Seminars in Hematology 58 (2021) 45–55

Contents lists available at ScienceDirect

Seminars in Hematology

journal homepage: www.elsevier.com/locate/seminhematol

DNA methylation inhibition in myeloma: Experience from a phase 1b study of low-dose continuous azacitidine in combination with lenalidomide and low-dose dexamethasone in relapsed or refractory multiple myeloma

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ARTICLE INFO

Keywords:
DNA methylation
Multiple Myeloma
Azacitidine
Lenalidomide
Dexamethasone

ABSTRACT

The DNA methyltransferase inhibitor azacytidine (aza) may reactivate pathways associated with plasma cell differentiation, cell cycle control, apoptosis, and immune recognition and thereby restore sensitivity to lenalidomide (len) and dexamethasone (dex) in relapsed and/or refractory multiple myeloma (RRMM). We aimed to develop an aza regimen that reaches epigenetically active levels 8 times in 28 days with less bone marrow toxicity than the myeloid malignancy standard of 7 consecutive doses to enable safe combination with len. Aza was escalated from 30 mg/m^2 once a week up to a predefined maximum of 50 mg/m^2 twice a week in combination with GFR-adjusted len (≥ 60 mL/min: 25 mg, 3059 mL/min: 10 mg) day 1 to 21 every 28 days and dex 40 mg once a week followed by a limited expansion study to a total N of 23 at the highest tolerated dose. Fifty-one patients (pts) with RRMM were screened, 42 were treated and 41 were evaluable for response based on at least 1 response assessment or progression after treatment start. The median number of prior lines of therapy was 5 (1-11) and 81% (34) were refractory to len and/or pomalidomide (pom). Two DLTs occurred in different cohorts, 1 neutropenic fever in 1/6 pts on the aza 40 mg/m^2 twice a week GFR ≥ 60 mL/min cohort and 1 GGT elevation in 1/6 pts on the aza 50 mg/m^2 GFR 30-59 mL/min cohort. An MTD was not reached and aza 50 mg/m^2 SC twice a week was chosen for the expansion study. At least possibly related Grade 3/4 AEs occurred in 28 pts (67%) with the following in > 1 pt: neutropenia (N = 16, 38%), anemia (N = 6, 14%), lymphopenia (N = 5, 12%), thrombocytopenia (N = 4, 10%), leukopenia (N = 4, 10%), febrile neutropenia (N = 4, 10%), fatigue (N = 3, 7%), fever (N = 2, 5%), and infection (N = 2, 5%). At a median follow up time for alive pts of 60.2 months (range: 36.1-82.5 months), the overall response rate (≥ partial response) and clinical benefit response

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https://doi.org/10.1053/j.seminhematol.2020.12.004

rate (≥ minor response) was 22 and 32%, respectively, with 4 very good partial responses (10%), 5 partial responses (12%), and 4 minor responses (10%). The median PFS was 3.1 months (95% confidence interval [CI]: 2.1-5.1 months), median TTP 2.7 months (95% CI: 2.1-7.5 months), and median OS 18.6 months (95% CI: 12.9-33.0 months). Achieving at least minor response and reaching TTP > 6 months was associated with approximately 35% lower median plasma levels of the enzyme that inactivates aza, plasma cytidine deaminase (CDA, $P < .0001$). Two of the len refractory pts achieved longer disease control than with any prior regimen and 1 responded immediately after progression on len, bortezomib, and prednisone. Analyses of the methylation state of over 480,000 CpG sites in purified myeloma cells at screening were possible in 11 pts and on day 28 in 8 of them. As in other studies, the majority of differentially methylated CpGs compared to normal plasma cells were hypomethylated in myeloma. Treatment decreased the number of CpGs that were differentially methylated in normal plasma cells by > 0.5% in 6 and by > 5% in 3 of the 8 pts, most pronounced in 2 pts with clinically convincing aza contribution who achieved a reduction in overall differentially methylated CpGs by 23 and 68%, respectively, associated with increased expression of immunoglobulin genes. The study demonstrated tolerability of twice a week SC aza at 50 mg/m^2 with len and dex in RRMM and suggested aza may help overcome the len/pom refractory state, possibly by activating differentiation pathways. Relatively low response rates and association of clinical benefit with low plasma levels of the aza inactivating enzyme CDA suggest the aza regimen will need to be optimized further and pt selection may be required to maximize benefit.

