c-IAP1), caspase activation pathway (caspase-8, 3, 9), tumor suppressor pathway (p53, p21), death receptor pathway (DR4, DR5), mitochondrial pathways, and protein kinase pathway (JNK, Akt, and AMPK).[81,82] Additionally, curcumin treatment has been reported to decrease global DNA methylation in a model of leukemia cells.[83] In another study aiming to assess the antitumor effect of curcumin on CC531 colorectal cancer cells both *in vitro* and *in vivo*, the results showed that this natural product reduced cell proliferation by more than 30% after 48 h and 50% after 72 h. The same study demonstrated, using a wound healing test, that curcumin inhibited migration. Finally, *in vivo*, curcumin reduced the tumor volume of liver implants of CRC cells by 5.6-fold.[84]

Tea polyphenols: EGCG, catechin and epicatechin

Several tea catechins and bioflavonoids have been studied for their ability to modulate DNA methylation catalyzed by prokaryotic SssI DNA methyltransferase and human DNMT1 (Fig. 3). According to the results of these studies, catechin and epicatechin inhibited DNMT1 in a dose-dependent manner with IC50 values ranging from 1.0 to 8.4 µM. The IC50 of EGCG, the most potent inhibitor, ranged from 0.21 to 0.47 µM.[85] Since EGCG is the most effective polyphenol, its mechanism of action was studied *in silico*. *In silico* studies demonstrated that the gallic acid moiety is responsible, in large part, for its high-affinity and direct inhibitory interaction with the DNMT1 catalytic site, and its interaction with the enzyme is stabilized by Mg^{2+}.[85]

Other *in vivo* and epidemiological studies have reported that tea polyphenols can inhibit the growth and metastasis of CRC through

anti-inflammatory, anti-oxidative, and pro-apoptotic effects. Some studies have shown that these natural molecules can influence several signaling pathways in tumor cells, such as mitogen-activated protein kinase pathway, phosphatidylinositol-3 kinase/Akt pathway, Wnt/β-catenin pathway, and 67 kDa laminin receptor pathway, leading to the inhibition of cell proliferation and apoptosis. Additionally, other studies have suggested that tea polyphenols can prevent the growth and metastasis of colorectal cancer by improving the immune response and decreasing inflammatory responses through the modulation of the gut microbiota composition.[86]

Quercitin

According to the results of a study aiming to evaluate the potential of quercetin, shown in the Figure 3, as an epigenetic modifier in cancer, this phytochemical decreases the activity of DNMTs, HDACs, and HMTs (histone methyltransferases) in a dose-dependent manner. The same study reported that quercetin decreased global DNA methylation levels in a dose- and time-dependent manner and restored TSG expression by demethylating their promoters. Additionally, an *in silico* study (molecular docking) showed that quercetin could act as a competitive inhibitor by interacting with residues in the catalytic site of several DNMTs and HDACs.[87]

The protective effect of quercetin on colon cancer was investigated in 45 rats using azotoxin methane (15 mg/kg s.c.) as a carcinogen. The results showed that quercetin reduces cytological changes in colon cancer cells, decreases beta-catenin and *Bcl-2* (anti-apoptotic gene) expression, and increases *caspase 3* (apoptotic gene) expression.[88]

Nanaomycin A

Nanaomycin A (see Figure 3 bellow) is an anthracycline antibiotic belonging to the quinone class. It was isolated from Streptomyces. Its mechanism of action depends on its reduction by respiratory chain-linked NADH or flavin dehydrogenase. The reduced form of this molecule produces singlet molecular oxygen (O_2^-) after its quick auto-oxidation by molecular oxygen, which is responsible for its antimicrobial activity.[89]

Since it induces antiproliferative activity against cancer cell lines, the epigenetic effect of nanaomycin A was first studied by an *in silico* screening method for the inhibition of DNMTs. Kuck *et al*., in a biochemical study, reported that this molecule interacts with the catalytic site of DNMT3B at specific AAs (Glu697, Arg731 and Arg733) of the enzyme binding pocket. It has been shown that this drug decreases the expression of DNMT1, 3A and 3B.[56] This molecule can also inhibit DNMT3B activity by reactivating the *RASSF1A* tumor suppressor gene, which reduces cell proliferation and viability.[56,90] In fact, *RASSF1A* (Ras association domain family 1), a pro-apoptotic gene involved in microtubule stabilization, is among the genes that are commonly silenced by methylation in CRC. The loss of this gene leads to an increase in RAS/RAF/MAP kinase signaling and death receptor-dependent apoptosis.[91]

