DNA Methylation for Bladder Cancer

Subjects: Biochemistry & Molecular Biology

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Bladder cancer (BC) is the tenth most frequent cancer worldwide and is associated with high mortality when diagnosed in its most aggressive form, which is not reverted by the current treatment options. The disruption of normal epigenetic mechanisms, namely, DNA methylation, is a known early event in cancer development. Consequently, DNA methyltransferase (DNMT) inhibitors constitute a promising therapeutic target for the treatment of BC.

bladder cancer DNA methylation DNA methyltransferases

1. DNA Methylation

DNA methylation is the covalent addition of a methyl group at the 5-position carbon of cytosine in a cytosine–phosphatidyl–guanine (CpG) dinucleotide [1]. Methylation occurs mainly at CpG islands—CpG-rich regions (at least 50% of cytosines and guanines) in the genome with a size larger than 200 bp [2][3]. Moreover, methylation can also be found in repetitive sequences such as retrotransposon elements and centromeres, in the X chromosome (leading to its inactivation) and genomic imprinting [4]. Approximately 29,000 CpG islands can be found in the human genome, most commonly in the promoter regions, close to the transcription starting site (TSS) or first exons [3]. Remarkably, 75% of cytosines in CpG dinucleotides dispersed throughout the genome are methylated, whereas cytosines of CpG islands located within gene promoters remain mostly hypomethylated [3][5]. Promoter DNA methylation is classically associated with transcription repression by inducing binding of transcriptional repressors or hampering binding of transcriptional factors [1][6]. In fact, a family of methyl-CpG-binding proteins (MBPs) intervenes in gene silencing by binding to methylated CpGs and recruiting histone modifier enzymes to establish histone post-translation modifications, which further sustain transcriptional repression [7]. DNA methylation can also be found in CpG island shores, 2-kb areas upstream of a CpG island, displaying lower CpG dinucleotide density. CpG island shore methylation is also associated with transcriptional repression [8]. On the other hand, methylation in the gene body was shown to stimulate transcription elongation and to have an impact on splicing, with exons disclosing higher methylation levels than introns [4][9]. Furthermore, tissue-specific methylation seems to be more frequent in intragenic CpG islands [10]. CpG islands in enhancers also influence gene regulation, i.e., hypermethylation is associated with loss of enhancer marks resulting in gene silencing [11].

2. DNA Methylation as a Therapeutic Target in BC

2.1. Preclinical Studies

A summary of the preclinical studies testing DNMT inhibitors in BC is depicted in **Table 1**. The effects of 5-aza were evaluated in cell lines in vitro and in a tumor xenograph model. 5-Aza was shown to inhibit cell proliferation and arrest cells at G0/G1, whereas volume and weight of tumor xenografts in mice were reduced. Interestingly, DNMT3a and DNMT3b expressions were also reduced after 5-aza treatment, causing the re-expression of hepaCAM, a TSG [12]. DAC treatment also led to an increase in hepaCAM expression in T24 and BIU87 cells, associated with arrest at G0/G1 phase [13]. In a canine model of invasive urothelial carcinoma, 5-aza disclosed anti-tumor effects, with 22.2% of the dogs demonstrating partial response and 50% depicting stable disease [14].

Table 1. Summary of pre-clinical studies targeting DNMTs in bladder cancer (BC).

Drug	Model	Concentration	Treatment Scheme	Effects	Year	Reference
DAC	T24	1 μM	1 day	↑ Gene expression related to IFN pathway	2002	[15]
DAC	T24	3 μM	1 day	No remethylation in CpG islands in the absence of cell division	2002	[16]
Hydralazine and procainamide	T24	10 μM	5 days	↓ *p16* and *RARβ* methylation levels ↑ p16 and RARβ expression	2003	[17]
DAC	TCC and UMUC	5 μM	n.a.	↑ MSH3 mRNA levels	2004	[18]
Zebularine	T24	100 μM	Every 3 days for 40 days	↓ Global methylation levels ↑ p16 expression	2004	[19]
DAC	J82C, T24C, TCC, and UMUC	5 μM	3 days	↑ Wif-1 mRNA expression levels	2006	[20]
DAC Zebularine	RT4 and T24	2 μM 100 μM	2 days Every 3 days for 7 days	↑ Cells doubling time ↑ APAF-1 and DAPK-1 expression	2006	[21]
S110	T24	0.1–10 μM	Every 3 days for 6 days	↓ Global methylation levels ↑ p16 expression	2007	[22]
DAC	BIU87	0.1–5 μM	3 days	Re-expression of RASSF1A	2009	[23]
DAC	BOY	1 μM	4 days	↑ COL1A2 expression	2009	[24]

