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Aurora Gonzalez-Fierro & Alfonso Dueñas-González

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REVIEW

Emerging DNA methylation inhibitors for cancer therapy: challenges and prospects

Aurora Gonzalez-Fierro[a] and Alfonso Dueñas-González[b]

aDivision of Basic Research, Instituto Nacional de Cancerología, México City, Mexico; bInstituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México UNAM/Instituto Nacional de Can cerología, México City, Mexico

ABSTRACT

Introduction: There is evidence of the association of DNA methylation alterations with cancer development and progression.

Areas covered: This review will briefly discuss the basis of epigenetics and the clinical results of first-generation DNA methyltransferase inhibitors (DNMTi) in myelodysplastic syndrome (MDS) and solid tumors, as well as the limited clinical information on second-generation DNMTi.

Expert opinion: Azacitidine and decitabine are FDA-approved for the treatment of MDS but show limited activity against solid tumors despite inducing gene promoter demethylation, gene reactivation, and global demethylation. Nevertheless, no data consistently shows that the response to these drugs is associated with any DNA methylation marker. It is key to increase clinical exploratory trials with existing and novel demethylating agents incorporating 'omics' technologies to identify DNA methylation 'drivers' or '`patterns' unique to specific malignancies and then proceed to clinical trials in highly selected patients. Ongoing studies with novel DNMTi would inform whether these agents overcome the pharmacological limitations of current DNMTi and improve their efficacy. Interestingly, solid pre-clinical data indicate that acquired DNA hypermethylation impedes PD-1 blockade-mediated T-cell rejuvenation and this phenomenon can be reverted with decitabine; moreover, this drug synergizes CTLA-4 blockade in vivo. This promising research avenue is now clinically being tested.

1. Introduction

The term 'Epigenetics' was coined in the 1940s when Conrad Waddington, a British embryologist, and geneticist [1], introduced the term 'epigenetics' to describe 'the interactions of genes with their environment, which brings the phenotype into being'. Upon this starting definition, the meaning of epigenetics has changed over time. Currently, the most widely accepted definition for epigenetics is the study of heritable phenotypic traits that result from modifications in gene expression without changing the DNA code [2]. It is remarkable that epigenetics has been increasingly studied as reflected by the thousands of publications on this topic in current years.

The human genome is organized into 23 chromosomes so that each diploid cell with 46 chromosomes contains approximately 6 billion base pairs of DNA. If this DNA would be in a linearized form, each cell would have around 2-m length of DNA. Such amount of DNA must be packaged into the nucleus; hence, histone proteins mainly are responsible for organizing the long fibers of DNA within the nucleus. Both, the DNA complexed with histones are the elements of chromatin and the nucleosome is considered the functional unit of the genome. Nucleosomes are formed by a histone octamer formed by dimers of H2A, H2B, H3, and H4 which are linked by histone H1. Approximately 147 bp of superhelical DNA is wrapped around the histone octamer forming the nucleosome core particle [3]. Epigenetics, therefore, can be referred to as the study of all the elements involved in the regulation of nucleosome. Functionally, these elements are highly interacting in order to respond to the cells' needs for proper regulation of gene expression in a time and cell-specific manner.

1.1. Brief historical overview of epigenetics

The concept of DNA methylation emerged in the 1970s. It was demonstrated that the addition of a methyl group at the fifth-position of the cytosine in a CpG dinucleotide could inactivate the expression of genes [4]. Thus, DNA methylation driven by DNA methyltransferase enzymes became the most important epigenetic factor [5]. Afterward, in the 1990s, the transcriptional regulatory effects by histone acetylases/deacetylases enzymes in lysine tails at the histone core of the nucleosomes, added another level of complexity [6]. In addition, it was uncovered that histone acetylases and histone deacetylases enzymes were also able to bind and regulate DNA methyltransferases and a family of proteins having a DNA methylation binding domain called methyltransferase binding domain proteins [7,8] The recognition that histone proteins can undergo modifications beyond acetylation/deacetylation such as methylation/demethylation, phosphorylation/dephosphorylation, ubiquitination, citrullination, and deimination [9–14] led to the discovery of the enzymes responsible for these modifications, as well as numerous interactions among