Resveratrol

Resveratrol is a natural polyphenol that has many biological activities. Recently, Resveratrol was shown to reactivate silenced tumor suppressor genes through decreasing DNMT expression. Additionally, synthesized derivatives of resveratrol (resveratrol-salicylate) could exhibit an important DNMT inhibitor effect. Some of these analogs selectively inhibit DNMT3. Additionally, the most active derivative showed an important cytotoxic effect (greater than that of resveratrol) against three human cancer cell lines: Hep-G2, SK-BR-3, and especially HT-29 (colorectal adenocarcinoma cells).[92,93]

Other natural molecules

Other natural molecules, such as fisetin,[94] myricetin,[95] theaflavin,[96] thearubigin,[96] trichostatin,[97] kazinol,[98] genistein, silibinin, luteolin, boswellic acid, mahanine and selenium, have been reported to exhibit a DNMT inhibitory effect,[99] which makes them good candidates for use as hypomethylating agents for CRC treatment (Table 2).

Hypermethylating molecules

TET Inhibitors (ten-eleven translocation inhibitors)

DNA methylation contributes widely to the dynamic chromatin states that impart precise epigenetic landscapes, which maintain cell type-specific transcriptional programs. Before the discovery of TET protein enzymes, it was believed that DNA methylation is an irreversible epigenetic event. These enzymes can modify methylcytosine and potentially erase DNA methylation.[100] They also play a key role in the efficient transcription of target genes necessary for the proliferation and survival of tumor cells.[101] TETs are a family of three proteins, TET1, TET2, and TET3, that are responsible for the catalysis of successive oxidation reactions of 5mC to 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine, respectively as seen in the Figure 4 bellow.[102,103]

As with DNMTs, the activity of TET enzymes, which are involved in removing epigenetic marks, is also a part of the disruption of epigenetic landscapes characterizing malignant transformation. Moreover, since TETs function is to demethylate CpG islands of genomic DNA, this could be responsible for, or at least increase, global DNA hypomethylation, which could lead to chromosomal instability. Consequently, we can hypothesize that this family of enzymes could be an interesting target for epigenetic modifiers in the treatment of cancer. Indeed, in principle, inhibition of TETs could lead to a decrease in global DNA hypomethylation and therefore prevent chromosomal instability. The fact that some TET inhibitors exhibit anticancer activity supports our hypothesis.[104,105]

Cytosine-based TET enzyme inhibitors

Some cytosine derivatives, shown in the Figure 5 bellow, have been synthesized by substitution at the 5 position and evaluated for their capacity to inhibit TET1 and TET2. The results showed that the derivative obtained by chlorination at this position, Bobcat212, had the most inhibitory effect, at 57% and 43%, on TET1 and TET2, respectively. Another interesting derivative, Bobcat339, which is substituted at the R2 position with 3-biphenyl, significantly increased the inhibitory effect of TET1 and TET2 without inhibiting DNMT3a.[106]

αKG-dependent dioxygenases

2-Hydroxyglutarate (2HG), N-oxalylglycine (NOG), and dimethyl fumarate (DMF) are known for their inhibitory effects on a variety of αKG-dependent dioxygenases.[107] Several molecules were synthesized by substitution of the C4 position with either -keto, -olefin, -methyl, or -cyclopropyl functional groups, and the C2 position was single or double substituted with -chloro, -fluoro, -hydroxy, -methyl, or –trifluoromethyl groups. These derivatives were subsequently tested *in vitro* and *in vivo* for their ability to induce cancer cell death and TET dioxygenase inhibition. These results suggested that TET inhibitors need to be further investigated as a new class of targeted agents for cancer treatment.[104]

Table 2. Some DNMT inhibitors used as potential epigenetic modifiers in CRC, classified according to their target, their origin, and their mechanism of action