Introduction

Multiple myeloma (MM) is a genetically [1] and epigenetically [2] heterogeneous malignant clonal disease of plasma cells with a lifetime risk in the US population of approximately 0.8% per estimates from 2015-2017 SEER data [3]. Genetic and epigenetic changes are involved in malignant transformation [4-12] from pre-malignant phase with readily detectable monoclonal gammopathy [13-16] and in the development of a treatment resistant state that is precluding cure for the overwhelming majority of patients with myeloma despite major advances in its treatment over the last 3 decades [17]. When treatment resistance to currently approved CD38 antibodies, IMiD compounds and proteasome inhibitors develops, dismal median survival of 6 months indicates an ongoing need for improved approaches [18].

So far insurmountable disease plasticity is based on the presence of genetically distinct subclones which evolve independent from each other [1,12,19], variable levels of genomic instability [20], and epigenetic changes that are driven by the mutational and micro-environmental background and, like genetic changes, selected for alterations that confer survival advantage under treatment-induced selection pressures [21]. Epigenetic modulation can occur due to active cell-cycling, mutations of protein homeostasis and histone modifying enzymes and other cellular processes [22]. While these effects can be transient, they promote changes in DNA methylation and thereby engrave suppression of processes that hurt clonal survival in a heritable fashion that is transmitted to cellular progeny predominantly through the combined action of DNA methyltransferase 1 (DNMT1) and UHFR1 with the latter involved in recognition of suppression marks and in activation of DNMT1 so that it can copy the methylation state during S-phase [22-25]. Additional pro-carcinogenic effects of aberrant DNA methylation include the following: methylated cytosine spontaneously deaminates to uracil and thymine, causing point mutations when thymine is erroneously repaired; hypomethylation of gene bodies destabilizes the nucleosome and by impairing access of enzymes required for RNA elongation and splicing can result in spurious transcription; hypomethylation can also activate transposable elements and impair access of DNA repair enzymes creating another mutagenic risk and if hypomethylation occurs in regulatory regions of oncogenes, their increased expression can promote tumor growth and survival [23,26,27]. An ideal therapeutic DNA methylation modulation in cancer would therefore consist of a normalization, and the ability of aza nucleosides to induce differentiation suggests this is possible [28,29].

Four independent studies that analyzed the myeloma DNA methylome have found global hypomethylation with less extensive regional hypermethylation compared to normal plasma cells as the predominant pattern [2,10,30,31]. Two studies that included more extensively pretreated patients found that methylation can increase during clonal evolution [10,31], especially to plasma cell leukemia [31]. The most comprehensive methylome analysis used the same array platform employed in this study in a population of over 100 pts plus whole genome methylome sequencing of 2 pts at the extremes of methylation. In addition, it included meticulous comparison to normal B-cell development stages and to other lymphoid malignancies. It found variability of the DNA methylome is greater in MM than in acute lymphoblastic leukemia, chronic lymphocytic leukemia and diffuse large B-cell lymphoma with, on whole genome methylation sequencing, between over 2 and 4 million differentially methylated CpGs that ranged from 50% hypermethylated to 90% hypomethylated compared to normal plasma cells [2]. Another striking finding was that hypermethylation of B-cell specific enhancers is a common finding in myeloma that is associated with their decommissioning suggesting an epigenetically defined block in differentiation [2]. Since many of the most active myeloma therapies target the plasma cell program, loss of differentiation may not only maintain self-renewal but may also provide a specific therapy-escape mechanism. Loss of differentiation is a described mechanism for MM resistance to the proteasome inhibitor bortezomib [32]. The activity of immunomodulatory imide group containing (IMiD) compounds, which target the myeloma differentiation and survival factor IRF4, on refractory myeloma cells in vitro has been shown to be increased by combination with the DNA methylation inhibitor azacytidine and the EZH2 inhibitor tazemetostat. This effect was independent of cereblon and felt to be related to epigenetic “re-programming” [33]. DNA demethylating treatment of cancer cells can reactivate genes involved in immune signaling [34,35] and evidence for an adaptive immune response has been shown in a study that used azacytidine followed by lenalidomide in patients with controlled myeloma suggesting DNA methylation inhibition may also augment the IL-2 driven immune stimulatory mechanism of action IMiD compounds [21,36-39]. Methylation of the RASD1 promoter has been found to mediate resistance of myeloma to dexamethasone [40]. Therapeutic benefit may therefore result from combination of DNA methyltransferase inhibition by azacytidine with lenalidomide and dexamethasone in relapsed and/or refractory myeloma. The standard azacytidine regimen for myeloid neoplasias which uses 75 mg/m^2 SC daily for 7 days every 28 days causes clinically significant bone