Article highlights

- The role of abnormal DNA methylation for cancer development and progression still is unclear. The ample heterogeneity of DNA methylation findings in cancer, namely global and regional DNA hypo and hypermethylation are often contradictory in regard to cancer causation or association.
- The first generation of azanucleosides azacitidine and decitabine, as single agents, are a well-established safe and effective therapy for MDS. However, no major responses have been observed with these agents in the treatment of solid tumors.
- Clinically, these agents are able to demethylate and reactivate the expression of tumor suppressor genes and/or induce global hypomethylation. Nevertheless, no consistent data show association of these biological effects with response in either hematological or solid neoplasias.
- Improved 'second generation' DNA demethylating agents are in development. The first results with guadecitabine are encouraging in both hematological and solid neoplasias.
- More research is needed to disclose predictive factors for response to existing and novel DNA demethylating agents.

histones and DNA itself. Protein complexes with nucleosome-remodeling activities [15] are equally recognized as important epigenetic players. Non-coding RNAs (micro RNA and lncRNA) are another set of epigenetic players that are themselves regulated by epigenetic machinery elements [16,17]. Lately, the discovery of DNA demethylases [18,19] – the ten-eleven translocation (tet1, tet2, and tet3) enzymes – which convert 5-methylcytosine to 5-hydroxymethylcytosine resulted in an unanticipated higher complexity of how epigenetics regulates of gene expression [20].

1.2. DNA methylation

DNA methylation involves the covalent addition of a methyl group to the carbon atom 5 of the cytosine pyrimidine ring in a CpG (cytosine-guanine) dinucleotide. In humans, using skeletal muscle as a tissue source, the global cytosine methylation is 4.59% though it is known that there is some variation depending on cell type [21]. Methyl groups in the dinucleotide CpGs of gene promoters regulate interactions between DNA and the transcription machinery of the cell and, in general, DNA methylation correlates with transcriptional silencing and typically occurs in DNA sequences that contain cytosines adjacent to a guanine nucleotide (known as a CpG site). Approximately 30 million CpG sites exist in the human genome [22] and methylation at these sites is associated with the silencing of the genes that are in proximity to the CpG islands. Normal differentiation requires proper DNA methylation/demethylation patterns, and abnormal DNA methylation limits the capacity of a cell to differentiate into tissue/cell-specific lineages and may ultimately induce a state of disease [23].

Mammalian cells possess multiple pathways to establish, maintain, and modify DNA methylation throughout the genome. Enzymes in mammalian cells, known as DNA methyltransferases, DNMT1, DNMT3A, DNMT3B, are responsible for establishing and maintaining DNA methylation while DNMT3L participate in DNA methylation in a catalytic independent manner. The DNMT2 catalyzes tRNA methylation [24,25] but its ability to methylate DNA is controversial [26].

Since DNA methylation is dynamic, mammalian cells also possess the ability to remove these marks. Passive DNA demethylation was the first to be described. As it is passive, it depends on DNA replication and cell division plus the subsequent lack of action of DNA methylation maintenance pathways. On the contrary, active DNA demethylation is replication-independent and occurs through the active enzymatic removal of the methylcytosine [27]. Among DNA demethylases, the enzyme activation-induced cytidine deaminase (AID) deaminate 5-mC yielding thymidine that is replaced by an unmethylated cytosine by the base-excision repair (BER) pathway. Thus, AID may promote aberrant gene expression by decreasing the promoter DNA methylation of specific genes [28,29]. The family of tet1, tet2, and tet3 (ten-eleven translocation) proteins are also considered active DNA demethylases. These enzymes carry out the hydroxylation of 5-mC to 5-hmC [30], 5-hmC, in turn, is replaced with an unmethylated cytosine by the BER pathway [31]. Recent data demonstrate that several proteins bind to 5-hmC, revealing the possibility that specific proteins may be able to interpret the 5-hmC epigenetic mark and subsequently influence chromatin structure and gene expression [32,33]. Taken together, the establishment and maintenance model of DNA methylation is likely an oversimplification of what actually occurs and all DNMTs in concert with tet enzymes, regulate DNA methylation levels through a dynamic equilibrium of site-specific gain and loss of methylation during development and health and disease conditions.

Abnormal DNA methylation signals may contribute to disease, and the reversible nature of epigenetic alterations makes the DNA methylation machinery an exciting therapeutic target including DNMTs, tet proteins as well as activation-induced cytidine deaminase (AID) protein [34]. In addition, methyl-CpG-binding domain (MBD) proteins which 'read' and interpret the methylation moieties on DNA, and are critical mediators of many epigenetic processes, may also targetable. These include at least MBD1, MBD2, MBD3, MBD4, and MeCP2 [35].