Drug	Model	Concentration	Treatment Scheme	Effects	Year	Reference
DAC	BOY, T24, and UMUC	10 µM	7 days	↑ FHL1 mRNA expression levels	2010	[25]
S110	Mouse tumor xenograft	10 mg/kg	Daily injection for 6 days	↓ Tumor growth rate ↑ p16 expression	2010	[26]
5-Aza	Dogs with naturally occurring invasive urothelial carcinoma	0.1–0.3 mg/kg	Two doses schedules: Everyday days 1 to 5 or days 1 to 5 and 15 to 19 Each cycle 28 days	22.2% Tumor partial response 50% Stable disease 22.2% Progressive disease	2012	[14]
DAC	BIU87 and T24	0.1–10 µM	3 days	Cell arrest at G0/G1 ↑ hepaCAM expression	2013	[13]
DAC	T24	0.25–2 µM	2 days [1]	↑ Maspin expression levels ↓ Cell proliferation, migration and invasion	2013	[27]
DAC	5637	1–3 µM	6 days	↑ GSTM1 expression	2014	[28]
DAC	EJ	1 µM	Every day for 3 days	↓ Cell tumorigenesis and invasiveness Cell arrest at G2/M ↑ BTG2 expression	2014	[29]
DNAzyme	T24	n.a.	n.a.	↓ Cell proliferation ↑ p16 expression	2015	[30]
5-Aza	BIU87, EJ, and T24 Mouse tumor xenograft	0.5–7 µM n.a.	1–4 days Every 3 days for 18 days	↓ Cell proliferation ↓ Tumor volume and weight	2016	[12]
DAC	T24 and J82	0.3 µM	1 day	↓ *RSPH9* methylation levels	2016	[31]
DAC	BLCAb001 (B01), BLCAb002 (B02),	0.1–1 µM	Every 2 days for 5 days	↑ NOTCH1 expression ↓ CK5 positive cells ↑ IL-6 release	2017	[32]

Non-toxic concentrations of DAC were used to treat four BC cell lines in order to evaluate the impact of hypomethylation in the BC cell transcriptome. Notch receptor 1 (NOTCH1) expression increased after treatment with DAC, in parallel with 50% demethylation of the promoter and enhancer regions. Interestingly, DAC-treated

Drug	Model	Concentration	Treatment Scheme	Effects	Year	Reference
	HT1376, and T24					[32]
DAC	T24	1 µM	1 day	↓ Methylation of 590 CpGs ↑ Methylation of 616 CpGs	2019	[33]

For migration and invasiveness assays. Abbreviations: 5-aza—5-azacytidine, APAF-1—apoptotic peptidase activating factor 1, BTG2-BTG anti-proliferation factor 2, CK5—Cytokeratin 5, CpG—Cytosine–phosphatidyl–guanine, COL1A2—collagen type I alpha 2 chain, DAC—decitabine, DAPK-1—death associated protein kinase 1; FHL1—four and a half LIM domains 1, GSTM1—glutathione S-transferase mu 1, IFN—Interferon, IL-6—Interleukin 6, MSH3—mutS homolog 3, n.a.—not available, NOTCH1—Notch receptor 1, RARβ—retinoic acid receptor beta, RASSF1A—Ras association domain family 1 isoform A, ↑—increase, ↓—decrease.

e effects,
e in cell
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state [32],
which may prevent BC progression [34]. DAC was also shown to inhibit cell proliferation, migration, and invasiveness, inducing apoptosis in T24 cells while increasing the expression of the TSG Maspin [27]. Another gene that appears to be regulated by methylation in BC is *BTG2*. After treating EJ cells with DAC, BTG anti-proliferation factor 2 (BTG2) expression increased mainly due to DNMT1 inhibition, and cell growth decreased. Furthermore, H3K9me2 levels decreased, whereas H3K4me3 increased, leading to an open chromatin state in the promoter and intronic region of *BTG2* [29]. Apoptotic peptidase activating factor 1 (APAF-1) and death associated protein kinase 1 (DAPK-1) expressions also increased after DAC treatment in both RT4 and T24 cells, whereas zebularine caused an increased expression of those genes in cell line T24, which is p53 mutated [21].

