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The clinical landscape of DNA methylation inhibitors in cancer therapy

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Abstract

As we expand our understanding of the complex entanglement between genetic and epigenetic abnormalities that shape the malignant cancer phenotype, epigenetic therapies gain more and more clinical relevance. Pioneering these pursuits is the first generation of DNA methyltransferase (DNMT) inhibitors (DNMTi), which incite global cellular DNA hypomethylation. This elevates tumour immunogenicity by the restoring antigen processing machinery, re-establishing cancer antigen expression, enhancing cytotoxic T cell tumour infiltration and inducing viral mimicry. The resultant anti-tumour response proved effective in eliminating certain myeloid malignancies, with DNMTis receiving FDA-approval for the treatment of multiple leukaemic disorders. However, their application in lymphomas and solid tumours demonstrated disappointing results, paralleling the outcomes obtained with other well-established epigenetic therapies. Because of this, the research focus has largely shifted from monotherapies to combinatorial approaches incorporating alternative oncotherapies. Preclinical research revealed that DNMTi-induced genome-wide demethylation can reverse chemoresistance and T cell exhaustion, thereby sensitizing refractory tumours to chemotherapeutic and immunotherapeutic interventions. They were also shown to synergize with other epigenetic therapies e.g. histone deacetylases (HDAC), lysine-specific histone demethylase 1A (LSD1) and enhancer of zeste homolog 2 (EZH2) inhibitors, greatly reducing tumour growth and progression in mouse models. The potency of combination treatments is evident in *in vivo* models, however translation into the clinic has yielded mixed results. Despite this, they wield immense potential that will require a great deal of clinical testing to be fully realized, although the application of powerful auxiliary technologies like nanoparticle-based drug delivery and CRISPR/Cas9 may accelerate this process.

1. The epigenome

Our understanding of epigenetics has steadily evolved since the concept was brought to light approximately 60 years ago, and its prominence in research has risen exponentially within the last decade. Currently we define epigenetics as the study of heritable and non-heritable changes in cell functions induced by modifications in gene expression unrelated to alterations in the nucleotide sequence. These modifications come in the form of marks that attach themselves either directly to DNA or to histone tails emanating from nucleosome complexes. As the definition suggests, these marks can persist through cell division and can even be passed down generationally.

Histone tails contain a variety of amino acid residues that become sites for epigenetic modifications, e.g. methylation, acetylation, phosphorylation and ubiquitination, which induce changes in nucleosome organization and manipulate the chromatin architecture, ultimately altering gene accessibility and transcription. On top of that, histone modifications also play a role in regulating other DNA processes, including replication and repair(1). Direct DNA modifications in eukaryotes are limited to the covalent binding of a methyl group to the fifth carbon of cytosine and to the nitrogen-6 position of an adenine, forming a 5-methylcytosine group (5mC) and N6-methadenine (6mA) respectively. (2,3). In the human genome, 5mC forms in approximately 1% of cytosines and occurs almost exclusively in regions with a CG dinucleotide combination (CpG)(4). Due to this, most CpG sites in the genome are heavily methylated, except for CpG islands, which are CpG-rich sections of DNA generally containing active gene promoters that are protected from DNA methylation. However, methylation of CpG sites within these promoter islands can result in stable silencing of the corresponding genes, underpinning the role of DNA methylation in regulating transcription(5). The frequency and genomic distribution of methylation can change during embryonic development and can be affected by exogenous factors throughout life, such as environmental agents, pharmaceuticals, diet, and aging. Furthermore, exposure to epigenetic carcinogens can spur abnormal DNA methylation patterns that give rise to cancer(6).

2. DNA methylation machinery

The process of DNA methylation is mainly built upon the interplay of three effector categories: chromosomal writers, readers and editors. Together, they form a highly dynamic and versatile cycle that regulates the global methylation of our genome.

2.1 Writers

In order to bind to DNA, epigenetic marks have to be delivered and affixed to regions of interest by specialized enzymes commonly referred to as writers. In the case of DNA methylation, this function is performed by the DNA methyltransferase (DNMT) family of proteins. Generally, DNMTs will obtain a methyl group from a S-adenosyl methionine (SAM) cosubstrate and catalyse its covalent binding to a cytosine base, forming 5mC (7) (Fig. 1A). The current known members of the DNMT family are: DNMT1, DNMT3A, DNMT3B, DNMT3L and DNMT3C. DNMT3A and DNMT3B are responsible for the de novo methylation of DNA following the mass demethylation event that occurs during zygote formation and early embryogenesis. They have a high affinity for unmethylated DNA and work to reconstruct the rich tapestry of methylation signatures as development progresses(8). DNMT1 is the most abundant DNMT in mammals and is largely involved in the maintenance of these methylation patterns following DNA replication. It achieves this by recognizing hemimethylated DNA post-replication and copying the methylation signatures from the template strand to the newly-synthesized strand, highlighting its role in facilitating the methylation pattern inheritance during cell division(9). Although DNMT1 has a much higher affinity for hemimethylated DNA, both DNMT3A and DNMT3B are likewise essential to the maintenance of methylation, as deletion of either or both of the enzymes results in the

progressive demethylation of a multitude of sequences in embryonic stem cells (ESC)(10). Unlike other members of the family, DNMT3L does not possess the active sites necessary for enzymatic activity, but it functions as a stimulator of the de novo DNMTs and was found to be essential for the methylation of imprinted genes in mouse germ cells(11,12). Finally, DNMT3C was recently discovered in rodents and has originally evolved from DNMT3B. It was found to aid in spermatogenesis through methylation of retrotransposons in male germ cells, thereby preventing their activation and interference with meiosis(13). The mechanisms that drive the recruitment of de novo DNMTs to unmethylated regions of DNA are not fully elucidated yet, however research has found that DNMT3A and DNMT3B are guided by specific post-translational histone modifications, such as H3K36me3, H4R3me2 and H3K9me2, or detect the absence of marks in certain locations, for instance H3K4me3, to induce gene repression (14–17).

2.2 Readers

DNA methylation marks can directly inhibit transcription by blocking the binding of transcription factors to promoter regions. This was shown to be the case for a subset of transcription factors e.g. CTCF and NRF1(18,19). In addition, they can also rely on the activity of specialized reader proteins, otherwise known as methyl-CpG-binding proteins (MBP). MBPs are generally categorized into three families, each one dubbed after the domain responsible for the methyl binding. The first family to be identified contains a methyl-CpG-binding domain (MBD) and its members are appropriately referred to as the MBD-containing proteins(20). As an example, methyl CpG binding protein 2 (MeCP2) is a MBD protein found in high concentrations in neurons. It is essential for the development of the central nervous system and synapse formation, with mutations in MeCP2 leading to neurodevelopmental disorders like Rett syndrome(21). Methyl-CpG-binding Zinc finger (MBZF) proteins rely on zinc finger motifs located at their C-terminus to bind methylated and unmethylated DNA(22). Transcriptional repressor KAISO is a MBZF protein that is implicated in driving a multitude of cancer-related processes upon overexpression(23). The last family employs a Set and RING-associated (SRA) domain to bind exclusively methylated CpG, with a stronger affinity for hemimethylated DNA(24). Ubiquitin-like with PHD and RING finger domains 1 (UHRF1) utilizes its SRA domain to facilitate the recruitment of DNMT1 to hemimethylated DNA following DNA replication(25). The broad function of all MBPs is to attract additional remodellers that can alter the architecture of chromatin and gene expression(26,27).

2.3 Editors

Prominent editors of 5mC are the ten-eleven translocation (TET) family of enzymes, which facilitate the demethylation of DNA. They achieve this through the progressive oxidation of 5mC first into 5-hydroxymethylcytosine (5hmC), then into 5-formylcytosine (5fC) and finally into 5-carboxycytosine (5caC)(28,29). Although all three members of the TET family: TET1, TET2 and TET3, have the capacity to catalyse oxidation reactions, the distribution of their distinct isoforms varies in a cellular context. Broadly, TET1 is expressed in ESCs and most somatic tissues, TET2 is likewise found in ESCs and plays an important role in heamatopoietic stem cell differentiation, while TET3 is absent in ESCs, but appears to be abundant in oocytes and neurons(30). Before and after the final TET-dependent oxidation step, the 5fc and 5caC intermediates can be recognized by thymine-DNA glycosylase (TDG), which excises the modified nucleobase. The vacancy can then be filled in with an unmodified cytosine ring through base excision repair (BER)(31). Alternatively, 5hmC, 5fC and 5caC can undergo oxidative deamination through the action of the AID/APOBEC family of enzymes, converting their internal cytosine bases into uracil and priming them for TDG-mediated base-excision repair, outlining another pathway for active DNA demethylation(32).