2. Evidences linking epigenetic alterations with cancer

Abnormalities in DNA methylation have long been associated with cancer. Both global hypomethylation and regional hypermethylation may play a prominent role in carcinogenesis, and their contribution shows scarcely defined boundaries. It has long been known that in cancer cells both alterations coexist: malignant tumors show global hypomethylation and regional hypermethylation however the meaning of this phenomenon remains to be clarified.

2.1. Global DNA hypomethylation promotes carcinogenesis

As early as 1983, Fearon and Vogelstein reported that DNA hypomethylation distinguishes genes of some human cancers from their normal counterparts [36] support for the carcinogenic effect of DNA hypomethylation was provided Gaudet et al., who generated mice having a hypomorphic DNA methyltransferase 1 (Dnmt1) allele, which reduces

Dnmt1 expression to 10% of wild-type levels resulting in substantial genome-wide hypomethylation in all tissues. Interestingly these mice developed aggressive T cell lymphomas and high frequency of chromosome 15 trisomy [37]. In another study, male F344 rats fed with a methyl-deficient diet for up to 36 weeks, had persistence and expansion of placental isoform of glutathione-S-transferase (GSTpi)-positive lesions. Interestingly, re-feeding rats with methyl-adequate diet beyond week 9, failed to revert liver carcinogenesis; thus, stable DNA hypomethylation is a promoting factor for the clonal expansion of initiated cells [38]. Radiation carcinogenesis has also been associated with stable loss of global DNA methylation and may contribute to cancer formation in radiation-targeted tissues [39]. Additional evidence on the tumor-promoting actions of global DNA hypomethylation come from the observations that murine embryonic stem cells nullizygous for Dnmt1 gene have significantly elevated mutation rates at both endogenous and exogenous genes, being gene deletions the predominant mutations [40] and complete double knockout of dnmt1 and dnmt3b in H116 human colon cancer cells reduces by 80% global DNA methylation accompanied by constitutive genomic instability manifested by chromosomal translocations [41]. Moreover, it has been reported that the methyl donor group S-adenosylmethionine (SAM) treatment in a model of breast cancer, causes a significant dose-dependent decrease in cell proliferation, invasion, migration, anchorage-independent growth and increased apoptosis in vitro, and these effects were recapitulated in vivo where oral administration of SAM reduced tumor volume and metastasis in a xenograft model, though no global levels of methylation were measured it is suggested that increasing global methylation may have antitumor effects [42]. Increased global DNA hypomethylation as evaluated by Alu repeats quantitation has been observed in distant metastatic and dedifferentiated thyroid cancer [43], and similar findings have been shown in gastric carcinogenesis [44]. Altogether these data are suggestive but not conclusive that global DNA hypomethylation cooperates for cancer development.

2.2. Global DNA hypomethylation protects from carcinogenesis

Intriguingly, a number of studies suggest that global DNA hypomethylation does the opposite. It has been reported that genetic reduction of DNA methylation levels suppresses the incidence, number and size of gastric tumors in two different mouse models for gastric tumorigenesis [45]. Cancer protecting effects have also been observed in other models of DNMT1 gene depletion. Dnmt1 hypomorphic mice have a reduction in pancreatic acinar cell tumor multiplicity [46]; suppression of polyp formation in Apc(Min/+) mice [47] and in a model of ApcMin-induced intestinal neoplasia in mice where a reduction in the DNA methyltransferase activity genetically and pharmacologically induced reduces the average number of intestinal adenomas from 113 in the control mice to only 2 polyps in the treated heterozygotes [48]. Thus, it remains to be determined the actual role of global DNA hypomethylation, with some studies suggesting it acts as carcinogen while another set of studies evidencing a protective effect.

2.3. Regional hypomethylation may promote carcinogenesis

Not only global DNA hypomethylation could be causally related to carcinogenesis. A number of studies also demonstrate that hypomethylation at promoters of oncogenes associated with oncogenic over-expression that at least in experimental models aids in cancer development or progression. Oncogenic ADP-ribosylating factor (ARF)-like 4c (ARL4C) overexpression in lung squamous cell carcinoma is associated with DNA hypomethylation in the 3′UTR of ARL4C gene [49]. Hypomethylation and increased expression of the oncogene ELMO3 have been observed in primary and metastatic tumors from lung cancer patients [50]. C-myc and c-jun are known oncogenes found to be over-expressed and associated with hypomethylation in a number of studies [51–53]. Several other oncogenes have also been found hypomethylated and over-expressed via diverse mechanisms in several tumor models including CTCF/BORIS, TERT, ras members, TKTL1, CD30, JunB, WNT5A, CRIP1, S100P, maspin, synuclein gamma, KLK4, and bcl-2 [54–65]. Regional hypomethylation is also observed in non-coding genes. For instance, hypomethylation of LINE-1 promoter activates an alternate transcript of MET oncogene in bladder cancer and a number of proto-oncogenes in colorectal cancer metastasis [66,67]. microRNAs are also found hypomethylated and hence overexpressed in several models, which indirectly activates oncogenic signals or interferes with tumor suppressor genes [68–71]. These studies are suggestive that not only global but regional hypomethylation at specific genes or non-coding elements may participate in cancer development.