The epigenetic regulation of Wnt inhibitory factor 1 (Wif-1), an antagonist of the Wnt pathway, important for carcinogenesis, was explored in four BC cell lines. DAC treatment led to increased Wif-1 mRNA levels with a simultaneous decrease in promoter methylation levels. Furthermore, Wif-1 expression was primarily regulated by DNA methylation and not genetic alterations [20]. DAC treatment in T24 cells induced the expression of several genes related to the IFN pathway, which could theoretically lead to inhibition of tumor cell growth [15]. Interestingly, Velicescu et al. showed that de novo methylation does not occur in non-dividing BC cells. Treating T24 cells with DAC allows for cell arrest at G0/G1 and determines how much time is necessary for re-methylation to occur. Indeed, no re-methylation was found in CpG islands, whereas various degrees of methylation reappeared in CpG poor regions. Furthermore, DNMT1 and 3b3 protein levels were not detected, whereas DNMT3a mRNA levels were maintained after day 10 of the experiment. This result demonstrates that DNMT3a might catalyze a de novo methylation in CpG poor regions outside the S phase of the cell cycle [16]. In another study, the carcinogen *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine) (BBN) was used to induce bladder tumors in mice. These tumors showed downregulation of several genes including GSTM1, which seems to be regulated, in part, by DNA methylation, since treatment with DAC in 5637 cells increased GSTM1 expression [28]. Kawakami et al. reported for the first time that *MSH3* epigenetic regulation by means of DNA methylation might contribute to gene silencing, being implicated in BC carcinogenesis [18].

Recently, a study comprising a wide range of different cancer cell lines, containing BC, showed that DNMT inhibitors, including DAC and 5-aza, increase methylation levels throughout the cancer epigenome. Specifically, in BC cell lines, DAC treatment increased methylation levels of 616 common CpGs and decreased methylation levels of 590 different CpGs, demonstrating that the DNMT inhibitor mechanism of action is complex and requires further exploration [33].

After treatment with S110, global methylation levels decreased, concomitantly with increased p16 expression [22]. The effects of S110 in vivo were also assessed in a tumor xenograft mouse model using EJ6 cells. Tumor-free animals tolerated S110 better than DAC, with less weight loss and mortality. S110 failed to cause reduction in

tumor sizes, yet their growth rate was lower and p16 expression was induced [26]. The development of DAC and 5-aza variants could potentially increase their half-life, improving bioavailability and therapeutic efficacy.

Procainamide and hydralazine inhibitory effects on DNA methylation were studied in T24 cells. Both compounds decreased the methylation levels of TSGs *p16* and *RARβ*, which associated with their re-expression, both at the transcript and at the protein levels. Interestingly, hydralazine treatment maintained p16 reactivation for longer than DAC [17]. A novel strategy to inhibit DNMT1, using an essential enzyme for cancer cell viability [35] highly expressed in BC [36], was proposed using DNAzymes. A DNAzyme is a stable DNA molecule with catalytic activity that targets specific RNA molecules leading to their destruction [30][37]. The DNAzyme DT433, constructed and selected to target DNMT1, displayed effects that were similar to those of 5-aza. Additionally, DT433 led to an increase in p16 expression and inhibition of cell proliferation [30]. Novel strategies applying repurposed drugs or DNAzymes to achieve DNMT inhibition constitute interesting alternatives to the demethylating drugs already approved. On the one hand, DNAzymes showed that increasing specificity toward the target may be achieved, whereas repurposed drugs, with negligible toxicity and known safety profiles, may be administered for longer periods and be considered as the next step for DNA methylation inhibition as anti-cancer therapy.