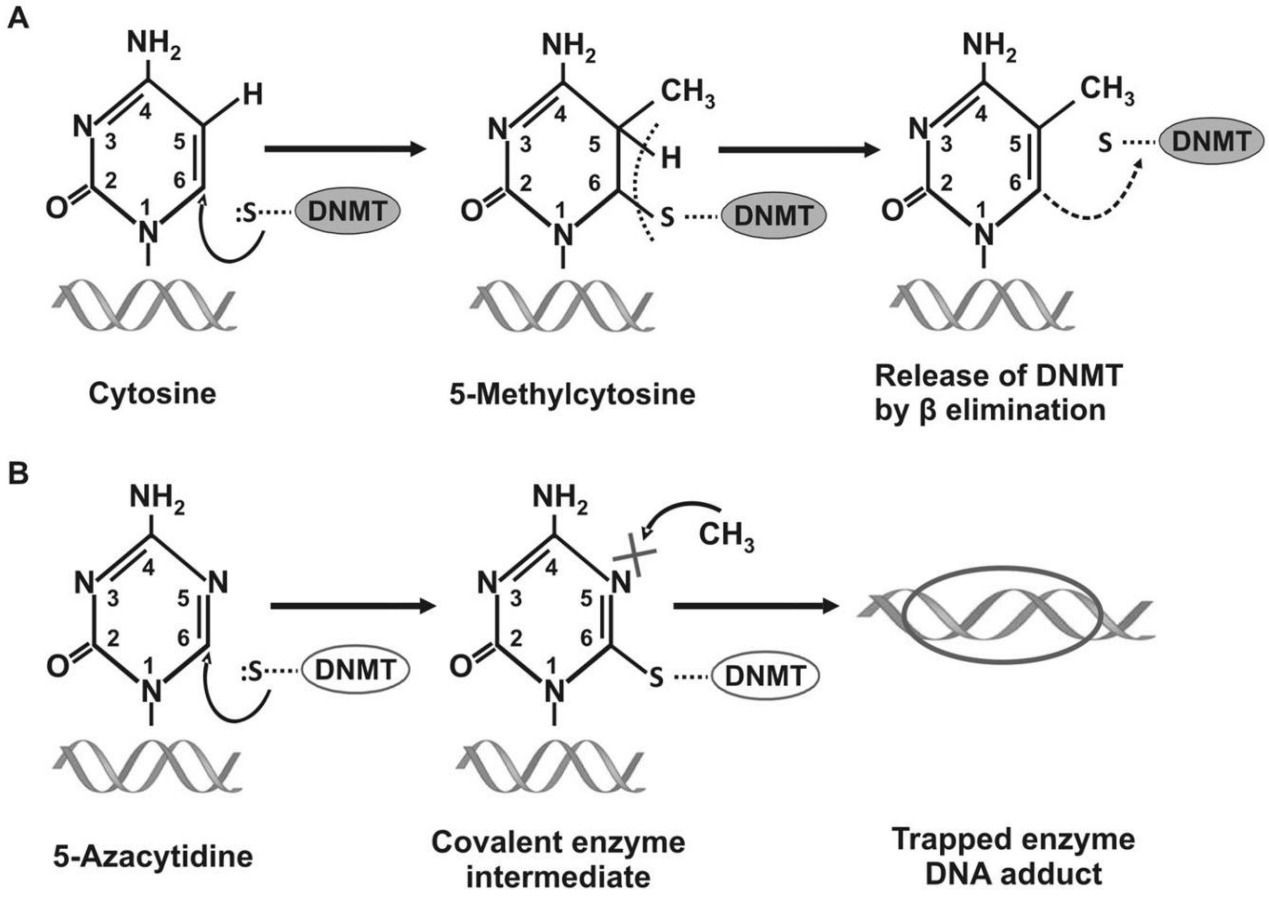

Figure 1. The mechanism of action of first-generation DNA methyltransferase inhibitors. **A)** DNA methyltransferases (DNMT) bind to cytosine in DNA and catalyse methylation of the 5th carbon in the pyrimidine ring, creating 5-methylcytosine. DNMT is then released from DNA through a β elimination reaction. **B)** First generation DNMT inhibitors (DNMTi) decitabine and 5-azacytidine are nucleoside analogues that replace cytosine residues in DNA. They facilitate DNMT binding, but block methylation mark attachment due to the presence of a nitrogen atom in 5-position. The difference in structure also mitigates β elimination-mediated release, which traps the enzyme in DNMT-DNA adducts that lead to DNMT degradation (Adapted from Gnyszka et al. 2013)(33).

3. Functions of DNA methylation in healthy tissues and cancer

3.1 Role of DNA Methylation in development

DNA methylation is imperative for the normal progression of key processes during development. Along with specific histone modifications, it contributes greatly to the phenomenon of genomic imprinting, described as the inherent epigenetic silencing of alleles based on their parental origin. The identity of the gene in question determines whether the maternal or paternal allele is repressed, while the other allele remains active. The process serves a vital dosage regulatory function in normal embryonic growth, with many postnatal diseases e.g. diabetes and obesity, being attributed to a breakdown in genomic imprinting (34,35). Abnormal localization of imprinting marks may also result in aberrant gene expression, leading to the development of debilitating genetic disorders during embryogenesis, like Prader-Willi syndrome or Angelman syndrome(36). Another developmental process that employs DNA methylation machinery is X-chromosome inactivation (XCI), by which a single copy of the X chromosome in female embryo somatic cells becomes permanently silenced. Essential for dosage compensation, XCI induces mass heterochromatization of the X chromosome marked for inactivation, utilizing the stable repressive properties of methylation marks. This entails a mass methylation of CpG islands, which, as mentioned prior, tend to remain unmethylated in active

chromosomes(37). Loss-of-function mutations in DNMTs can result in overactivation of XCI machinery, leading to heterochromatization of active X chromosomes and silencing of X-linked genes, possibly resulting in cell death and embryo loss(38). Additionally, DNA methylation is also instrumental in the silencing of transposable elements (transposons), which are sequences of DNA that can switch their positions within the genome. In this manner, transposons are able to generate chromosomal deletions, inversions, insertions, translocations and duplications that could potentially prove mutagenic(39). The dysregulated expression of transposable elements can contribute to a number of neurodevelopmental disorders, diseases such as haemophilia and Alzheimer's disease, as well as a strong predisposition for cancer(40–43). To avoid large-scale gene disruptions and maintain genomic stability, the DNA methylation effectors collaborate with non-coding RNA to suppress the expression and transposition of these elements(39).

3.2 Tumorigenic effects of DNA methylation

While the significance of the DNA methylation machinery in upholding genomic integrity has been established, it is important to note that cytosine methylation can lead to mutagenesis. Methylated CpGs can undergo deamination prior to any TET-induced oxidation events, in which case the cytosine base is converted into a thymine base, instead of the usual uracil. Although the G-T mismatch can be detected and repaired by TDG, the thymine base renders the process error-prone, potentially resulting in the permanent loss of that particular CpG dinucleotide(44). On a broader scale, the high tendency of 5mC residues to act as mutational hotspots contributes to the continuous depletion of CpG sites from our genome, on top of the potentially oncogenic consequences stemming from the single nucleotide polymorphisms in coding or gene regulatory regions (45).

3.2.1 Protein-coding RNA promoters

Abnormal activity of DNMTs can result in the hypermethylation or hypomethylation of gene promoters, both of which can produce detrimental outcomes in the host. In the case of hypermethylation, DNMTs invade the normally secure CpG islands and block the activity of promoters, potentially silencing the expression of active tumour suppressor genes (TSG). As an example, the expression of DNA repair gene breast cancer gene 1 (*BRCA1*) is often lost in ovarian and breast cancers due to promoter hypermethylation(46). In a similar vein, the promoter of cell cycle regulator *p16* often becomes hypermethylated in gastric cancer(47). Interestingly, reports state that the epigenetic silencing of tumour suppressors rarely coincides with their genetic inactivation(48). However, it is not uncommon for the hypermethylation of a TSG promoter to stimulate mutagenesis in other genes. For instance, the hypermethylation-mediated silencing of DNA repair gene O6-methylguanine DNA methyltransferase (*MGMT*) in head and neck squamous cell carcinoma (HNSCC) increases the susceptibility of *p53* to genetic mutations(49). Gastric and colorectal cancer (CRC) research demonstrated that the downregulation of mismatch repair genes e.g. MutL protein homolog 1 (*MLH1*) through promoter methylation can lead to high degrees of microsatellite instability, promoting further hypermethylation of CpG islands and giving rise to what is commonly called the CpG island methylation phenotype (CIMP)(50,51). Furthermore, mass methylation of CpG islands has been shown to contribute to the loss of imprinting of insulin-like growth factor 2 (*IGF2*), which consequently becomes overexpressed and precipitates the onset of gastric, colorectal, breast and a number of other cancers(52,53).

Hypomethylation of promoters, although far less researched than its hypermethylated counterpart, has also been implicated as a driver of oncogenesis, mainly stemming from the concomitant upregulation of oncogenes and proto-oncogenes. Notably, some cancers display considerable losses in methylation throughout tumour progression, with many metastatic lesions exhibiting greater levels of hypomethylation in comparison to their tumours of origin. This can be observed in metastatic non-small cell lung carcinoma

(NSCLC) and metastatic osteosarcoma, driven by the overexpression of engulfment and cell motility 3 (*ELMO3*) and Iroquois homeobox 1 (*IRX1*) oncogenes respectively(54,55).

3.2.2 Non-coding RNA promoters

Non-coding RNA (ncRNA) are RNA molecules that do not get translated into proteins. Despite that, many of them perform crucial functions at the RNA level and are directly involved in a multitude of cellular processes that are vital for the maintenance of normal cell function. Examples include transfer RNA (tRNA) and ribosomal RNA (rRNA), which are essential for translation, small nuclear RNA (snRNA), which form parts of the spliceosome complex, small nucleolar RNA (snoRNA) that regulate chemical modifications of other RNAs, and finally long non-coding RNA (lncRNA), microRNA (miRNA) and small interfering RNA (siRNA) that regulate gene expression(56). miRNA are the most abundant small ncRNA subtype and their expression is tissue-type specific, suggesting susceptibility to epigenetic regulation, further reinforced by their tendency to be imprinted(57,58). Building on that, studies have determined that the aberrant methylation of miRNA promoters can lead to tumorigenesis. The hypermethylation of the *miRNA-124a* promoter in acute lymphoblastic leukaemia (ALL) reduced the transcription of the miRNA, leading to the upregulation of its direct target, cyclin-dependent kinase 6 (CDK6), inciting the activation of the oncogenic CDK6-retinoblastoma (Rb) pathway. In a similar vein, the tumour suppressor activities of *miRNA-199a* can also be suppressed through promoter methylation, augmenting the progression and invasion of testicular, gastric and ovarian cancers(59–61). In the following years, researchers demonstrated that *miRNA-506* and *miRNA-124* impede the progression of colorectal cancer by inhibiting DNMT1 and DNMT3b activity(62). An ensuing breast cancer study discovered that the epigenetic silencing of *miRNA-506* resulted in overactivation of DNMT1 and the subsequent hypermethylation of the *MEG3* lncRNA promoter, amplifying cancer migration and invasion(63). Besides that, researchers found that the hypermethylation-induced silencing of the lncRNA *MORT* induces immortalization in a wide spectrum of human tumours(64). Recent microarray analysis revealed a correlation between epigenetic silencing of *SNORD123*, *U70C* and *ACA59B* snoRNA genes and incidence of oncogenesis across a variety of human cancers, possibly revealing an unbeknown antitumoral role of snoRNAs(65).