marrow suppression [41] as an overlapping toxicity with lenalidomide. This may in part be dose related since peak exposures are expected to reach around 10 times higher concentrations than required for epigenetic effects [42,43]. In addition, continuous DNA methylation inhibition during the first 7 days of a cycle is expected to maintain the immature state of normal hematopoietic stem cells and may therefore result in neutropenia due to delay in maturation [44] adding to toxicity from high peak exposure.

Based on these considerations, we decided to evaluate the safety and preliminary efficacy of up to twice a week azacytidine dosing, up to a dose of 50 mg/m^2, in combination with standard doses of lenalidomide and dexamethasone in pts with relapsed or refractory myeloma.

Methods

Patients

The study enrolled pts with relapsed or refractory MM who were 18 years or older with an Eastern Cooperative Oncology Group performance status (ECOG-PS) of 0-2 and measurable disease at baseline defined as a serum m-spike $\geq$ 1g/dL, a urine m-spike $\geq$ 200 mg/24 hours, serum free light chains $\geq$ 100 mg/L (provided the kappa/lambda ratio was abnormal), or bone marrow plasma cells $\geq$ 30%. Additionally, adequate hematologic (absolute neutrophil count $\geq$ 1500 /mm^3 and platelet count $\geq$ 75,000/mm^3), renal (calculated creatinine clearance (Cockroft-Gault) $\geq$ 30 mL/min), and hepatic (total bilirubin $\leq$ 1.5 x ULN, serum AST and ALT levels $\leq$ 2 x ULN) function was required. Patients were excluded if they had $\geq$ grade 3 peripheral neuropathy, prior history of a desquamating rash while taking IMiD compounds or previous lenalidomide intolerability. The protocol for this study was designed in accordance with the general ethical principles outlined in the Declaration of Helsinki. The study was approved by the Cleveland Clinic and University Hospitals Cleveland Medical Center Institutional Review Boards and was registered at www.clinicaltrials.gov as NCT01155583. All pts gave informed consent.

Study Design

The study used a 3 + 3 dose escalation design to determine the highest tolerated azacytidine dose up to a maximum of 50 mg/m^2 SC twice a week initially with lenalidomide 25 mg daily from day 1-21 every 28 days and dexamethasone 40 mg once a week in pts with GFR $\geq$ 60 mL/min (estimated by the Cockroft-Gault formula). After confirmation of tolerability of the 2 highest azacytidine dose levels (DLs) in pts with GFR $\geq$ 60 mL/min, these 2 DLs were assessed for tolerability in pts with GFR 30-59 mL/min who received standard GFR-adjusted lenalidomide at 10 mg day 1-21 every 28 days. In DL 1 & 2 azacitidine was given weekly at 30 and 40 mg/m^2 SC, respectively, in DL 3 to 5 twice a week at 30, 40, and 50 mg/m^2, respectively. Declaration of the maximum tolerated dose (MTD) or the highest tolerated low dose required treatment of at least 6 pts per GFR cohort at that DL with no more than 1 DLT observed per cohort. The MTD was defined as the dose level below which DLTs occur in 2 of up to 6 pts treated during the DLT period of 28 days. An expansion by 10 response evaluable pts was planned at the MTD or the highest tolerated low dose if no MTD was observed.