2.2. Combination Studies

The anti-tumor effects of 5-aza, trichostatin A (TSA), and FK228 (the latter is a class I histone deacetylase (HDAC) inhibitor) were evaluated individually and in combination in a set of BC cell lines, as well as xenograft and orthotopic mouse models. The combination 5-aza and FK228 was shown to be toxic for 90% of the BC cells, inducing apoptosis and decreasing the G2/M cell population. The same combination in in vivo models led to a decrease in tumor size, mainly due to the effect of FK228 alone [38]. Combination treatments using 5-aza and TSA also reduced the cell number and a shift in the expression of proteins that regulate the cell cycle in canine BC cell lines [39]. More recently, combination of DAC and entinostat, another HDAC1 inhibitor, was tested in bladder cell lines, including two cisplatin-sensitive (J82 and RT112) and one cisplatin-resistant (J82CisR) cell lines, as well as one urothelial cell line isolate from normal tissue (HBLAK). The combination treatment did not revert cisplatin resistance of J82CisR, although treatment with both drugs promoted cell growth arrest. Additionally, increased apoptosis was observed, related to caspases 3 and 7, prompting cell arrest at G2/M transition. Forkhead box O1 (FoxO1) expression increased after the combination treatment, as well as BIM and p21, along with a decrease in survivin expression [40].

Several strategies using epigenetic drugs were devised to overcome chemoresistance and increase therapy success. Ramachandran et al. showed that pre-treatment with 5-aza followed by cisplatin or docetaxel exposure increased cytotoxicity in BC cells. Moreover, in UMUC3 cells that developed resistance to cisplatin or docetaxel in vitro, pre-treatment with 5-aza resulted in 44% and 55% of cytotoxicity in cisplatin- and docetaxel-resistant cells, respectively, in opposition to 2% with no pre-treatment [41]. Pre-treatment with DAC also enhanced cytotoxicity of cisplatin and doxorubicin in BC cells. Furthermore, RASSF1A expression was observed concomitantly with activation of the Hippo pathway [42]. Interestingly, UMUC14, RT4, 96-1, and 97-1 cell lines displayed low sensitivity to cisplatin, being associated with high *HOXA9* methylation levels. This was also verified in MIBC patients in which

high *HOXA9* methylation levels were associated with resistance to chemotherapy. In line with other studies, DAC led to a 4–5-fold decrease in half maximal inhibitory concentration (IC_{50}) for cisplatin, vinblastine, doxorubicin, and etoposide in BC cells [43]. Wu et al. demonstrated for the first time that DAC treatment reduced the cancer stem-cell population in mice. Specifically, in a BNN-induced mouse model of BC, DAC alone or in combination with cisplatin or gemcitabine led to a decline in the keratin 14 (KRT14)+ cell population, which originates from the bladder urothelium [44]. Consistently, the percentage of SRY-box transcription factor 2 (SOX2)+ cell population assumed to be responsible for the spread of the tumor [45] was also lower. Curiously, the percentage of such cells increased in mice treated with chemotherapy only [46]. These results were replicated in patient sample-derived xenografts, demonstrating that a combination treatment of DAC with chemotherapeutic agents might constitute a valuable option for BC therapy [46]. Another study showed that a quadruple combination therapy comprising gemcitabine, cisplatin, DAC, and TSA increased apoptosis via cyclin D1 (CCND1) downregulation, DNA fragmentation, and caspase 3 expression in T24 cells. On the other hand, a cell proliferation reduction and lower BCL2L1 mRNA levels were observed after treatment [47].

A novel dual inhibitor, CM-272, targeting G9a, a histone methyltransferase that catalyzes H3K9me2, and DNMTs demonstrated activity against a wide range of cancer cells [48]. Specifically, treatment of hematologic malignancies cell lines with concentrations in the nanomolar range led to a global decrease in H3K9me2 and 5-methylcytosine levels, a reduction in cell proliferation, induction of cell-cycle arrest and apoptosis, and a decrease in TSG promoter methylation. Interestingly, CM-272 also induced an IFN-γ type I response and immunogenic cell death. In an in vivo mouse model, CM-272 was safe to administer, with an increased overall survival of the treated mice [48]. Furthermore, CM-272 was also active against hepatocellular carcinoma cell lines [49]. Recently, the effects of CM-272 were evaluated in in vitro and in vivo models of BC. CM-272 in combination with cisplatin led to inhibition of cell proliferation, which was also verified in a BC xenograft mouse model, leading to a decreased tumor growth, whereas apoptosis and autophagy were increased [50]. These effects were also observed in a quadruple-knockout transgenic mouse model of advanced BC. Remarkably, BC cells treated with CM-272 showed upregulation of genes linked to the immune response, including IFN-α and γ, and tumor necrosis factor (TNF)-α, probably through induction of an endogenous retrovirus response [50]. Taking into account these results, the combination of CM-272 with anti-PD-L1 in the quadruple-knockout mouse model was explored. A sustained response to the combination treatment was observed, with the number of animals developing tumors or metastases being lower in the combination group when compared with the group treated with anti-PD-L1 monotherapy [50].