3.2.3 Intragenic methylation

Although DNA methylation blocks transcription initiation, it does not inhibit transcription elongation. In fact, the H3K36me3 histone modification associated with active transcription seems to facilitate the methylation of intragenic DNA loci through the recruitment of DNMTs(66). Genome-wide epigenomic studies reveal a positive correlation between intragenic DNA methylation and gene expression, in both healthy and cancer tissues(67,68). Gene body hypomethylation establishes alternative start sites that can be recognized by transcription machinery, thereby introducing a degree of volatility into the process. The non-canonical transcript variants produced as a result of this may encode protein isoforms with oncogenic properties(69). On top of that, intragenic hypomethylation can reverse the heterochromatization of localized transposable elements, such as *LINE1*, which can induce insertional mutations that drive the progression of a multitude of cancer types(70–72).

3.2.4 Enhancers

A large-scale microarray analysis comparing the methylomes of healthy mature B cells and chronic myeloid leukaemic (CLL) cells revealed that DNA hypomethylation of enhancer sites was the most frequent difference between the two populations(73). Similarly to promoters, the methylation of enhancers attenuates the expression of related genes, but research finds enhancer methylation to be an even more potent predictor of gene expression(74,75). Reinforcing that, a recent lung squamous cell carcinoma (LUSC) study determined

that the methylation of enhancer regions plays a more prominent role in tumorigenesis than the methylation of promoters(76). Although these are established and confirmed patterns, more work has to be done in order to elucidate the complex interplay between DNA methylation, enhancers, and transcription machinery, and to identify a potential causality between enhancer methylation and enhancer regulation.

4. DNA methylation as a target for cancer therapy

4.1 Interrogating the cancer methylome

Development and rapid advancement of DNA sequencing technologies enabled genomic profiling of a large collection of tissue samples and generation of concise databases that permit an extensive comparison of healthy and affected tissues. This form of lateral analysis exposes the medley of genetic aberrations that drive or contribute to cancer pathophysiology and progression. Amidst this sequencing revolution, an array of protocols have been developed for the interrogation of the cellular epigenome, which in combination with high-throughput sequencing technology, can help further elucidate complex tumorigenic mechanisms. Bisulphite conversion sequencing (BS-seq) is currently deemed to be the gold standard for genome-wide DNA methylation profiling. It relies on the bisulfite-induced deamination of cytosine nucleobases, converting unmethylated cytosines into uracil, while leaving 5mC groups unaffected(77). The onslaught of epigenomic analysis in the wake of this technology, such as the large-scale multi-dimensional analysis of 33 distinct cancer types by The Cancer Genome Atlas (TCGA) project, revealed tissue-specific alterations in DNA methylation profiles. Coupling this data with their other sequencing and cataloguing efforts, including the ascertainment of tumour grade and patient prognosis, grants additional insights into the inner machinations of varying cancer types, allowing for expanded stratification of tumour subgroups and inference of subgroup-specific therapeutics(78,79).

Aberrant DNA methylation signatures are some of the most common oncogenic alterations and can be encountered quite early in development(80). Methylation marks are ubiquitous and highly stable; hence, they can serve as convenient biomarkers for specific cancer subtypes. As an example, CIMP-positive CRC consistently exhibits hypermethylation of the *p16*, *MLH1* and thrombospondin (*THBS1*) promoters(81). On the other hand, CIMP in hepatocellular carcinoma (HCC) has been characterized by a wide array of methylation-induced gene silencing patterns, with varying panels of affected genes being linked to cancer progression and diverse tumour characteristics. Researchers work to disambiguate these methylation biomarkers by finding links between individual alterations and patient clinicopathological parameters e.g. *SOCS1* promoter methylation was correlated with age, tumour size and tumour progression(82,83), while methylation of *GSTP1* was associated with gender, alcohol consumption and reduced overall survival(84,85). Building up on that, some groups are starting to stratify CIMP-positive HCC subgroups based on their tendency to induce somatic mutations(86). Ultimately, the aim is to elucidate the mechanisms underlying hypermethylation-induced cancers, while inferring the prognostic and diagnostic values of related biomarkers and perhaps developing suitable therapies. Currently, numerous in vitro diagnostic (IVD) tests based on the detection of hypermethylated DNA in cancer have been developed, encompassing colorectal, breast, liver, bladder, and cervical malignancies, with multiple CRC IVDs achieving FDA pre-market approval. Despite this, clinical translation is proving to be arduous, demanding tremendous time and monetary investments(87).

4.2 DNMT inhibitors as oncotherapies

Our frontline assets in combating CIMP and promoter hypermethylation-related malignancies are DNMT inhibitors (DNMTi). The two major DNMTis actively being employed in the clinic are azacitidine (5-aza) and decitabine (DAC). Both drugs function as cytosine analogues, allowing them to become incorporated within DNA in place of regular cytosine groups. The analogues facilitate the binding of DNMTs; however, they prevent

their methylation activity and block the beta elimination reaction that releases DNMTs from DNA, covalently trapping and sequestering the enzyme on the DNA double strand (Fig. 1B). The abducted DNMT hinders DNA functionality, setting off DNA damage signals that degrade the enzyme. As a result of this, methylation signatures cannot be passed down during DNA replication and gradually become depleted from the system(88). 5-aza is a ribonucleoside, meaning it can integrate itself into both DNA and RNA. Approximately 80-90% of azanucleosides become incorporated in RNA, where they alter the structure of rRNAs and tRNAs, leading to malfunctions of polyribosomes and tRNA acceptor stems, thereby inhibiting translation initiation in affected cells(89). At higher doses, the breakdown of protein synthesis machinery can generate lethal levels of cytotoxicity. Through the action of ribonucleotide reductase (RR) enzymes, the remaining 10-20% of azanucleosides are converted into deoxyribonucleosides, which can insert themselves into the DNA sequence(90). On the other hand, DAC consists of deoxyribonucleosides and does not require RR to become incorporated into DNA. Paralleling 5-aza, higher dosages of DAC can also lead to cytotoxic cell death, as the accumulation of DNMT-DNA adducts can stall replication fork formation and impede DNA synthesis (91). Due to their mechanisms of action, DNMTis require target cells to possess the capacity to divide in order to be efficacious. Their efficiency, and hence cytotoxicity, is enhanced in rapidly dividing cells, underpinning their potent anti-neoplastic effects(92).

Both 5-aza and DAC have been approved by the FDA for the treatment of myelodysplastic syndrome (MDS)(93,94). 5-aza has also been approved for the treatment of juvenile myelomonocytic leukaemia (JMML) in paediatric patients, while DAC, in combination with cytidine deaminase inhibitor (CDAi) cedazuridine, was approved for the treatment of chronic myelomonocytic leukaemia (CMML) (95). More recently, 5-aza has been FDA-approved for the treatment of acute myeloid leukaemia (AML), in combination with BCL-2 antagonist venetoclax, as well for remission maintenance therapy(96,97). On top of the two prevalent DMNT inhibiting drugs, purine nucleoside DNMTi clofarabine has been approved by the FDA as alternative treatment for ALL(98), along with arsenic trioxide, which has been FDA-approved as a therapy for relapsed or refractory acute promyelocytic leukaemia (APL)(99). Evidently, DNMTis have proven to be effective in myeloid malignancies, spurring many clinical trials to investigate their efficacy in lymphoid neoplasms(100–105). Monotherapeutic approaches yielded disappointing results and displayed tenuous clinical benefits in patients(100,102), prompting the emergence of combinational therapies(103,105,106). Similar patterns were observed in a variety of solid tumours, as DNMTi monotherapies demonstrated low response rates, marginal clinical benefits and potent adverse effects, aggravated further by the rapid development of drug resistance(107–110). Recent efforts combining the transient hypomethylation effects of 5-aza and DAC with alternative oncotherapies, e.g. immunotherapy or chemotherapy, displayed promising synergistic effects in patients with cervical, ovarian, oesophageal, gastric, colorectal and metastatic skin cancers, which will be expanded upon later in the review(111–115).