2.4. Regional hypermethylation may promote carcinogenesis

There are numerous reports demonstrating that tumor suppressor genes belonging to nearly every cancer pathway or function category have silenced or diminished expression due to abnormal promoter hypermethylation. Many of these reports are summarized in reviews [72–74]. In fact, these findings were the rationale behind the clinical development of DNA methyltransferase inhibitors. The first discoveries of promoter methylated tumor suppressor genes were made using the candidate gene approach. Greger et al., and Sakai et al. [75,76], were the first to demonstrate silencing of retinoblastoma gene by promoter methylation in primary tumors as a 'non-genetic' structural defect mechanism of allele inactivation according to the 'two-hit' Knudson hypothesis [77]. In 2001, Esteller et al. performed a comprehensive analysis of promoter hypermethylation changes in 12 genes (p16, p14ARF, p15, p73, APC, BRCA1, hMLH1, GSTP1, MGMT, CDH1, TIMP3, and DAPK). Each of these genes was rigorously characterized for association with abnormal gene silencing from over 600 primary tumor samples representing 15 major tumor types. They found a unique profile of promoter hypermethylation for each human cancer in which some gene changes are

shared and others are cancer-type specific. These data illustrated that epigenetic inactivation may affect genes implicated in key cancer molecular pathways such as cell immortalization and transformation cycle, DNA repair, cell adherence, metastases, and metabolic enzymes among others [74]. The main findings of this study were also somehow replicated by Costello et al., using a 'non-candidate approach'. They performed a global analysis of the methylation status of 1,184 unselected CpG islands in 98 primary human tumors using restriction landmark genomic scanning. They reported an average of 600 CpG islands found methylated in tumors, and again, methylation patterns that were shared within each tumor type, together with patterns and targets that displayed distinct tumor-type specificity. The functional consequences of such methylation were inferred from treating glioma cell lines with known methylated and silenced genes with decitabine. Overall, 6 out of 16 genes (38%) were fully or partially reactivated while 10 genes (63%) were unaffected by the demethylation treatment in all five cell lines, regardless of methylation status [78].

3. Clinical development of hypomethylating therapy

The azanucleotides azacitidine and decitabine were initially studied as classical cytotoxics for solid tumors and leukemia [79–82]. It was not until the discoveries of Jones et al., on the effects of these drugs upon DNA methylation [83], that low-dose schedules were used to exploit their hypomethylating activity in a number of phase I and II trials. These trials culminated in their FDA-approval for myelodysplastic syndrome based on the results of randomized phase III trials in 2004 and 2006, respectively, [84–87].

3.1. Randomized phase III trials with azacitidine in high-risk MDS

The first randomized trial of azacitidine performed in 191 MDS patients to compare this drug with best supportive care (BSC) as no standard of therapy for MDS existed. Azacitidine was administered 75 mg/m^2/d subcutaneously for 7 days every 28 days. Among patients assigned to azacitidine there were 60% of responses (7% complete, 16% partial and 37% hematological improvement). Responses were statistically significantly different as compared to BSC (p < 0.001) as was also the median time to leukemic transformation or death (21 vs 13 months, p = 0.007). Although no overall survival difference was reported, the median survival for azacitidine was 20 months (95% CI, 16 to 26 months) whereas it was only 14 months (95% CI, 12 to 14 months) for BSC (p = 0.10). The lack of survival difference was attributed to the 53% of patients of BSC that crossed-over to azacitidine, though a 6-month landmark analysis did show statistically significant superior survival for azacitidine. Quality of life (QOL) was significantly improved in patients treated with azacitidine, specifically regarding physical functioning, dyspnea, and psychosocial distress even after controlling for red blood cells transfusions. In addition, QOL was also improved in the patients of BSC after crossing-over to azacitidine [84].