Study Objectives and Endpoints

The primary objective of this study was to determine the safety and tolerability of the regimen in pts with GFR $\geq$ 60 mL/min and GFR 30-59 mL/min and used safety endpoints with CTCAE version 4.03 for grading of toxicities. Secondary objectives included evaluation of response, progression-free-survival (PFS), and overall survival (OS) using uniform response criteria by the international myeloma working group criteria (reported here according to the latest version from 2016 [45]) and PFS and OS measured from study entry. Additional objectives included assessment of association between plasma levels of the azacytidine inactivating enzyme cytidine deaminase (CDA) and efficacy as well as investigation of the effect of treatment on global DNA methylation and gene expression profiles in pts where purified myeloma cells (the protocol required a purity of at least 70%) of sufficient quantity for Illumina HumanMethylation450 and HT-12 v4.0 BeadChip arrays, respectively, could be run. Safety assessments were performed at screening and throughout the study. For the first 6 cycles, assessments were conducted weekly for the first cycle then at the start of the subsequent cycles. During maintenance, safety assessments were done monthly for the first 6 cycles and then every other month. They included history and physical examination including vital signs, complete blood count, and metabolic panel for adverse event (AE) detection and monitoring and measurement of vital signs. Response was evaluated by the treating investigator at the beginning of each of the first 6 cycles and then monthly while on maintenance. Response categories included stringent complete response, complete response (CR), very good partial response (VGPR), partial response (PR), and minor response (MR). Since this study used no minimal residual disease assessment, MRD categories were not used. Prior to publication, response assessment was verified by the principal investigator (senior author) and the lead author and corrected if necessary (1 PR was downgraded to MR, 1 SD upgraded to MR). Blood samples for correlative studies were obtained weekly during the first cycle, then every 28 days for the first 6 cycles. Bone marrow aspirates were obtained at screening in all pts and on day 28 unless pts refused or a medical rationale existed to not proceed.

Drug administration

Azacitidine was administered SC at least 5 days apart in weekly dosing cohorts and at least 48 hours apart in twice a week dosing cohorts in combination with GFR-adjusted lenalidomide po day 1 to 21 every 28 days and dexamethasone 40 mg po weekly for 6 28-day cycles followed by maintenance with lenalidomide at the last tolerated dose. For pts who experienced disease stabilization with less than PR after 6 cycles, continuation of combination therapy was allowed per physician discretion. All pts received aspirin 81 mg to 325 mg daily for venous thromboembolism prophylaxis or more intensive anticoagulation with enoxaparin or warfarin if they had risk factors for venous thromboembolism.

Correlative studies

Plasma CDA activity was measured by quantifying the conversion of cytidine to uridine at 37°C with a previously described high performance liquid chromatography method [46] at Zymo Research Corp., CA in a blinded fashion. Each measurement was done in triplicate with a uridine standard curve to allow quantitation of enzyme activity after initial calibration with recombinant CDA enzyme. Bone marrow aspirates were processed the same day with Ficoll-Paque density gradient isolation of mononuclear followed by Miltenyi CD138 magnetic bead purification according to the manufacturer's instructions. If at least approximately 1 million CD138 purified cells could be obtained, the sample was processed further. DNA and RNA were isolated using Qiagen AllPrep DNA/RNA mini kit according to the manufacturer's instructions and stored at -80 C or processed immediately. RNA was transcribed into cDNA using

Superscript III (Invitrogen) within 30 days and EZ DNA Methylation kits from Zymo Research were used for bisulfite conversion of DNA. After confirmation of DNA and RNA purity and quantity using NanoDrop, samples were submitted to the Cleveland Clinic core facility for Illumina HumanMethylation450 and HT-12 v4 BeadChip array hybridization with quality control before investigational analyses were performed on genome studio version 2011.1. Normal bone marrow mononuclear cells for separation of CD138 positive and negative fractions of 3 healthy donors was obtained commercially from ALLCELLS and processed the same way or used without purification as normal bone marrow control. The purity of plasma cells was evaluated by flow cytometry and staining for CD138 and intracellular kappa and lambda. In myeloma samples, a purity of 90% and higher was achieved while normal plasma cells had a purity of 70% by CD138 staining after 2 rounds of magnetic bead purification. As additional control for DNA methylation studies human myeloma cell lines MM-1S and MM-1R were purchased from ATCC, grown in RPMI 1640, supplemented with 10% fetal bovine serum, penicillin G (50 units/ml), and streptomycin (50 µg/mL) and cultured at 37°C, 5% CO^2 in humidified air.

Statistical analyses

Toxicity and efficacy outcomes were summarized using frequencies and percentages. Overall survival (OS) and progression-free survival (PFS) were estimated using the Kaplan-Meier method. The difference in CDA activity between pts who achieved TTP greater and less than 6 months was analyzed by a 2-tailed Mann-Whitney test using Prism 8 software.