Despite the significant advances achieved in 5-aza and DAC therapeutics, the drugs are regarded as highly toxic and chemically unstable, often marked by low bioavailability. In response to these limitations, researchers have developed alternative cytidine nucleoside analogues that could represent the next generation of DNMTis(33). Zebularine was the first novel cytidine analogue to be developed after the initial pair, differentiating itself through the possession of a longer half-life and strongly reduced toxicity, both *in vitro* and *in vivo*(116,117). Although zebularine has not seen involvement in any clinical trials, preclinical studies have demonstrated its efficacy as a demethylating agent with significant anti-neoplastic attributes(118,119). Furthermore, zebularine was shown to potentiate the effects of other epigenetic therapies, including DAC(118,120). A major drawback of first generation DNMTis is their susceptibility to deamination by cytidine deaminases (CDA), which pharmacologically inactivates them, resulting in reduced drug longevity in plasma and tissues with high levels of CDA(121,122).Next generation DNMTi, guadecitabine, is a dinucleotide consisting of decitabine and deoxyguanosine, which confers the analogue component with a resistance to CDA-mediated degradation. The clinical efficacy of guadecitabine has been confirmed in MDS and AML, however its high cost, as well as its negligible improvements to overall patient survival when compared to 5-aza and DAC, depreciate its value in the clinic(123–125). On top of the two mentioned here, a

multitude of nucleoside analogues have demonstrated certain improvements over first generation DNMTis in a preclinical setting, with some being currently engaged in clinical trials e.g. 4'-thio-2'-deoxycytidine (TdCyd), RX-3117 and 5-Fluoro-2'-deoxycytidine(126).

Besides the nucleoside analogue DNMTis, other drug types have been found to possess the capacity to inhibit DNMT activity. The recently discovered non-covalent DNMT1 inhibitor GSK-3484862 was used to induce the DNA demethylation of erythroid progenitor cells, demonstrating more controlled levels of cytotoxicity compared to DAC(127). Some of these DNMTis have reached phase III in clinical trials, such as the antihypertensive hydrazaline in ovarian cancer and the polyphenol resveratrol in lymphangioleiomyomatosis (LAM), but none of them have been clinically approved as of this review(126,128,129).

4.3 Targeting the cancer epigenome with DNMT inhibitors

The cancer epigenome often displays sweeping losses in DNA methylation, excluding CpG island regions, which often undergo extensive cancer-specific hypermethylation. Silencing of CpG island promoters can lead to the inactivation of essential TSGs and DNA repair genes, inciting tumour development and progression. DNMTis counteract this by inducing global DNA hypomethylation, restoring the expression of tumour suppressors and epigenetically reprogramming cancer cells to discontinue their aberrant self-renewal and invasion patterns. This is achieved in part through the conversion of immune cold tumours, which evade and suppress the patient immune response, into immune hot tumours, which are exceedingly immunogenic and often bear a higher mutational burden(130) (Fig. 2). The escalation in immunogenicity enhances cancer cell recognition and expedites adaptive immune system activity, thereby initiating a potent anti-tumour response.

In-depth molecular dissection of the tumour microenvironment reveals the multifaceted contribution of DNMTis to this phenotypic conversion. DNA hypomethylation upregulates major histocompatibility complex-class I (MHC-I) expression, restoring antigen processing and presentation functions in cancer cells and the TME(131,132). This is supplemented by the increased expression of tumour-specific cancer testis antigens (CTA), e.g. melanoma-associated antigen 1 (*MAGE1*), which MHC-I can present to CD8+ cytotoxic T cells (CTL) to augment immune recognition of cancer cells and initiate an anticancer immune response(133,134). Antigen presentation kickstarts CTL maturation, after which CTLs will migrate to the region of interest to target CTA-expressing cells, however they are rarely able penetrate the dense tumour foci and tend to colocalize with the peripheral extracellular matrix (ECM)(135). DNMTis upregulate the expression of CXC chemokine receptor 3 (*CXCR3*), as well as its ligands CXC chemokine ligand 9 (*CXCL9*/*CXCL10*/*CXCL11*), potentiating CD8+ T cell penetration of the tumour lesion and increasing its internal concentration of effector and memory CTLs(131). Immune cold tumours are characterized by the accumulation of immunosuppressive cell populations in their foci, e.g. tumour-associated macrophages (TAM) and myeloid-derived suppressor cells (MDSC), which maintain tumour integrity by inhibiting lymphocyte function. DNMTis help counteract this by hypomethylating promoters for tumour necrosis factor α (*TNFα*), restoring cytokine production and promoting TAM depletion from the tumour core(136). Without the immunosuppressive influence of TAMs, tumour infiltrating CTLs are more inclined to target and lyse tumour cells. Concurrently, DMNTis lead to the demethylation of endogenous retrovirus (ERV) sequences, which are elements of retroviral genomes that can integrate themselves into germline DNA, thereby becoming endogenous in the human genome. They comprise approximately 5-8% of our genome, where they are ubiquitously distributed, excluding regions of satellite DNA and transcription start sites(137). ERV transposons tend to be methylated to maintain their transcriptional quiescence, however treatment with DNMTis rapidly reactivates their expression, initiating an endogenously stimulated viral mimicry immune response. The subsequent surge in ERV double-stranded RNA (dsRNA) localized in the cytosol is detected by intracellular pattern recognition receptors (PRR), such as melanoma differentiation-associated protein 5 (MDA5) or retinoic acid-inducible gene I (RIG-I), which activate IkB kinase-ε (IKKε) and TANK-binding kinase 1(TBK1) through the action of aggregated mitochondrial antiviral-signalling proteins (MAVS)(138,139). TBK1 and IKKε activate interferon regulatory factors 3 (IRF3) and 7 (IRF7),

which form a transcriptional complex with NF-kB to induce the expression of type I interferons (IFN-I)(140). IFN-I promote the expression of antigen presenting machinery components through the JAK-STAT pathway and enhance the production of CXCL9/CXCL10/ CXCL11, reinforcing the immunogenic effects of DNMTis(141,142). Furthermore, IFNs-I induce the expression of interferon stimulated genes, e.g. STING, which in turn will expedite IFN-I generation through a positive feedback mechanism, exponentially intensifying the antitumour response(143).

Studies show that tissue-specific variations in chromatin organization, including those found in tumour, are caused in part by concomitant differences in methylation status. Cohesin is a protein complex that facilitates DNA looping and the subsequent formation of topologically associated domains (TAD), which are usually defined by CTCF transcription factors acting as insulator proteins(144). TADs become sequestered from neighbouring genomic regions, limiting interactions of DNA regulatory elements only to other elements within the same TAD and restricting cross-TAD interactions between regulatory elements(145,146). CTCF is sensitive to DNA methylation, therefore influencing TAD formation. Aberrant hypermethylation of these sites can lead to major disruptions in chromatin topology, resulting in potentially oncogenic interactions between previously unassociated regulatory elements(147). Furthermore, researchers linked losses in CTCF activity to unique tissue-specific hypermethylation signatures associated with prostate and breast cancer progression(148). DNMTi hypomethylation can restore cohesin- and CTCF-mediated TAD formation, reforming proximal topological structures and halting tumorigenic enhancer/silencer activity(147).

4.4 DNMT inhibitors - a double-edged sword

Besides boosting the patient's immune activity and inhibiting tumorigenic enhancer activity, DMNTis can also have a profound effect on genomic stability. They aid DNA damage response by re-establishing the expression of DNA repair genes and reinstating stalled DNA damage surveillance mechanisms. These antimutagenic effects are further reinforced by the concomitant re-expression of TSGs, which diminish the DNA replication stress produced by active oncogenes(149,150). However, DNMTi-induced global hypomethylation also entails renewed expression of previously silenced oncogenes, instituting a balancing act between drug benefits and detriments. Research largely demonstrates that genome-wide hypomethylation results in a net increase in chromosomal instability, exacerbating cancer initiation and progression(151,152). Simultaneously, the increased mutational burden generated because of this, coupled with the cytotoxic potential of DNMTis, strongly promotes cancer cell apoptosis. The collateral toxicity, combined with the toxicity generated by a myriad of off-target effects, can give rise to a multitude of severe haematological adverse effects, such as myelosuppression, bleeding or anaemia, often alongside nausea, vomiting and fatigue(126).

The efficacy of cytidine nucleoside analogues is limited to dividing cells, specifically cells in S-phase, which is vital for their incorporation into the DNA sequence. This limitation offsets DNMTi adverse effects by introducing a more focused therapeutic window and curtailing unwanted hypomethylation. Cancer cells lack cell cycle control and undergo accelerated cell division, rendering them more prominent targets of this therapy(92). Unfortunately, only a portion of cancer patients responds to hypomethylating agents, and it is quite common for treatment-sensitive patients to lose responsiveness over time, ultimately developing a secondary resistance that greatly reduces their overall survival(153). Patient prognosis can worsen in cases of cross-resistance, where treatment with one DNMTi induces resistance to other hypomethylating agents, often leaving patients with no approved follow-up treatment options(154,155).

5. DNMT inhibitor combination therapies

DNMTi monotherapies demonstrate limited clinical benefits in lymphoma and solid tumour patients. Even in myeloid malignancies, where their bioactivity is the highest, patient response is often poor and resistance is commonplace; ergo, the interest in determining their combinational potential. DNMTis were found to sensitize tumours to several clinically relevant therapies, in some cases even augmenting their potency, underpinning the promise of hypomethylating agents as adjuvant oncotherapies.