The second randomized trial compared azacitidine (same schedule of the first trial) against conventional care (BSC, low-dose cytarabine, or intensive chemotherapy as selected by investigators before randomization). This study accrued 358 patients. After a median follow-up of 21.1 months (IQR 15.1–26.9), the median overall survival was statistically significantly higher for azacitidine, 24.5 months (9.9-not reached) versus only 15.0 months (5.6–24.1) in the conventional care group (hazard ratio 0.58; 95% CI 0.43–0.77); stratified log-rank p = 0.0001). At last follow-up, there were 82 deaths in the azacitidine group as compared to 113 in the conventional care group. Kaplan–Meier estimates at 2 years, showed that survival rates were 50.8% (95% CI 42.1–58.8) versus 26.2% (18.7–34.3) (p < 0 · 0001) in the azacitidine versus conventional care groups, respectively, [85]. QOL was not assessed in this trial; however, the higher rate of responses and hematological improvement suggest that patients most likely had their QOL improved.

3.2. Randomized phase III trials with decitabine in high-risk MDS

In the first randomized phase III trial of decitabine, 170 patients received either decitabine at 15 mg/m^2 given intravenously over 3 h every 8 h for 3 days (at a dose of 135 mg/m^2 per course) and repeated every 6 weeks, or BSC. The response rate to decitabine was statistically significant as compared to BSC. The overall response rate was 17% (8% PR and 9% CR), whereas no patient achieved response in arm of BSC, p < 0.001. Additionally, 12 (13%) patients treated with decitabine showed hematological improvement that was associated with transfusion independence. Overall survival was not statistically different between arms. However, there was a trend for a longer median time to acute myelogenous leukemia (AML) progression or death compared with patients who received BSC; for those with International Prognostic Scoring System intermediate-2/high-risk disease; for patients with de novo disease, and in treatment-naive patients [86].

The second randomized trial was done in 233 patients, and the primary endpoint was overall survival. Decitabine was administered at the same dose and schedule but infused over 4 h and the control arm was BSC. Again, responses favored the decitabine arm. These were CR (13% v 0%), PR (6% v 0%), hematologic improvement (15% v 2%), stable disease (14% v 22%), progressive disease (29% v 68%), hypoplasia (14% v 0%), and unevaluable (8% v 8%) for decitabine and BSC, respectively. There was no increase in overall survival with decitabine (median OS, 10.1 vs 8.5 months, [HR], 0.88; 95% CI, 0.66 to 1.17; two-sided, log-rank p = 0.38). Progression-free survival (PFS), but not AML-free survival (AMLFS), was significantly prolonged with decitabine versus BSC (median PFS, 6.6 versus 3.0 months, respectively; HR, 0.68; 95% CI, 0.52 to 0.88; p = 0.004; median AMLFS, 8.8 versus 6.1 months, respectively; HR, 0.85; 95% CI, 0.64 to 1.12; p = 0.24). Nevertheless, the rate of transformation to AML at 1 year was statistically significantly reduced, 33% in BSC and 22% with decitabine. The most common toxicity for decitabine is myelosuppression and infection which are also observed in untreated patients. In this study, 47.4% of decitabine patients

had grade 3–4 infection with neutropenia versus 35% in BSC arm, likewise, grade 3–4 febrile neutropenia was most frequent (25.4% vs 7.1%) in decitabine and BSC groups, respectively. Both randomized decitabine trials formally assessed QOL which was significantly improved in patients of decitabine arms [87].

3.3. Meta-analysis of azacitidine and decitabine in MDS

A systematic review and network meta-analysis comparing azacitidine and decitabine for the treatment of myelodysplastic syndrome has been performed. After review of literature, authors were left with only the four trials aforementioned. The results of the meta-analysis show that compared to BSC, azacitidine was significantly associated with lower mortality (RR = 0.83, 95% CI 0.74–0.94, I^2 = 89%) whereas decitabine did not significantly reduce mortality (RR = 0.88, 95% CI 0.77–1.00, I^2 = 53%). However, both drugs are associated with higher partial and complete response rates compared to BSC. Indirect comparisons were not statistically significant for all the studied outcomes, except for complete response where azacitidine was less likely to induce complete response compared to decitabine (RR = 0.11, 95% CI = 0.01–0.86, this at very low-certainty evidence). Overall, it is clear that both azacitidine and decitabine are associated with improved outcomes compared to BSC. In the absence of head-to-head comparisons, both drugs are well-established standard of care for high-risk MDS and the choice of either should be driven by patient preferences, adverse effects, drug availability, and cost [88].