DNMT i	Target	Nucleoside/non nucleoside	Mechanism of action	Natural/synthetic
Azacitidine	Global DNMT	Nucleoside inhibitor	Incorporation into the RNA and/or genomic DNA	Synthetic
Decitabine	Global DNMT	Nucleoside inhibitor		Synthetic
S110 (derivative of Decitabine)	Global DNMT	Nucleoside inhibitor,		Synthetic
Curcumin	Global DNMT	Non-nucleoside inhibitor,	Direct inhibitory interaction of the catalytic site	Natural
EGCG	DNMT1	Non-nucleoside inhibitor,		Natural
Catechin	DNMT1	Non-nucleoside inhibitor,		Natural
Epicatechin	DNMT1	Non-nucleoside inhibitor,		Natural
Quercetin	DNMT1	Non-nucleoside inhibitor,		Natural
Fisetin	DNMT1	Non-nucleoside inhibitor,		Natural
Resveratrol	DNMT	Non-nucleoside inhibitor		Natural
Genistein	DNMT1	Non-nucleoside inhibitor		Natural
resveratrol-salicylate derivatives	DNMT3B			Synthetic
Myricetin	DNMT1	Non-nucleoside inhibitor,		Natural
			block the binding of DNMTs to DNA	
Procainamide	DNMT1	Non-nucleoside inhibitor,		Synthetic
RG108 (found by virtual screening)	DNMT1	Non-nucleoside inhibitor,		Synthetic
Procaine	Global DNMT	Non-nucleoside inhibitor,		Synthetic
SGI-1027 (lipophilic, quinoline-based compound)	Global DNMT	Non-nucleoside inhibitor,	competitive inhibitor of SAM	Synthetic
Zebularine	Global DNMT	Nucleoside inhibitor,	form a covalent complex with DNMT and cytidine deaminase in DNA	Synthetic
Laccaic Acid A	DNMT1		DNA-competitive inhibitor	Synthetic
MG-98 (20 bp anti-sense oligonucleotide)	DNMT1	Non-nucleoside inhibitor,	antisense oligonucleotide binding DNMT1	Synthetic
Theaflavin 3, 3′-digallate N6	DNMT3A	Non-nucleoside inhibitor,	dietary polyphenols from black tea and coffee	Natural
Thearubigin	DNMT3A	Non-nucleoside inhibitor,		Natural
Nanaomycin A	DNMT3B	Non-nucleoside inhibitor,	quinone antibiotic	Natural
Trichostatin A	DNMT3B	Non-nucleoside inhibitor		Natural
Kazinol C	DNMT	Non-nucleoside inhibitor	Pro-apoptotic (via AMPK activation)	Natural

AMPK, AMP-activated protein kinase; CRC, colorectal cancer; DNMT, DNA methyltransferase; EGCG, epigallocatechin gallate; SAM, S-adenosyl-L-methionine.

C35

Forty TET inhibitors were designed using a virtual ligand screening pipeline (Lvspipe), and tested *in vitro* for their inhibitory activity. The results demonstrated that C35, shown in the Figure 5 below, can inhibit the catalytic activities of both TET1 and TET3 with IC50 values of 3.48 μM and 2.31 μM, respectively. Using molecular modeling, the same study reported that there is an interaction between C35 and the TET2 catalytic domain.[108]

Proposed epidrug combinations

The epigenetic modifiers discussed in this article could be used alone or in combination. Combinations of molecules with different mechanisms of action are widely used to treat different diseases, especially cancer.[109] Here, we propose some potential combina-