2.3. Clinical Studies

A non-randomized multicenter phase II study evaluated the effects of FdCyd in THU [51][52]. The safety, maximum tolerated dose (MTD), pharmacokinetics, and pharmacodynamics of this combination were determined in a previous phase I clinical trial (FyCyd 100 mg/m^2/day and THU 350 mg/m^2/day) [53]. Patients with metastatic or unresectable breast cancer (n = 29), head and neck cancer (n = 21), non-small-cell lung cancer (n = 25), and urothelial cell carcinoma (n = 18) which endured progression after at least one line of standard therapy were included in this study. The combination was well tolerated, and urothelial carcinoma patients showed some clinical responses, with an objective response rate (ORR) of 5.6%, a progression-free survival (PFS) of 3.6 months, and a

four-month PFS probability of 42%. Furthermore, increased p16 expression was observed in cytokeratin-positive circulating tumor cells (CTCs) of some patients, although it did not associate with clinical response (**Table 2**) [52].

Table 2. Clinical trials in BC using DNMT inhibitors.

Drug	Phase (ID)	Status	Enrollment	Schedule	Results	Period	Reference
5-Aza and sodium phenylbutyrate	I (NCT00005639)	Completed	Patients with diagnosis of a refractory solid tumor malignancy with no curative options including BC (*n* = 34)	Regimen A: Low-dose of 5-aza with intermittent phenylbutyrate 400 mg/m^2/day over 24 h on days 6 and 13. Regiment B: 5-aza 75 mg/m^2/day for 7 days, followed by two different doses of phenylbutyrate starting on day 8 and continuing for 7 days. Each cycle lasts 35 days for A and B. Regiment C: 2 different daily doses of 5-AC for 21 days and phenylbutyrate 400 mg/m^2/day over 24 h once per week. Each cycle lasts 42 days.	Three doses were well tolerated. Common toxicities included bone marrow suppression-related neutropenia and anemia. One patient showed stable disease; the remaining did not show any clinical response.	2000–2005	[54]
DAC	I (NCT00030615)	Completed	Advanced metastatic solid tumor patients after other standard therapies fail including BC (*n* = 24)	DAC intravenous (IV) over 30 min on days 1–5 weekly for 4 weeks. Course repeated every 6 weeks in the absence of disease progression or	Not available	2001–2008	[55]

Drug	Phase (ID)	Status	Enrollment	Schedule	Results	Period	Reference
				unacceptable toxicity.			
FdCyd and THU	II (NCT00978250)	Completed	Metastatic or unresectable solid tumors including urothelial transitional cell carcinoma (*n* = 18), whose disease progressed after at least one line of standard therapy.	FdCyd (100 mg/m^2/day) by 3 h intravenous infusion and THU (350 mg/m^2/day) 20% as a bolus, with the remaining co-administered with FdCyd over 3-h infusion on days 1–5 and 8–12 of each 28-day cycle.	Co-administration with THU was shown to increase the area under the curve of FdCyd more than 4-fold. Combination was well tolerated. ORR of 5.6%, PFS of 3.6 months, and 42% of 4-month PFS probability for urothelial cancer patients.	2009–2019	[52][56]
CC-486, carboplatin, and paclitaxel protein-bound particles (ABI-007)	I (NCT01478685)	Completed	Patients with relapsed or refractory solid tumors including urinary bladder neoplasms (*n* = 169)	Arm A: CC-486 (doses between 100–300 mg) was administered orally daily either 14 or 21 days. Carboplatin was given by intravenous (IV) infusion once every 21 days Arm B: CC-486 (doses between 100–300 mg) was administered orally daily for either 14 or 21 days ABI-007 was administered by intravenous (IV) infusion on	CC-486 dosed 14/21 days was tolerated as a priming agent with carboplatin and ABI-007. Both combinations show evidence of clinical activity.	2011–2015	[57][58]