4. DNA demethylating agents for solid tumors

Despite the strong and vast preclinical evidence that DNA demethylating therapy should be active in solid tumors, particularly with low-dose regimens, clinical results with these agents (mostly azacitidine and decitabine) are unsatisfactory.

Recently, a systematic review of DNA demethylating therapy against solid tumors was published. The review focused on the outcome of DNA demethylating therapy upon clinical response, methylation and the effects in the immune system. The DNMTi included in the review were azacitidine, decitabine, hydralazine, procaine, MG98, guadecitabine, and zebularine. However, 33 of the 58 studies combined a DNA methylation inhibitor with classical chemotherapy agents, histone deacetylase inhibitors, interferon, interleukin 2, anti-EGFR agents, antiandrogen therapy and cell immunotherapy. Among the 55 studies (out of 58) for which clinical outcome was available, 13 had complete response (CR), 35 had partial responses (PR) and 47 reported stable disease (SD). In all but one study, progressive disease was observed. The effects upon DNA methylation and the immune system were not available in all studies. A decrease in global methylation was observed in 15 out of 17 studies whereas gene promoter demethylation and re-expression was reported in 13 out of 15 studies. Fourteen studies showed immune-related responses such as re-expression of cancer-testis antigens and up-regulation of interferon-related genes. While these results, in general, indicate that DNA demethylating therapy works in a variety of malignant solid tumors studied including melanoma, breast, gastrointestinal, lymphoma, hepatocarcinoma, ovarian, cervical, renal, prostate among others, it must be stressed that from these 58 studies, only 22 used demethylating drugs as a single agent. As shown in Table 1, among the 21 studies (one excluded because it was a case report of a single patient) totalizing 816 patients, no complete responses were observed; the mean partial response rate was 7.3%, 95CI: 2.18–12.52; mean stable disease rate was 14.95 SD 16.2, 95CI: 7.28–22.72, and the mean progressive disease rate 76.80 SD 29.39, 95CI: 63.04–90.56. In 11 studies azacitidine was used, decitabine in seven and MG98 (a DNMT1 antisense oligonucleotide) in three studies. Responses rates were not different for azacitidine and decitabine in regard to the dose (high cytotoxic dose) or low-dose (demethylating regimens). From this review, it seems

Table 1. Clinical studies of DNA methylation inhibitors as single agents.

Agent, number of patients	Cancer	CR (%)	PR (%)	SD (%)	PD (%)	ORR (%)	PFS (m)	OS (m)	Year	Dose
Azacitidine, 27	Breast	0	7	15	78	7	–	–	1974	High
Azacitidine, 17	Germ cell tumor	0	0	0	100	0	–	–	1993	High
Azacitidine, 28	Gastro-intestinal	0	4	29	67	4	–	–	1972	High
Decitabine, 12	NSCLC	0	0	50	50	0	–	6.7	1997	High
Azacitidine, 26	Melanoma	0	0	19	81	0	–	–	1978	High
MG98, 15	Renal	0	0	40	60	0	–	–	2006	–
Decitabine, 82	Various	0	7	22	79	7	–	–	1987	High
Decitabine, 19	Various	0	0	0	0	0	0.9	1.2	2003	Low
Azacitidine, 22	Various	0	9	0	91	9			1974	Low
Decitabine, 8	Various	0	13	0	88	13	–	–	2014	Low
Azacitidine, 6	Various	0	17	0	83	17	–	–	1975	High
MG98, 33	Various	0	3	3	94	3	–	–	2009	–
Azacitidine, 167	Various	0	3	10	146	3	–	–	1977	High
MG98, 14	Various	0	7	14	79	7	–	–	2003	–
Decitabine, 20	Various	0	5	0	95	5	–	–	1986	High
Azacitidine, 87	Various	0	2	2	95	2	–	–	1977	High
Azacitidine, 29	Various	0	45	21	34	45	–	–	1972	High
Azacitidine, 150	Various	0	7	44	49	7	–	–	1977	Low
Azacitidine, 21	Various	0	0	14	86	0	–	–	1976	High
Decitabine, 8	Various	0	25	0	75	25	–	–	2005	Low
Decitabine, 25	Various	0	0	16	84	0	–	–	2006	Low

Summary of responses with DNA methylation inhibitors as single agent in solid tumors. No. Pts: Number of Patients; CR: Complete Response; PR: Partial Response; SD: Stable Disease; PD: Progressive Disease; ORR: Overall Response Rate; PFS: Progression-Free Survival; OS: Overall Survival. Data taken from reference 89.

that any single agent demethylating drug has poor antitumor activity despite they induce changes in global and gene-specific demethylation and gene re-expression [89].