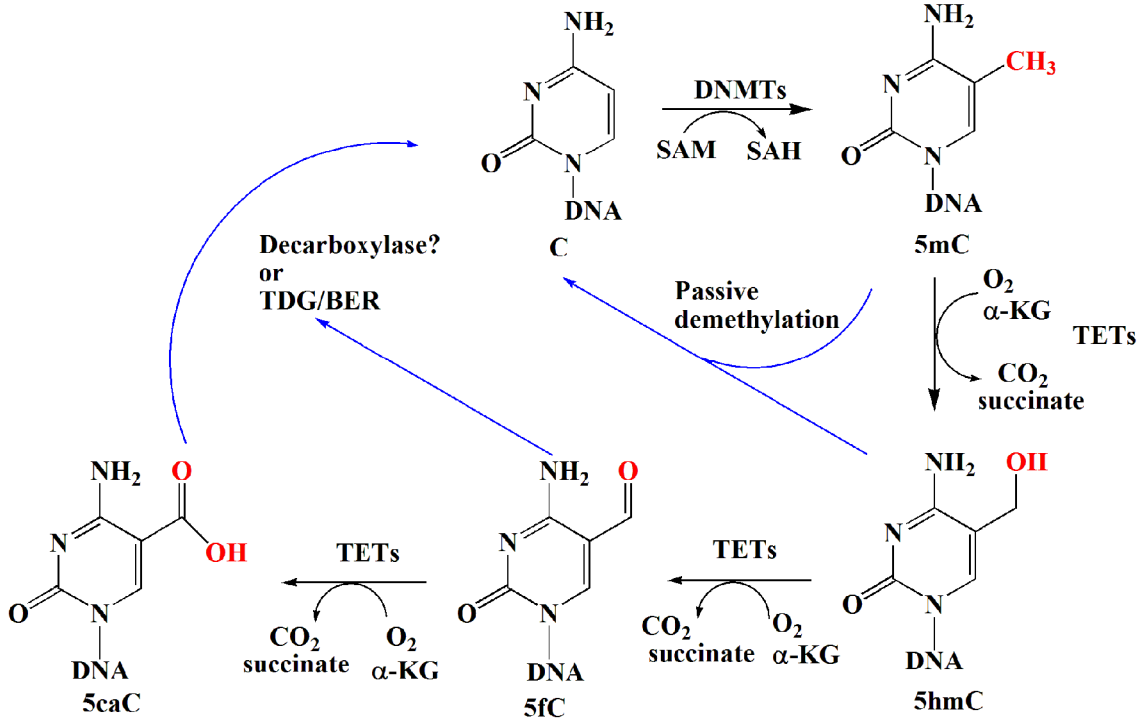

Fig. 4. Successive oxidation reactions of 5mC. DNMT, DNA methyltransferase; SAM, s-adenosylmethionine; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; 5-fC, 5-formylcytosine; 5-caC, 5-carboxycytosine; TET, ten-elleven translocation, 5hmC, alpha ketoglutarate; TDG, thymine DNA glycosylase; BER, base excision repair.

tions of epidrugs that could be useful for the treatment of CRC. These combinations are designed according to different parameters, as shown in the Table 3.

Conclusions

Currently, epigenetic alterations are being increasingly explored as targets in the treatment of cancer, especially CRC. This is because epigenetic modifications are reversible, which means that they can be fixed by molecules called epigenetic modifiers. Among these epigenetic alterations that could lead to CRC carcinogenesis, alterations in the methylation of gene promoters are the most studied, which prompted researchers to look for molecules targeting these alterations. As we have detailed in this article, many natural and synthetic molecules could be interesting candidates as epigenetic modifiers targeting the alteration of methylation in CRC. Two categories of these molecules have been described. The first group consists of DNMT inhibitors, which are hypomethylating (or demethylating) agents that can restore the expression of genes silenced by hypermethylation of their promoters. In the second group, we identified TET inhibitors that could prevent chromosomal instability through decreasing the global hypomethylation of genomic DNA. Finally, these epidrugs could be tested alone, in combination with each other, or with other conventional chemotherapeutic drugs. Although several DNMTis are used in the clinic, there are still some limitations of DNMTis, such as toxicity and lack of selectivity, that could be overcome by strategies like combination with other molecules and/or modification of their chemical structure to develop novel molecules with more efficacy and less limitations.

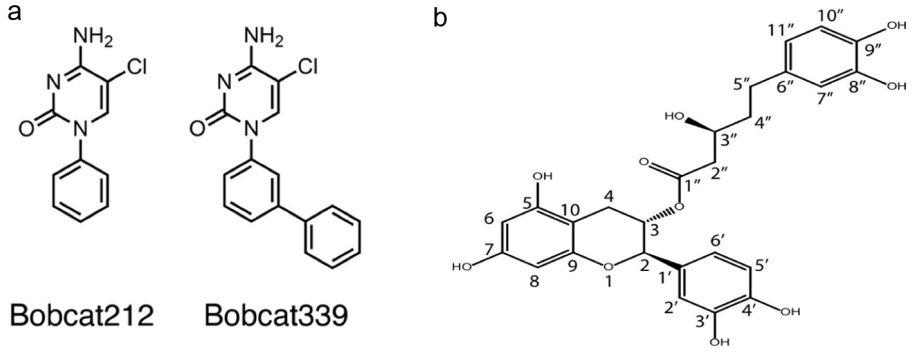

Fig. 5. Hypermethylating molecules. (a) Cytosine derivatives; (b) C35.

Table 3. Some proposed combinations of epigenetic modifiers targeting alterations in DNA methylation in CRC based on their mechanism of action

Parameter	Combination	Example of combination
Targeted enzyme (DNMT)	Inhibitor of DNMT1 + Inhibitor of DNMT3	Procainamide + nanaoycin A Laccaic acid + Trichostatin + Thearubigin
Mechanism of action	Incorporation into the RNA and/or genomic DNA + Direct inhibitory interaction of the catalytic site	Decitabine + curcumin
	block the binding of DNMTs to DNA + competitive inhibitor of SAM	Procaine + SGI-1027
Source	Natural + Synthetic	Decitabine + EGCG Quercetin + resveratrol-salicylate Zebularine + Epichatechin
Chemical structure	Nucleoside + Non-nucleoside	S-110 + Procainamide 5-Azacytidine + RG-108

CRC, colorectal cancer; DNA, deoxyribonucleic acid; DNMT, DNA methyltransferase; EGCG, epigallocatechin gallate; SAM, S-Adenosyl methionine.

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Conflict of interest

The author has no conflict of interest related to this publication.

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