5.1 Combination with targeted cancer therapies

Researchers speculated whether the global epigenetic alterations induced by DNMT inhibition could enhance the efficacy of targeted molecular therapies, such as tyrosine kinase inhibitors (TKI). Despite strong initial response rates, targeted interventions often fail in advanced cancers, as tumours tend to evolve a resistance that eventually leads to relapse. The mechanisms contributing to secondary resistance are poorly understood and vary depending on drug targets, but they generally involve a multifactorial adaptation built on an interlaced lattice of genetic and epigenetic aberrations. More concretely, aside the heterogeneity generated by the mutational status of a driver oncogene e.g. epidermal growth factor receptor (*EGFR*), the epigenetic signatures associated with its promoter were also determined to contribute to primary and secondary resistance against EGFR-TKIs. As an example, analysis of 3 NSCLC cell lines with varying levels of sensitivity to EGFR-TKI gefitinib revealed a positive correlation between EGFR promoter methylation and gefitinib resistance. Unlike gefitinib monotherapy, the combination of DAC and gefitinib in refractory cells inhibited their proliferation and increased the incidence of apoptosis, hinting at drug synergy(156). Analysing liquid biopsies taken from a cohort of 127 NSCLC patients, researchers linked intrinsic resistance to EGFR-TKIs gefitinib, erlotinib and afatinib to increased methylation of Homeobox B9 (*HOXB9*) gene enhancers, potentially revealing a predictive function for the methylation status of HOXB9 regulatory elements. Another correlation was drawn between EGFR-TKI resistance and the aberrant hypermethylation of *PTEN* promoters, which augments the phosphorylation of AKT, as well as the subsequent activation of cyclin-D1 and intracellular adhesion molecule-1 (ICAM-1), thereby accelerating tumour progression and cancer cell migration(157). Additionally, studies have linked the aberrant hypermethylation of *RASSF1A*, *GADD45β*, *KL* and *S100P* promoters to EGFR-TKI resistance, further exposing potential vulnerabilities to DNMT inhibition(158–160). As of this review, no confirmative clinical studies have been put in motion, but DNMTi co-administration could theoretically delay the onset of TKI resistance, although tumour heterogeneity will surely hinder the efficacy of this combinational therapy. However, addressing epigenetic resistance factors can elevate our comprehension of refractory mechanisms, paving the way for further advancements.

As stated previously, DNMTis generate DNMT-DNA adducts that produce genome-wide DNA lesions, which contribute to the drugs' antineoplastic efficacy. The genomic instability induced by the resultant accumulation of DNA single- (SSB) and double-strand breaks (DSB) is counteracted by poly (ADP-ribose) polymerases (PARP), preservers of genomic stability that can detect DNA damage and initiate a variety of repair pathways, based on the nature and extent of the damage. Generally, upon detection of direct SSBs, PARPs recruit the scaffold protein X-Ray Repair Cross Complementing 1 (XRCC1), which in turn recruits a number of polypeptides that facilitate each stage of DNA repair, specifically DNA end processing, gap filling and ligation(161). DAC treatment boosts PARP activity, leading to increased expression of XRCC1 and promoting its abundant colocalization with trapped DNMT1 foci for the purpose of augmenting SSB repair(162). PARPs are also highly sensitive for DSBs, inciting their repair through the synthesis of negatively charged ADP-ribose polymers, thereby inducing local histone displacement(163). This creates a platform for the recruitment MRE11, Nibrin (NBS1) and Rad50 proteins, forming the MRN complex that boosts DNA repair fidelity by promoting homologous recombination (HR) repair in DSBs(164,165). PARP inhibitors (PARPi), e.g. olaparib and talazoparib, trap PARPs at DNA damage sites and mitigate their catalytic activity, consequently diminishing XRCC1-mediated SSB repair. The resultant accumulation of SSBs leads to higher rates of replication fork

stalling, causing them to degrade into more unstable DSBs(162,167). Concomitantly, PARP inhibition prompts the substitution of high-fidelity HR with low-fidelity non-homologous end joining (NHEJ), which can generate tumoricidal levels of genomic instability in HR-deficient cancer cells e.g. human breast and ovarian tumours with BRCA1/2 inactivating mutations(168). This synthetic lethality can be further exacerbated through the coadministration of DNMTis, as XRCC1 factors lose their ability to efficiently detect and clear DNMT-DNA adduct-induced damage, resulting in a buildup of SSB and DSBs that dramatically diminishes cell survival. A panel of AML cell lines was treated with a combination of DAC and olaparib to confirm their synergistic production of DSBs and the significant increase in cell death, as compared to DAC monotherapy(162). A follow-up phase I clinical trial utilized a combination of DAC and talazoparib in relapsed/refractory AML, employing a 3+3 dose escalation strategy. The combination was well tolerated, with 8% of patients demonstrating complete remission with incomplete count recovery and 12% presenting with haematologic improvement. Despite the low response rates, researchers noted a link between mutational signatures and treatment efficacy, advocating for a patient stratification approach(169). Currently recruitment is underway for a phase I clinical trial combining talazoparib with ASTX727, a fixed-dose mixture of DAC and cedazuridine, in triple negative or hormone resistant/HER2-negative metastatic breast cancer(170). It is important to note, that samples with low BRCA1/2 expression stemming from promoter hypermethylation may be inherently resistant to the treatment combination, as the DNMT-induced demethylation of these promoters will work to restore the process of HR, counteracting the effects of the PARPi(168).

5.2 Combination with chemotherapy

DNMTis promote global chromatin relaxation, generally potentiating the efficacy of genotoxic chemotherapy. Platinum-based chemotherapies, e.g. cisplatin and carboplatin, are currently some of the most utilized anti-cancer agents, producing a strong initial response in most cancer patients, which will likely diminish over the course of treatment as many patients become resistant. Researchers working with cisplatin-refractory cancer cell lines ascertained that the factors differentiating cisplatin-sensitive and cisplatin-resistant tumours may arise from differences in the epigenome, with BS-seq revealing 14 gene promoters that undergo hypermethylation in resistant cell lines. 6 of the genes were found to be inducible by cisplatin in sensitive cells, suggesting that chronic cisplatin exposure generates selection pressure that can lead to aberrant epigenetic silencing of genes and a subsequent development of chemotherapy resistance(171). DNMTi-mediated hypomethylation promotes global chromatin relaxation and reactivation of relevant gene promoters, priming tumours for chemotherapy and countering chemoresistance by restoring essential oncoprotective signalling pathways. A study on nasopharyngeal carcinoma (NPC) employed DAC to inhibit the hypermethylation of *ARNTL*, which mitigates the dysregulated expression of cyclin-dependent kinase 5 (CDK5), thus inhibiting NPC proliferation and sensitizing the tumour to cisplatin treatment both *in vitro* and *in vivo*(172). DAC-induced demethylation of the MLH1 promoter was shown to confer cisplatin sensitivity to ovarian cancer and HNSCC cells, as well as HNSCC xenografts(173,174). In the realm of platinum-resistant high grade serous ovarian cancer, sequential treatment with 5-aza and carboplatin decreased tumour proliferation and enhanced cancer cell cytotoxicity(175). DAC also restores *RASSF1A* expression in bladder cancer cells, activating the Hippo signalling pathway, which inhibits tumour growth and promotes apoptosis, while simultaneously enhancing cisplatin cytotoxicity(176).

Besides platinum-based drugs, DNMTis were also shown to have a potentiating effect on a variety of alternative types of chemotherapy. As an example, DAC-mediated *RASSF1A* re-expression also sensitized bladder cancer cell lines to doxorubicin, an anthracycline anticancer antibiotic that interferes with DNA damage repair by inhibiting topoisomerase II and driving controlled cancer cell death(176,177). Treatment of chemoresistant ovarian cancer cell lines with DAC was shown to reverse their resistance to paclitaxel, a taxane that inhibits microtubule depolymerization and blocks mitotic progression, through the upregulation of negative growth factor Transforming growth factor-beta-inducible gene-h3 (*TGFBI*)(178,179). Gastric cancer cells and xenografts resistant to irinotecan, a topoisomerase I inhibitor that induces replication fork arrest,

demonstrated increased expression of tumour suppressor RUNX3 following DAC treatment, which resensitizes them to irinotecan and promotes cancer cell apoptosis (180,181). Finally, administration of DAC within *in vitro* and *in vivo* basal-like bladder cancer models re-established the transcription of *SOCS3*, which inhibited STAT3-mediated self-renewal of cancer stem cells, rendering them tumour sensitive to nucleoside analogue gemcitabine (182).

The synergistic cytotoxicity derived from DNMTi pre-treatment displayed robust potential for the evolution of clinical chemotherapeutic strategies. A pilot clinical trial combined a range of DAC concentrations with fixed doses of cisplatin, administered consecutively in a small sample size of patients with varied solid tumours, demonstrated a disappointingly limited response rate(183). A follow-up study with cervical cancer patients introduced a 21-day cyclical regime with 3-day alternating infusions of DAC and cisplatin, which yielded a partial response in 38.1% and stabilized the cancer in 23.8% of enrolled patients(111). Two separate phase II clinical trials involving platinum-resistant ovarian cancer patients employed low- and high-dose DAC prior to carboplatin administration. Lower dosage patients displayed higher response rates compared to the carboplatin monotherapy control group, while high DAC dosages impeded the chemotherapeutic effects and raised the incidence of adverse effects, such as neutropenia and carboplatin hypersensitivity(112,184). Differences in the treatment plans preclude a direct comparison between the two studies, however it is pertinent to note that a majority of subsequent studies employing first generation DNMTis utilized low drug dosages, at least in part to minimize the occurrence of off-target effects (102,113,174,185). One of the more recent clinical trials achieved an objective response rate (ORR) of 40.74% in platinum-refractory ovarian cancer patients, through the combination of a low-dose DAC pre-treatment and the subsequent coupled administration of carboplatin and paclitaxel(113). In the realm of lymphoma, a combination of oral 5-aza with R-CHOP, an amalgamation of 5 chemotherapies often utilized in lymphoma treatment, produced an exceptional 94.9% ORR in diffuse large B-cell lymphoma (DLBCL) patients, with 88.1% of patients experiencing a complete response(103). Despite these great improvements, the objective efficacy of this combinatorial therapy still leaves a lot to be desired in a majority of cancer types, with many recent clinical trials struggling to recreate the outstanding results obtained by their predecessors(186–188). To that end, researchers are investigating potential interventions that could further synergize with this treatment and obtain greater clinical benefits.