5. New DNA methylation inhibitors

Azacitidine and decitabine the oldest DNMTi are nucleoside analogs that intercalate with the DNA and upon their incorporation during S-phase of the cell cycle, form an irreversible complex with the DNMTs, leading to enzyme degradation. A limitation of these drugs is their short half-life of about 30 min which limits exposure of diseased cells to the drug, potentially abrogating their effectiveness [90,91]. On these bases, nucleoside analogs with longer half-lives are being sought that could theoretically improve response to therapy by enhancing incorporation of the active agent into dividing cells. Several novel nucleosides are in development. Zebularine, a less toxic cytidine analog was proven to be an effective and less toxic DNMTi in preclinical models. However, its clinical development was halted due to its extremely low bioavailability in rhesus monkeys [92].

Second generation nucleosides analogs are in clinical development. Of these, guadecitabine (SGI-110) administered subcutaneously has already being tested in phase I and II clinical trials. In the phase I trial on 93 patients with AML and MDS testing several schedules and doses, guadecitabine at 60 mg/m^2 daily for 5 days was well-tolerated, easily administered, and biologically and clinically active in both MDS and AML. The most common grade ≥3 adverse events were febrile neutropenia (41%), pneumonia (29%), thrombocytopenia (25%), anemia (25%), and sepsis (17%). As expected, a dose-related DNA demethylation was observed in the daily regimen reaching a plateau in demethylation at 60 mg/m^2. Though the study was not designed to evaluate the efficacy, 6 out 19 MDS patients (31.5%) and 6 out 74 AML patients (8%) had response [93]. An update of the aforementioned trial [93] reported the results only of the phase II part in 107 patients with treatment-naive AML randomized to either 5-day (60 or 90 mg/m^2) or 10-day (60 mg/m^2) both in a 28-day schedule (54 and 53 patients) in 5-day or 10-day, respectively, was reported. At a median follow-up of 953 days (IQR 721–1040), the composite complete response rates were 54%, 59%, and 26% in the 5-day at 60 mg/m^2, 5-day at 90 mg/m^2 and 10-day 60 mg/m^2, respectively. Though authors state that treatment had tolerable toxicity, the most common grade 3 or worse events were febrile neutropenia, thrombocytopenia, neutropenia, pneumonia, anemia, and sepsis regardless of the 5-day or 10-day schedule. Overall, 23 (22%) patients died because of adverse event, all of them in the 10-day cohort [94]. An open-label phase III study that enrolled 815 patients is ongoing, comparing guadecitabine versus treatment choice in adults with previously untreated AML who are not considered candidates for intensive remission induction chemotherapy (NCT02348489).

Two phase I studies with guadecitabine combined with chemotherapy have been reported. A study of guadecitabine with irinotecan in 22 metastatic colorectal cancer patients exposed to irinotecan used four dose levels of guadecitabine and irinotecan with or without growth factor support (GFS). The most common grade 3–4 toxicities were neutropenia 77%, neutropenic fever (23%), leukopenia 50%. The conclusion of this study was that 125 mg/m^2 with GFS resulted safe and tolerable with early indication of benefit, 12 out of 17 evaluable patients had stable disease as the best response. Circulating tumor DNA showed a decrease in global demethylation by LINE-1 after treatment [95]. A second study in 20 cisplatin-resistant ovarian cancer patients with a median of previous regimens of 7 (1–14) were treated with guadecitabine at 45 mg/m^2 plus carboplatin AUC5. The dose of guadecitabine was deescalated to 30 mg/m^2 and carboplatin at AUC4 after the first cohort of six patients who presented dose-limiting neutropenia and thrombocytopenia. There were indications of activity, among the 20 patients, three patients had a partial response (PR), and six patients had stable disease (SD) for more than 3 months, for an overall response rate (ORR) and clinical benefit rate of 15% and 45%, respectively. Evidence of demethylation was also observed in LINE-1 from PBMCs and promoter demethylation/gene reexpression in paired tumor biopsies/ascites [96]. A study of guadecitabine with sorafenib for advanced hepatocellular carcinoma is ongoing (NCT01752933). There is another second-generation nucleoside analog in clinical development, the cytidine analog 4'-Thio-2'-deoxycytidine is undergoing clinical trial for advanced solid tumors (NCT02423057). A second cytidine analog (RX-3117) is also being evaluated in a phase 1b/2a multicentric study combined with abraxane for patients with metastatic pancreatic cancer (NCT03189914).