Results

Participant characteristics

Overall, 51 patients were screened and 44 were enrolled between July 2010 and April 2016. Data were analyzed July 31, 2020. Two pts did not start treatment since eligibility criteria for neutrophils and progression, respectively, were not met. Baseline characteristics of treated pts are shown in Table 1. The median age was 62.5 (range: 39-88) and the median number of prior lines of therapy was 5 (range: 1-11). Forty-one pts (98%) had received an IMiD, and 34 (81%) were refractory to lenalidomide and/or the more potent pomalidomide according to international myeloma working group definitions [47], meaning failure to achieve at least MR or progression on or within 60 days of last therapy. Thirty pts (75%) had myeloma refractory to the proteosome inhibitors bortezomib and/or carfilzomib and 29 (69%) were refractory to both lenalidomide and/or pomalidomide and bortezomib and/or carfilzomib. Two pts entered the study with secondary plasma cell leukemia.

Safety and tolerability

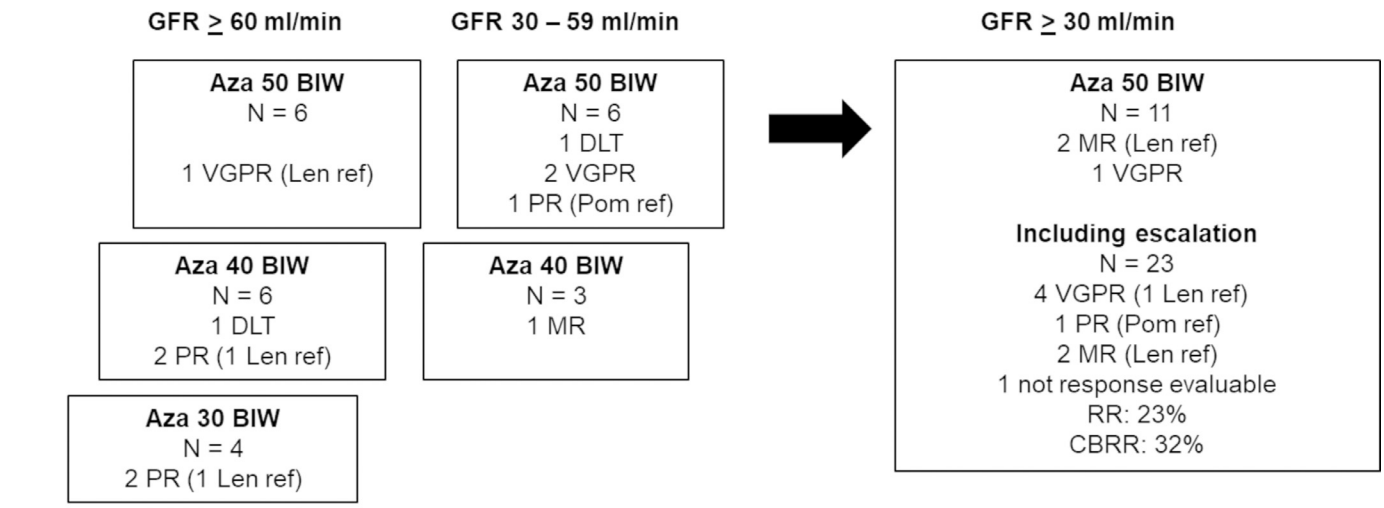
The median number of cycles of therapy received was 3 (range: 1-36) and 8 pts completed the first 6 cycles of aza, len and dex. The median number of cycles of aza, len, and dex was 3 (range: 1-6). Two DLTs were observed, 1 neutropenic fever in the GFR ≥ 60 mL/min 40 mg/m^2 SC twice a week cohort and one possibly related GGT elevation in the GFR 30-59 mL/min 50 mg/m2 SC twice a week cohort (Fig. 1 and Suppl. Table 1). Suppl. Table 1 shows AEs that were felt by the treating physician to be at least possibly related to study drugs, for the dose level selected for expansion (50 mg/m^2 twice a week) and for lower dose levels combined. Overall, 53 Grade 3-4 AEs were observed across all dose levels in 28 participant (67%) and G3-4 AEs that occurred in more than 1 participant were neutropenia (N = 16, 38%), anemia (N = 6, 14%), lymphopenia (N = 5, 12%), thrombocytopenia (N = 4, 10%), leukopenia (N = 4, 10%), febrile neutropenia (N = 4, 10%), fatigue (N = 3, 7%), fever (N = 2, 5%), and infection (N = 2, 5%). Grade 3-4 neutropenia was reported more frequently for pts treated at 50 mg/m^2 twice a week than at lower doses (52 and 21%, respectively) while G3-4 thrombocytopenia was comparable between lower dose levels and the dose level selected for expansion (11 and 9%, respectively). Febrile neutropenia occurred in 2 of 6 pts treated at 40 mg/m^2 twice a week and 2 of 23 pts (9%) treated at 50 mg/m^2. Injection site reactions were reported only for pts treated at 50 mg/m^2 twice a week (N = 6, 26%) and were all of mild (Grade 1) intensity.