5.3 Combination with other epigenetic therapies

Having established the crucial role of aberrant epigenetic regulation in tumorigenesis, researchers sought to uncover epigenetic manipulations that could hinder or halt tumour progression, as the complex and multifaceted nature of the cancer epigenome inherently advocates for combinational interventions to maximize treatment efficacy. The most prevalent coupling of epigenetic therapies is the combination of DNMT and histone deacetylase (HDAC) inhibitors (HDACi), which has recently begun undergoing clinical translation. The rationale behind this approach considers that transcriptionally repressed heterochromatin is not only characterized by pervasive DNA methylation, but also a deficiency in histone tail acetylation. Acetylation neutralizes the positive charge of histone lysine residues, thereby decreasing the interaction between histone N termini and negatively charged DNA components, causing chromatin to adopt a more relaxed and transcriptionally active conformation(189). HDACs act to remove the acetyl functional group from histone tails, promoting heterochromatization and gene repression, which can be counteracted through HDACis e.g. vorinostat and romidepsin. Like DNMTis, single-agent HDACis display limited oncotherapeutic competencies, since individually they only manage to re-express a portion of epigenetically silenced TSGs(190–196). To that end, scientists contemplated the benefits of the combined inhibition of both transcriptional repressors. This is outlined in a NSCLC study, where the coadministration of 5-aza and pan-HDACi ITF-2357 lead to additional re-expression of several heavily hypermethylated TSGs, increased expression of CTAs and heightened ERV-induced IFN-I signalling (166). On top of that, the dual inhibition downregulated levels of *MYC*, an oncogenic transcription factor implicated in the initiation and progression

of multiple cancer subtypes(197). In mouse models, MYC dampens host antitumour response by upregulating the expression of immune checkpoint proteins programmed death-ligand 1 (PD-L1) and cluster of differentiation 47 (CD47) in the TME, which cooperate with a congregation of TAMs within the tumour core to enable evasion of both the innate and adaptive immune systems(198)(Fig. 2). Simultaneous inhibition of DNMTs and HDACs surpasses their individual monotherapies, as it depletes PD-L1 and CD47 from the TME, while greatly reducing accumulation of both TAMs and MDSCs at the tumour lesion, thereby promoting superior CTL tumour infiltration and M1 macrophage-mediated cancer cell phagocytosis. Additionally, repression of *MYC* lead to increased expression of MHC-I components and chemokines, such as CCL5, potentiating antigen presentation and further enhancing CTL intra-tumoral infiltration(166,199–201). Ultimately, researchers determined that the application of a HDACi, preceded by a low-dose DNMTi treatment, greatly augmented the latter's antiproliferative effects in cancer cell lines, and helped mitigate the tumour burden and metastasis in mouse xenografts (166,202–205). A phase II study combined oral 5-aza with romidepsin in treatment-naïve or relapsed/refractory peripheral T cell lymphoma (PTCL) patients, achieving an overall ORR of 61% and giving rise to two ongoing follow up studies(206). Unfortunately, these robust benefits rarely manifest in other clinical trials, as ORRs in solid tumours remain staggeringly low and few to no improvements have been observed in myeloid malignancies, although many trials are still underway(207–211).

Chromatin immunoprecipitation (ChIP) microarray analysis of patient-derived prostate cancer cells revealed a population of transcriptionally silenced promoters with low levels of DNA methylation, but enriched for repressive H3K27me3 histone post-translational modifications(212). Furthermore, gene expression analysis of cells post-DNMTi monotherapy revealed that many promoters undergoing demethylation remain inactive, caused in part by an epigenetic switch that imparts H3K27me3 marks onto relevant histone regions(213). Placement of these methylation marks is executed by polycomb repressive complex 2 (PRC2), catalysed by its enhancer of zeste homolog 2 (EZH2) subunit, making it an attractive drug target(214). A phase I clinical trial employing a single EZH2 inhibitor (EZHi) GSK126 was launched in 2014, but had to be terminated prematurely due to insufficient evidence for clinical benefit. Since then, the synergistic anti-tumour effects of DNMT and EZH2 dual inhibition have been established in a number of in vitro and in vivo cancer models(215–217). Mechanistic analysis of the combined therapy revealed increased TSG expression, viral mimicry activity and immunogenicity in comparison to DNMTi monotherapy. Nonetheless, the clinical efficacy of this combination has not been investigated as of this review.

Although histone methylation can repress gene expression, specific methylation signatures can also activate transcription, drawing attention to histone demethylases e.g. lysine-specific histone demethylase 1A (LSD1). LSD1 demethylates mono- and di-methylated H3K9 and H3K4 lysines, which are known promoter-activating signatures(218). Accordingly, LSD1 overexpression has been implicated in the formation and progression of an assortment of cancer types, often being associated with poor prognosis in patients(218–222). To that end, researchers devised an abundance of reversible and irreversible LSD1 inhibitors (LSD1i), many of which are currently employed in clinical trials. LSD1i clorgyline was found to synergize with 5-aza, as it helps re-establish the expression of many abnormally silenced TSGs and CTAs by enriching the chromatin for H3K4me2 and H3K4me1. Ultimately the dual inhibition bolsters chromatin relaxation and strongly contributes to the restoration of MHC-I antigen presentation machinery in bladder cancer cells, sensitizing cells to additional genotoxic and immunotherapeutic strategies(223). Their combined efficacy is further reinforced in a 2018 study, where LSD1i TPC-144 sensitized DNMTi-resistant human AML cells lines and mouse xenografts to DAC treatment(224).