A number of DNA methylation inhibitors are in preclinical development and are not discussed here. These include CP-4200, an elaidic acid ester of azacytidine; the naturally occurring flavonoid Epigallocatechin-3-gallate (EGCG); the polyphenols theaflavin 3, 3'-digallate and thearubigins from black tea; the quinoline derivative, SGI-1027; the quinone antibiotic, nanaomycin A, and a phthalimido-L-tryptophan derivative, RG-108 [20].

6. Conclusion

DNA demethylating therapy with the azanucleotides azacitidine and decitabine has become the standard of care of MDS, a clinical entity where abnormalities in DNA methylation seem to play an important role in its molecular pathogenesis. Hard data indicates, however, that while these drugs undoubtedly improve clinical conditions and lead to better QOL, their effect on overall survival was clearly seen in only one study with azacitidine. On the other hand, their efficacy in solid tumors as a single agent, either used at high (cytotoxic) or low (demethylating) doses is yet to be demonstrated. No randomized trials of any DNMTi as a single agent in solid tumors has been done and a number of phase I, II trials, complete response rates are inexistent while partial responses and disease stabilization rates are around 7% and 15%, respectively. These poor results in solid tumors, however, clearly improve when DNMTi are used combined with chemotherapy, molecular targeted therapy or immunotherapy.

7. Expert opinion

The simple view that DNMTi could be effective anticancer therapy based on the reactivation of tumor suppressor genes silenced by promoter methylation (regardless of the potentially deleterious- drug-induced global DNA hypomethylation in tumors) may not be correct. So far, there is evidence that DNMTi do induce gene promoter demethylation and gene reactivation, as well as global demethylation. Nevertheless, no data consistently show that response to these drug types is associated with any DNA methylation marker. The search for more effective DNMTi is ongoing. Having 'first generation' demethylating drugs approved and accepted as a standard of care for MDS is a vivid demonstration that we are moving forward. Technological advances are rendering a new wave of potentially more selective and efficacious DNMTi drugs. Preliminary data do suggest that 'second generation' DNMTi guadecitabine is safe and effective for MDS and AML and it is also tested in combination with chemotherapy for solid tumors. The cytosine analog 4'-thio-2'-deoxycytidine is another 'second generation' DNMTi that is already being in clinical trials. The clinical testing of a number of non-nucleosides analogs with high selectivity for DNMT enzymes which are not cytotoxics on their own, would allow to generate more evidence on the oncogenic role of DNA hypermethylation.

Nevertheless, we should be reminded that it is still unclear the role of altered DNA methylation in cancer. Global hypomethylation in some models promotes and in other models protects cancer development whereas both regional hypomethylation and hypermethylation may promote carcinogenesis. The complexity of DNA methylation makes hard to arrive at educated guesses on how to modulate this cascade of events by pharmacological means: that is, how to best make use of pharmacological interventions to modify in a beneficial way the processes of cancer without at the same time, producing undesirable epigenetic changes that could favor disease development or progression.

Despite the challenges imposed by this complexity it is very important to greatly increase the number of small clinical exploratory trials with existing drugs and novel DNMTi both as single agents and in combination with existing cancer therapeutic agents. These exploratory trials should incorporate pharmacogenetic, pharmacogenomics and other 'omics' technologies to identify predictive factors for response and/or toxicity, to eventually personalize the use of DNMTi. The other approach, which also can be cataloged as personalized medicine, is to continue the study of diseases to find out DNA methylation 'drivers' or 'methylation patterns' unique to specific malignancies and then proceed to clinical trials in a highly selected population of patients. On the other hand, a very promising research avenue for DNA demethylating therapy is the use of DNMTi with checkpoint inhibitors. Solid preclinical data indicate that acquired DNA hypermethylation impedes PD-1 blockade-mediated T-cell rejuvenation. Interestingly, this phenomenon can be efficiently reverted by prior use of decitabine in mice model. Moreover, decitabine not only enhances lymphocyte function in a murine model of ovarian cancer, but also synergizes CTLA-4 blockade. In fact, a number of clinical studies are ongoing combining DNMTi (decitabine, azacitidine, guadecitabine) with several checkpoint inhibitors such as ipilimumab, nivolumab, and pembrolizumab.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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