Table 1
Baseline characteristics of patients.

	N = 42 (%)
Age (years)	
Median	62.5
Range	39-88
Female	23 (55)
Male	19 (45)
White	38 (90)
African American	4 (10)
ECOG Performance Status	
0	19 (45)
1	17 (40)
2	6 (14)
Immunoglobulin type	
IgG (2 with secondary plasma cell leukemia)	19 (45)
IgA	11 (26)
IgD	1 (2)
Light chain	11 (26)
ECOG-PS	
0	19 (45)
1	17 (40)
2	6 (14)
ISS stage at entry	
Stage I	16 (38)
Stage II	12 (29)
Stage III	11 (26)
Missing data	3 (7)
Disease Manifestations	
Kidney	7 (16)
Lytic lesions	31 (72)
Hypercalcemia	7 (16)
Anemia	26 (60)
Prior proteasome inhibitor therapy	38 (90)
Bortezomib	38 (90)
Carfilzomib	11 (26)
Previous IMiD® therapy	41 (98)
Lenalidomide	36 (86)
Pomalidomide	6 (14)
Thalidomide	20 (48)
Prior high-dose chemotherapy with autologous hematopoietic cell transplantation	11 (26)
Lenalidomide and/or Pomalidomide refractory	34 (81)
Bortezomib and/or Carfilzomib refractory	31 (74)
Refractory to Lenalidomide and/or Pomalidomide AND Bortezomib and/or Carfilzomib	29 (69)

Treatment allocation and outcome

Fig. 1 shows the allocation of pts to treatment cohorts with responses per cohort and dependent on refractory state to lenalidomide and/or pomalidomide. When all dose levels were analyzed together, the overall response rate (ORR) was 22% with 4 VGPRs and 5 PRs in 41 response evaluable pts. The clinical benefit response rate (CBRR) was 32% which included 4 MRs. At the dose level selected for expansion, ORR and CBRR were similar at 23 and 32%, respectively. When response evaluable pts with disease refractory to lenalidomide and/or pomalidomide (N = 33) were analyzed separately according to frequency of aza administration, the ORR was 0% and CBRR 17% in 6 pts treated with weekly aza while twice a week aza cohorts combined (N = 27) reached an ORR of 15% and a CBRR of 26%. Among 8 pts with disease that was nei-



	All response evaluable	Lenalidomide and/or Pomalidomide refractory			Not Lenalidomide or Pomalidomide refractory
	N = 41	Aza QW N = 6	Aza BIW N = 27	Aza 50 BIW N = 17	All Aza BIW N = 8
Best overall response					
VGPR	4	0	1	1	3
PR	5	0	3	1	2
MR	4	1	3	2	0
Response Rate	22%	0%	15%	12%	63%
Clinical Benefit Response Rate	32%	17%	26%	24%	63%

Fig. 1. Treatment allocation and response dependent on Lenalidomide and/or Pomalidomide refractory state. Response rate = PR or better; Clinical Benefit Response Rate = MR or better. Abbreviations: Aza = azacytidine; Aza 30, 40, 50 = azacytidine subcutaneous at 30, 40, 50 mg/m^2; BIW = twice a week at least 48 hours apart; GFR = glomerular filtration rate per Cockroft-Gault formula; MR = minor response; PR = partial response; QW = once a week at least 5 days apart; VGPR = very good partial response.

ther lenalidomide nor pomalidomide refractory, 3 achieved VGPR and 2 PR for an ORR and CBRR of 63% (Fig. 1). Narrowing the response analysis in len and/or pom refractory disease to pts who were treated with 50 mg/m^2 aza twice a week (N = 17) found similar ORR and CBRR as for the combined analysis of twice a week aza treated pts with 12 and 24%, respectively. The median time to response was 29 days (range 27 to 71), median time to best response was 60 days (range 27 to 616) and median duration of response was 125 days (30 to 1058). The median PFS was 3.1 months (95% CI: 2.1-5.1 months), median TTP 2.7 months (95% CI: 2.1-7.5 months, Fig. 2), and median OS 18.6 months (95% CI: 12.9-33.0 months). Reasons for discontinuation from study were disease progression (N = 27, 63%), participant withdrawal (N = 7, 16%), AEs (N = 4, 7%), difficulty getting to the treatment center (N = 2%), participation in an allogeneic stem cell transplant study (N = 1, 2%) and physician decision due to declining performance status (N = 1, 2%).