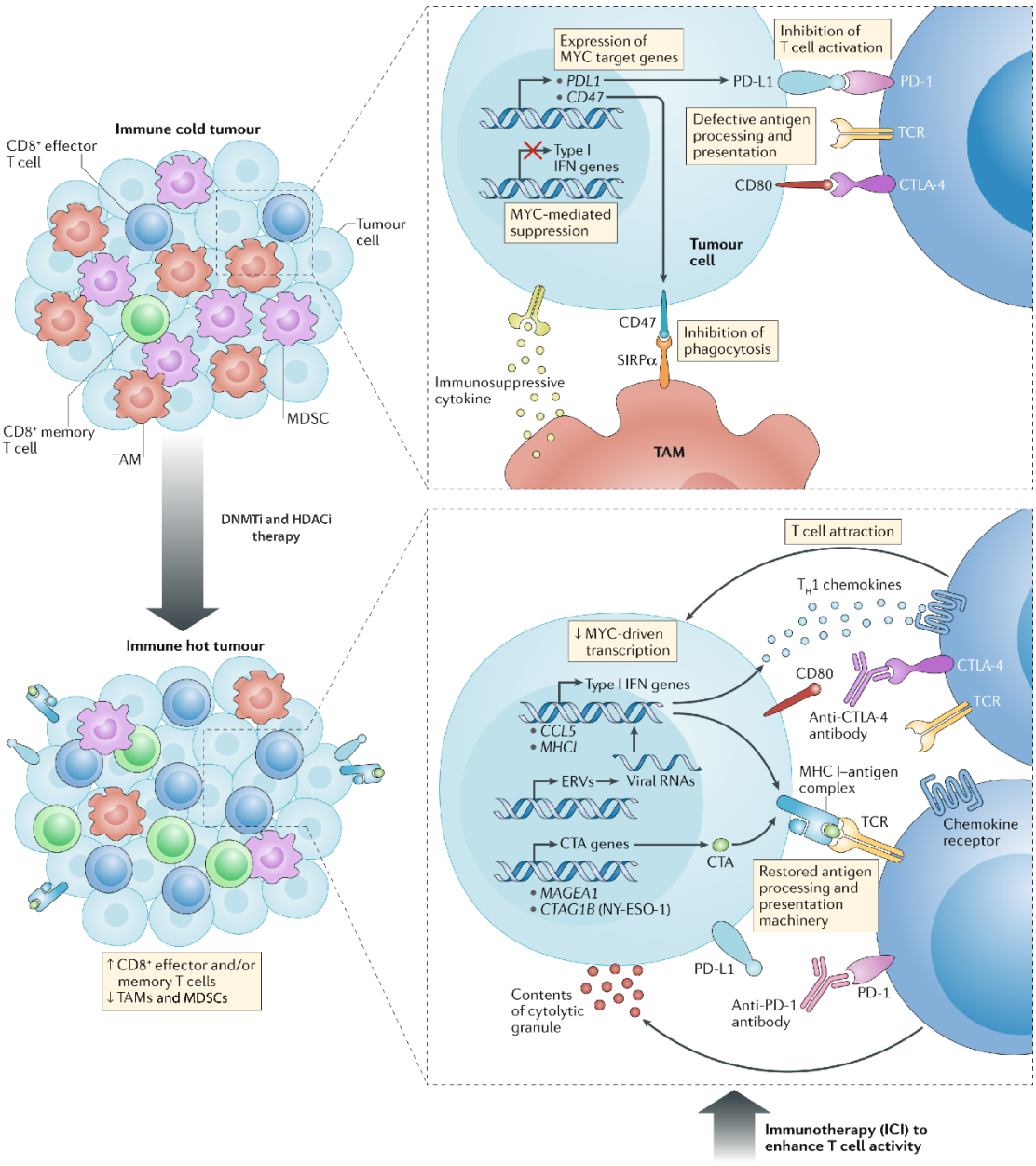

Figure 2. Combinatorial effects of epigenetic therapy and immunotherapy on tumour immunogenicity and T cell activity. Immune cold tumours accumulate tumour associated macrophages (TAM) and myeloid-derived suppressor cells (MDSC), simultaneously limiting their internal abundance of CD8+ effector and memory T cells. Activity of the MYC oncogene downregulates type I interferon (IFN-I)-mediated gene expression and upregulates expression of immune checkpoint proteins programmed death-ligand 1 (PD-L1) and CD47, which collaborate with other checkpoint proteins e.g. CD80, to inhibit innate and adaptive immune responses. Coadministration of DNA methyltransferase (DNMT) and histone deacetylases (HDAC) inhibitors (DNMTi & HDACi) raises tumour immunogenicity, thereby converting immune cold tumours into immune hot ones. Their dual inhibition represses MYC activity, thereby augmenting the antigen-presenting capacity of major histocompatibility complex I (MHC-I) and boosting chemokine production e.g. chemokine CC-chemokine ligand 5 (CCL5). Epigenetic therapies also re-establish the expression of tumour-associated cancer testis antigens (CTA) e.g. MAGE Family Member A1 (MAGEA1) and CTAG1B. Chemokines promote T cell recruitment and tumour infiltration, while the restored antigen processing and presentation machinery potentiates immune recognition. Finally, DNMTi activates endogenous retroviruses (ERV) transcription, leading to the cytoplasmic aggregation of viral RNAs that initiate and propagate IFN-I activity. Additional application of immune checkpoint inhibitors (ICI) e.g. anti-programmed cell death protein 1 (PD-1) and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibodies enhances T cell activity and counteracts the T cell exhaustion induced by sustained activity of immune checkpoint proteins and co-inhibitory receptors. Resultant immune hot tumours display higher internal concentrations of rejuvenated CD8+ effector and memory T cells, as well as reduced levels of immunosuppressive TAMs and MDSCs, which greatly augments immunogenicity and reduces tumour burden. Adapted from Topper et al. 2020(166). *TCR, T cell receptor; Th1, T helper 1; SIRPα, signal regulatory protein α.*

5.4 Combination with immune checkpoint inhibitors

One of the main methods cancer cells utilize to evade host immune response involves the upregulation of immune checkpoint receptors programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), along with their corresponding ligands PD-L1, CD80 and CD86. These elements can accumulate and interact with each other in the TME, promoting immune tolerance and T cell exhaustion, resulting in tumour immune evasion(225,226). Their immunosuppressive effects can be countered with immune checkpoint inhibitors (ICI), which block the ligand binding of PD-1 and CTLA-4 receptors, restoring the adaptive immune system's capacity to identify cancer cells. Clinical trials employing ICIs in solid tumours have demonstrated promising response rates, with several PD-1 and PD-L1, as well as a single CTLA-4 inhibitor, receiving FDA approval for treatment of a multitude of cancer types, including melanoma, NSCLC, triple-negative breast cancer (TNBC), gastric cancer, oesophageal adenocarcinoma, hepatocellular carcinoma, and many others(227). Notably, the immunotherapy yielded disappointing results in AML and MDS patients, likely due to their poor inherent immunogenicity and sustained T cell exhaustion phenotype(228–230).

Exhaustion in CTLs represents an altered differentiation state that serves as a major component of immune tolerance. It arises due to persistent antigen exposure, along with sustained expression of co-inhibitory receptors and immune checkpoint proteins. As a result of this, T cells acquire epigenetic alterations and unique transcriptional signatures that incite progressive loss of effector functions, a decline in proliferation, and metabolic reprogramming, which prevents their transition into CD8+ memory T cell-associated quiescence(231). Instead, CTLs differentiate into a state of hyporesponsive exhaustion, which is forcibly maintained through a positive feedback loop that promotes endogenous generation of immune checkpoint receptors (232). The exhausted phenotype dysregulates T cell cytokine signalling, as production of potent pro-inflammatory cytokines like interleukin-2 (IL-2) and TNFα is heavily diminished, alongside a significant depletion in IFNγ activity. Moreover, exhausted CTLs displayed reduced expression of CD122 and CD127, which facilitate antigen-independent self-renewal in memory CTLs through the binding of binding IL-15 and IL-7 respectively. Loss of CD122 and CD127 activity forces exhausted CTLs to rely on antigen stimulation for continued survival, eventually leading to antigen addiction and impaired proliferation(233). Studying the epigenetic changes driving the CTL effector-to-exhaustion transition, researchers discovered that DNMT3a plays a major role in the methylation-induced silencing of vital genes regulating effector function, proliferation and cytokine production, while simultaneously inhibiting ICI-mediated CTL reinvigoration(234). Confirming this observation, treatment with DAC demonstrated the capacity to dismantle some of these epigenetic irregularities and upregulate the production of immune checkpoint factors, sensitizing tumours to PD-1 blockade and augmenting the rejuvenation potential of exhausted CTLs(234)(Fig. 2). Furthermore, DNMTis raise tumour immunogenicity by inducing viral mimicry, promoting TAM extravasation, enhancing memory/effector CTL tumour infiltration and reactivating the MHC-I antigen presentation machinery, thereby creating a greatly synergistic platform for ICI coadministration(131,134,136,235).

DNMTi and ICI combination demonstrated significant efficacy within an in vitro T cell and colon cancer cell coculture system, managing to jumpstart the cytotoxicity and expansion of exhausted T cells, leading to an enhanced antitumour response(236). The dual inhibition prompted large-scale TME remodulation in mouse models, which boosted the potency of their adaptive immune system, inhibited tumour growth and prolonged overall survival(236–238). Currently there are many ongoing clinical trials involving this drug combination, with several having reached completion and demonstrating mixed feasibility. A phase I dose-escalation trial applying guadecitabine and CTLA-4 inhibitor ipilimumab in metastatic or unresectable melanoma was tolerable in patients and displayed a promising ORR of 26%(239). Combination of pembrolizumab and guadecitabine in mixed ICI-resistant solid tumours, along with the combination of pembrolizumab and oral 5-aza (CC-486) in advanced refractory NSCLC, displayed some resistance reversal, albeit improvements in response rates and progression-free survival remain minimal(240,241). A phase II study administering CC-486 and PD-L1 inhibitor durvalumab in immune cold colorectal, breast and ovarian cancer patients failed to obtain a single clinical response, with tumour biopsies revealing a clear lack of

genome-wide DNA hypomethylation, despite DNMTi treatment(242). Interestingly, coupling DNMTi and ICIs in AML patients has led to some varied outcomes, with one trial achieving an ORR of 33%, while another had to be terminated early due to the expiration of a majority of the patient cohort from disease progression and adverse effects(243–245). A phase II multicohort study investigated the efficacy of 5-aza combined with pembrolizumab in relapsed/refractory AML patients and newly diagnosed older AML patients, attaining ORR rates of 12.5% and 48.1% respectively(246). The overarching sentiment shared between these studies pushes forward biomarker-specific patient stratification as a vital strategy for treatment optimization.

5.5 Triple therapies

Combinatorial repression of epigenetic machinery has displayed some significant antineoplastic effects, but their profuse potential becomes even more apparent when coupled with more aggressive forms of oncotherapy, e.g. chemotherapy or immunotherapy. Although the dual inhibition of HDAC and DNMT lacked impactful efficacy in most patients, the treatment was generally well tolerated, which opened avenues for triple therapies. Compared to monotherapy, their combined activity induces superior chromatin relaxation, robustly priming the tumour for genotoxic chemotherapy. A phase II clinical trial in relapsed/refractory ALL patients incorporated the epigenetic repressors with a 5-step chemotherapeutic regimen, achieving an ORR of 46.2%(247). A follow up study focused on pediatric patients with relapsed or refractory B-cell ALL obtained a similar ORR of 39%, however it had to be terminated prior to completion due to the prevalence of infectious toxicity(248). The combination of DNMTi and HDACi also surpasses their individual activities by reducing the levels of MDSCs within the tumour lesion, in doing so significantly raising tumour immunogenicity(199). The concomitant depletion of *MYC* expression potentiates IFN-I signalling, which promotes CTL tumour infiltration through chemokine production, further sensitizing cells to ICI application(166)(Fig. 2). Coadministration of epigenetic therapies with ICI greatly reduced tumour burden and boosted overall survival in breast, ovarian and colorectal cancer mouse models(249,250). A study centered around a single DLBCL patient with a *p17* deletion, who failed to respond to the initial three lines of therapy, reported partial remission as a result of DNMTi/HDACi and PD-1 inhibitor triple therapy(251). A phase I trial involving 27 advanced CRC patients, the combination of CC-486, romidepsin and pembrolizumab was determined to be safe and well tolerated, although it only incited a partial response in a single patient. Another clinical trial incorporating 5-aza, entinostat and nivolumab in metastatic NSCLC has recently achieved completion, however the results have not been published as of this review (NCT01928576). Besides that, there are two more ongoing clinical trials employing this triple therapy template in advanced NSCLC and relapsed/refractory PTCL (NCT03220477, NCT03161223). In a similar vein, the combination of GSK126 and 5-aza improved the efficacy of PD-L1 inhibitors in ovarian cancer mouse xenografts, mitigating tumour burden and growth(252).

Development of ICI resistance in many solid cancers can severely limit treatment options for patients, drawing interest towards the search for novel ICI resensitization methods. The coadministration of 5-aza and carboplatin in ICI-resistant melanoma patients displayed the potential to prime tumours for an anti-PD-L1 rechallenge with avelumab. However, the study only achieved an ORR of 10%, with two patients attaining partial response(253). A phase I/II study involving CRC and NSCLC cohorts combined 5-aza and pembrolizumab with epacadostat, an inhibitor of immune checkpoint protein indoleamine-pyrrole 2,3-dioxygenase (IDO1), obtaining a disappointing ORR of 5.7% and outlining the persistence of ICI resistance. Researchers discovered that PD-1/PD-L1 blockade resistance could potentiallly be driven by a reactive upregulation in CTLA-4, which was detected in biopsies of non-responder AML patients following 5-aza and nivolumab reatment. The existence of this compensatory mechanism was reinforced through the analysis of patient bone marrow aspirates, which revealed elevated levels of CTLA-4-expressing CD4+ T cells and CTLs (243). To counteract this, a new paradigm combining PD-1/PD-L1 and CTLA-4 blockade was introduced, supplemented with DNMTi to potentiate their efficacy. This is exemplified in a mouse melanoma study, which combined guadecitabine with anti-CTLA-4 and anti-PD-1 antibodies, resulting in significantly reduced tumour

burden, compared to other treatment permutations, and paralleling a significant decrease in tumour metastasis when compared to pure ICI therapy. Currently there are two trials investigating the clinical benefits of this drug combination in AML and MDS patients (NCT02397720, NCT03600155).