Drug	Phase (ID)	Status	Enrollment	Schedule	Results	Period	Reference
				two of every three weeks Arm C: CC-486 (doses between 100–300 mg) was administered orally daily for either 14 or 21 days.			
RX-3317	I (NCT02030067)	Completed	Patients with advanced or metastatic solid tumors including advanced BC (*n* = 124)	A cycle was 4 weeks, with up to 8 cycles. RX-3117 dosing was given 3 times each week for 3 weeks followed by 1 week off treatment. All subjects were followed for at least 30 days after the last dose of RX-3117.	Not available	2013–2019	[59]
CC-486	I (NCT02223052)	Completed	Subjects with hematologic or solid tumor malignancies including BC patients (*n* = 89)	Arm 1: Two 150-mg tablets of CC-486 on day 1 and 1 × 300 mg CC-486 on day 2 Arm 2: 1 × 300 mg tablet of CC-486 on day 1 and 2 × 150 mg CC-486 on day 2.	Not available	2014–2018	[60]
SGI-110, gemcitabine, and cisplatin	Ib/IIa (2015-004062-29)	Recruiting	Urothelial BC patients with stages T2-4aN0M0 (*n* = 20)	Arm 1: SGI-110 days 1–5 at the determined dose, gemcitabine 1000 mg/m^2 days 8 + 15, cisplatin 70 mg/m^2 day	Not available	2015–present	[61]

Drug	Phase (ID)	Status	Enrollment	Schedule	Results	Period	Reference
				8. 3–4 cycles of 21 days each. Arm 2: Gemcitabine 1000 mg/m^2 days 8 + 15, cisplatin 70 mg/m^2 day 8 3–4 cycles of 21 days each for both arms. 3–4 cycles of 21 days each.			
75 approved agents	II (NCT02788201)	Completed	Patients with a diagnosis of metastatic, progressive urothelial carcinoma of the bladder, urethra, ureter, or renal pelvis (n = 8)	COXEN algorithm was used to determine the best therapy from among 75 FDA-approved agents (single agent or combination). Patients had regular visits for blood, urine, and tumor scans.	Not available	2017–2019	[62]
Azacitidine, pembrolizumab, and epacadostat	I (NCT02959437)	Completed [54]	Subjects with advanced or metastatic solid tumors including BC patients (n = 70)	Five doses of azacitidine were administered by subcutaneous injection or intravenously (IV) over days 1 to 7 in cycles 1 through 6. Pembrolizumab was administered in a 30-min IV infusion every 3 weeks on day 1 of each 21-day cycle. Epacadostat	Not available	2017–2020	[63]

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whereas three patients displayed partial response, with both combinations disclosing clinical value. Interestingly, peripheral blood mononuclear cells (PBMCs) were found to be hypomethylated. A recommended phase II dose (RP2D) study will comprise an expansion of cohorts and the combinations of 300 mg of CC-486 with carboplatin and 200 mg of CC-486 with ABI-007 [58].

Other clinical trials are at this date assessing the clinical benefit of combining immune checkpoint inhibitors with epigenetic drugs. In a recently completed study, the combination of pembrolizumab, epacadostat [indoleamine 2,3-dioxygenase 1 (IDO1)-selective inhibitor], and 5-aza was assessed in a phase I and II trial enrolling 70 patients, including urothelial cancer patients [63]. Furthermore, in a currently recruiting phase II clinical trial, the biological

Drug	Phase (ID)	Status	Enrollment	Schedule	Results	Period	Reference
				tablets were administered orally twice daily.			[64]
Atezolizumab and guadecitabine	II (NCT03179943)	Suspended	Recurrent/advanced urothelial carcinoma (stage IV) patients who previously progressed on checkpoint inhibitor therapy with anti-PD-1 or PD-L1 therapy (n = 53)	Atezolizumab is administered intravenously on day 1 and day 22 of a 6-week cycle for a period of 8 cycles. Guadecitabine is administered subcutaneously on days 1 through 5 of the 6-week cycle for a period of 4 cycles.	Not available	2017–estimated end 2022	[64]

Abbreviations: 5-aza—5-azacytidine; DAC—decitabine.

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