Outcome Dependent on Plasma CDA Activity

To explore whether the known rapid and variable metabolism of azacytidine [42,48] might affect outcome, we analyzed the enzyme responsible for its inactivation, plasma CDA, at screening and on treatment in 39 response evaluable pts. Three pts entered the study as backfill after completion of CDA analyses; 2 had myeloma refractory to len and none of the 3 pts achieved MR or better or TTP > 6 months. Patients who achieved at least MR or TTP > 6 months had significantly lower median CDA levels throughout the study than pts who achieved no response or who experienced progression within 6 months (Fig. 2B, C). For the entire study population with CDA assessments (N = 39), median CDA values in pts who achieved at least MR were 1169 mU/mL compared to 1476 mU/mL in nonresponders (P= .036, Mann-Whitney Test) and 973 mU/mL in pts who reached TTP > 6 months compared to 1507 mU/mL in pts with TTP < 6 months (P < .0001, Mann-Whitney Test). In pts with disease refractory to len and/or pom, median CDA levels were 945 and 950 mU/mL for pts who achieved at least MR and TTP > 6 months, respectively, compared to 1488 and 1487 mU/mL in nonresponders and earlier progressors, respectively (P < .0001 for both comparisons, Mann-Whitney test). Treatment had no significant effect on plasma CDA activity with median pretreatment and on-treatment CDA values of 1513 and 1411 mU/mL, respectively, (p = 0.6262, Mann-Whitney Test) but intra-individual variability of CDA levels at different time points was observed with a median standard deviation of 34.7% (range 2.3 – 87.4%). A single measurement may therefore not always adequately predict aza metabolism.

Table 2 shows clinical characteristics of pts who achieved TTP > 6 months or at least MR. While the study was not designed to test the contribution of treatment components, review of individual pt histories added to the impression from CDA analyses that