Finally, researchers have also implemented targeted molecular therapeutics into these triplet combination strategies. For instance, first generation DNMTis are known to be moderately unstable and lacking in longevity due to their rapid deamination by CDA. Thus, the epigenetic potency of DAC in colorectal adenocarcinoma cells was tested in combination with HDACi 4-phenylbutyric acid (PBA) and the CDAi tetrahydrouridine, resulting in enhanced drug potency and an augmented mitigation of cell proliferation(254). An alternative strategy involves the re-expression of typically silenced cancer biomarkers, exposing tumours to classical targeted therapies. This tactic was employed in a TNBC clinical trial, where the combined inhibition of DNMTs and HDACs, through the application of DAC and LBH589 respectively, was meant to reactivate epigenetically silenced oestrogen receptors, rendering the tumour vulnerable to tamoxifen. Unfortunately, this study was terminated due to slow accrual of participants (NCT01194908).

6. Future perspectives

Despite the vast potential displayed by first generation DNMTis, some of their fundamental drawbacks have still not been addressed sufficiently. Their broad pleiotropic effects render them inherently unspecific, made worse by their low stability and longevity *in vivo*(33). These problems are being partially addressed with next generation DNMTis, e.g. guadecitabine and zebularine, however strides have also been made in the development of new encapsulation and delivery methods, with the intent of improving therapeutic indices. A 2020 study has developed a unique method to improve upon all of the aforementioned drawbacks of first generation DNMTis, while simultaneously expanding their combinatorial potential(255). They utilized Zn^{2+} cofactor cations as prosthetic groups to combine 5-aza and vorinostat into supramolecular nanofibers through sonication-induced assembly. These constructs were regularly internalized in gastric cancer cells, generating cytotoxicity that was superior to free mixture vorinostat and 5-aza. Moreover, the nanofibers displayed improved antineoplastic effects in tumour-bearing mice following their intravenous tail injections, greatly reducing tumour growth and elevating drug specificity through the engagement of the enhanced permeability and retention (EPR) effect. EPR takes into the account the compromised architecture of solid tumour vasculature and its extensive production of permeability factors, which facilitates the extravasation of macromolecules from the tumour vascular bed that could not take place in healthy tissue. The nanofibers can then accumulate in the tumour tissue and interstitium, compounding the efficacy, localization, and specificity of the epigenetic repressors. Additionally, tumours do not possess a functional lymphatic system and cannot effectively drain their interstitial fluid, enhancing nanofiber drug retention, as well as their passive targeting(256). Unfortunately, EPR introduces a high degree of inter-patient heterogeneity, and is rarely effective in haemotologic and metastatic cancers(257). Regardless, this nanofiber technology is an innovation in the combinatorial paradigm of epigenome-based oncotherapeutics and it holds substantial promise for future use in the clinic.

In the realm of drug delivery systems, nanotechnology-based agents have continuously demonstrated significant advantages when compared to conventional methods. This technology is based on the utilization of nanoparticle platforms as capsules for the targeted delivery and controlled dispensation of one or multiple therapeutic agents. The platforms come in various forms, including polymeric, inorganic, viral and lipid-based, each family possessing unique properties that can be exploited. Nanomedicine wields the potential to considerably elevate the efficacy of many pharmaceutical remedies, including epigenetic therapies, by improving their chemical stability and bioavailability, while also boosting internalization rates and specificity through EPR(258). As of this review, several lipid-based and a single albumin-based platforms have been approved by the FDA, with some polymeric platforms attaining approval in Korea and Japan(259). To take full advantage of the benefits EPR provides, nanoparticles need to remain in plasma for an extended period,

which can be disrupted by circulating opsonins, that can bind nanostructures and induce their digestion through phagocytosis. Immune detection can be avoided if nanoparticles are conjugated with polyethylene glycol (PEG) chains, CD47 or hematocyte cell membranes purified from the host. Besides EPR-mediated passive specificity, nanoparticles also allow for active cancer cell targeting through incorporation of tumour-targeting ligands that guide internalization. These can be antibodies, peptides, glycoproteins or polysaccharides that detect tumour-specific biomarkers e.g. antigens and tumour-associated carbohydrates, or factors that are overexpressed in cancer e.g. receptor tyrosine kinases(260). A more recently developed subtype of nanocarriers, referred to as nanogels, allow for the release of encapsulated drugs upon exposure to specific environmental stimuli, such as temperature, light, magnetic field, pH, or recognition of specific biomolecules(261). pH-sensitive nanogels containing 5-aza stabilized through PEG conjugation were administered in breast cancer cells and mouse xenografts, demonstrating superior bioavailability, drug solubility, specificity and overall efficacy to the free drug solution(262). Pretreatment of chemoresistant breast cancer cells with a combination of DAC and vorinostat incorporated in nanogels greatly sensitized them to doxorubicin treatment, with the chemotherapeutic drug proving even more potent if loaded in its respective nanogels(263). Nanoparticle-mediated epigenetic therapies are yet to be applied in patient cohorts. Despite the clear benefits afforded by this method, there are important adverse effects that have to be considered prior to translation, such as excess genotoxicity and oxidative stress generated through overproduction of reactive oxygen species(264).

Continued research and characterization of cancers reveals more about the pathophysiological significance of epigenomic aberrations in tumour initiation and progression, further contributing towards the building interest in combinatorial therapeutic strategies incorporating tumour epigenome manipulation. Progressive translation and optimization of these approaches should reveal tolerable drug combinations that are effective in human models. Omics-based analysis of tumour biopsies obtained from the responding and non-responding patients could bolster our knowledge of epigenetic biomarkers, opening doors for personalized medicine strategies. Application of nuclease dead CRISPR/Cas9 systems (dCas9) as a versatile targeting tool for epigenome editing would allow the examination of said biomarkers in a biochemical and phenotypic context, providing a more accurate modality for disease modelling. As an example, dCas9 loaded with appropriate single guide RNA (sgRNA) could be fused with a TET effector enzyme, which would allow for targeted demethylation of hypermethylated promoters and enhancers, thereby providing evidence for the tumorigenic potential of specific epigenetic aberrations(265,266). Besides that, dCas9 can be utilized as a locus-specific targeting module for many epigenetic writers and editors, e.g. DNMTs, HDACs, EZH2 and LSD1, with a majority of these being actively utilized to interrogate the epigenomes of cancer cell lines(267–270). Recent studies have applied these fusion proteins *in vivo*, e.g. in mice and chicken embryos, which generated promising results despite some clear limitations, such as poor specificity and off-target effects (271,272).

Besides the divulging of key biomarkers, dCas9-mediated locus-specific epigenetic manipulation could prove to possess more practical applications. The efficacy of DNMTis largely stems from their genome-wide reactivation of ERV expression, which cannot be attained with the scope of dCas9, however it could facilitate the epigenetic activation of TSGs, or epigenetic silencing of oncogenes, to impede the advancement of tumorigenic processes like chemoresistance. To that end, this technology could theoretically be administered in conjunction with conventional chemotherapy to boost treatment efficacy. On top of that, dCas9 may also be conjugated with kinase dead DNMT molecules, which could stably block the binding of functional DNMTs to DNA. This blockade could be applied after an initial DNMTi treatment, compensating for their limited longevity by sustaining the activity of previously silenced TSGs for prolonged periods.

7. Conclusions

In recent years, the focus of cancer drug target research has partially shifted from growth factor receptors and intracellular signalling molecules to tumorigenic aberrations in chromatin structure. Epigenetic signatures, especially DNA methylation, are somatically heritable and contribute greatly to drug resistance and immune evasion. The work reviewed here outlines the circumstances of targeting DNA methylation in cancer and the corresponding alterations in the TME that elevate tumour immunogenicity and activate a magnified anti-tumour immune response. The effectiveness of DNMTis is limited in lymphoid and solid tumour patients, due to the emergence of severe adverse effects at higher dosages, prompting the movement to explore the synergistic potential of combinatorial strategies. DNMTis potentiate the efficacy of many conventional chemotherapies, immunotherapies, targeted therapies, and epigenetic therapies in preclinical models, by mitigating chemotherapy and drug resistance, while alleviating T cell exhaustion. Notably, they also exhibit their greatest efficacy when combined with these oncotherapies. Various blends of dual and triple therapies involving DNMTis are currently being tested in the clinic, with most cohorts consisting of patients that have relapsed from, or are resistant to, a prior treatment, although some emerging trials are starting to employ them as first line treatments. So far, their potency has been markedly fluctuating between various cancer types, although at least a minor improvement in patient response rates is observed in most cases. The future acceptance of these approaches relies on continuous testing and careful observation of their efficiency, which in time should reveal optimal combination options, or at least propagate corresponding stratification strategies. This process should be aided by the development and distribution of the next generation of DNMTis, which improved upon a lot of the limitations posed by the first generation. On top of that, utilization of secondary tools such as nanofibers, nanoparticle-based delivery and dCas9-based epigenome editing will open new doors for these polytherapies, further expanding their already great pool of clinical potential.

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