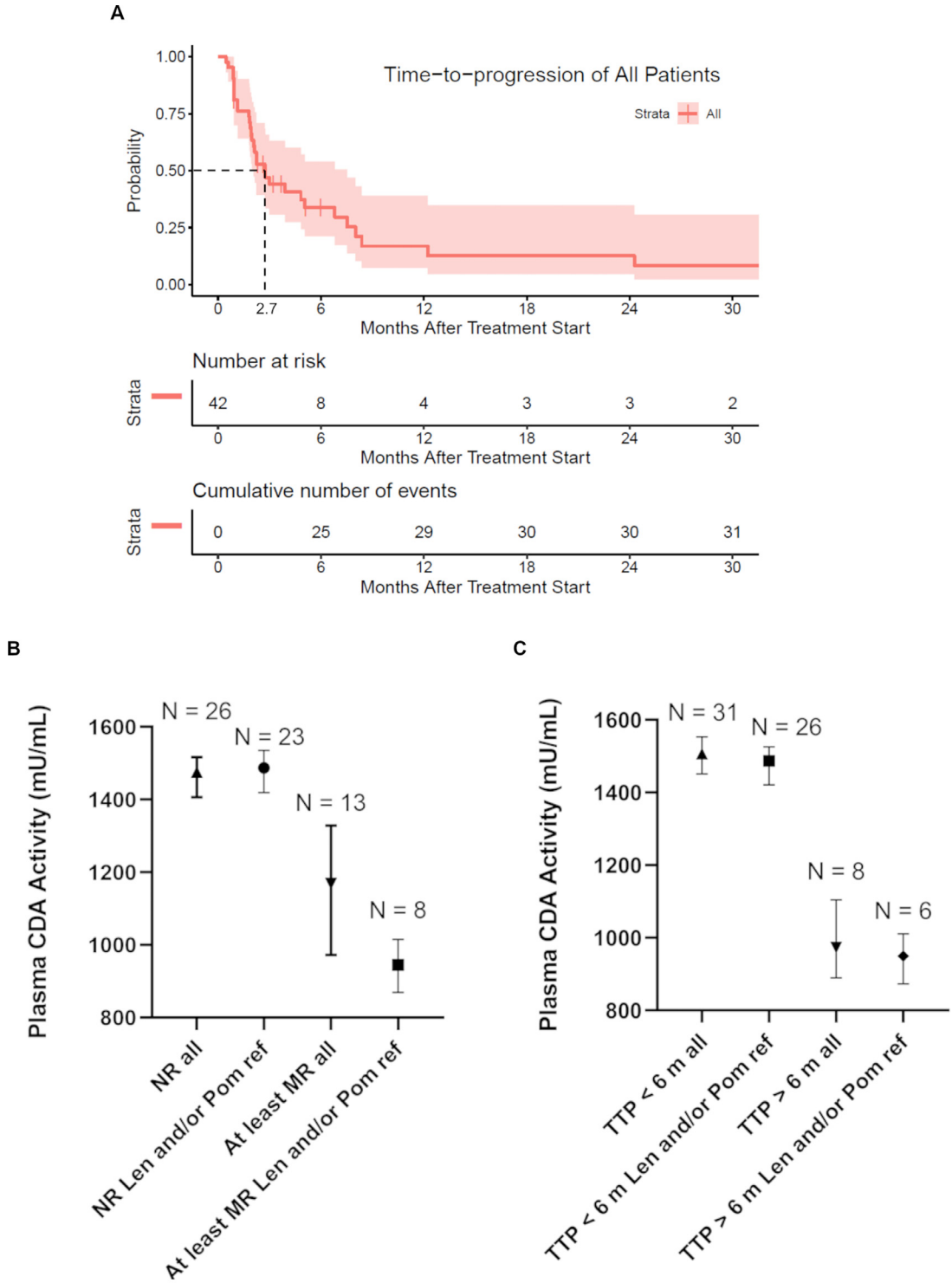


Fig. 2. Time to progression and association of plasma cytidine deaminase activity with outcome. (A) Time to progression of the entire study population. The shaded area displays the 95% confidence interval. (B) and (C): Association of median plasma cytidine activity throughout the study with achieving response (B) and time to progression greater than 6 months (C). Error bars display 95% confidence intervals. Three patients entered the study after completion of cytidine deaminase activity analyses and did not reach at least minor response or TTP > 6 months. Abbreviations: CDA = cytidine deaminase; Len = Lenalidomide; m = months; MR = minor response; NR = no response; Pom = Pomalidomide; TTP = time-to-progression; ref = refractory.

Table 2
Characteristics of patients who reached TTP > 6 months or at least MR.

PID	DL (mg/m^2)	BoR	TTP or time to exit (mos)	Median CDA (mU/mL)	Len or Pom ref?	Btz or Cfz ref?	Ref at study entry?	# prior Rx lines
32	Aza 50 BIW	MR	32	822	Y	Y	Y	3
104	Aza 50 BIW	VGPR	32	1401	N	N	N	2
18	Aza 40 BIW	PR	24.2*	1249	Y	Y	Y	4
23	Aza 50 BIW	VGPR	12.2	870	Y	Y	Y	4
14	Aza 40 BIW	SD	8.6	1577	Y	Y	Y	6
102	Aza 40 BIW	MR	7.8	641	Y	Y	Y	6
30	Aza50 BIW	VGPR	7.6	969	N	N	N	2
9	Aza 30 BIW	PR	6.8**	1353	Y	Y	Y	8
107	Aza 50 BIW	PR	5.3	1942	Y	N	Y	2
36	Aza 50 BIW	MR	4.8	1661	Y	Y	Y	5
108	Aza 50 BIW	VGPR	3.7	1978	N	N	N	2
15	Aza 40 BIW	PR	3.6	2009	N	Y	Y	1
10	Aza 30 BIW	PR	3.4	1869	N	N	N	1
1	Aza 30 QW	MR	3.1	1528	Y	Y	Y	8

Abbreviations: PID = participant identification; DL = dose level; BoR = best overall response; TTP = time to progression; mos = months; Median CDA = median plasma cytidine deaminase activity throughout the study; Len = lenalidomide; Pom = pomalidomide; ref = refractory according to IMWG criteria (best response less than MR or progression on or within 60 days of last therapy); Btz = bortezomib; Cfz = carfilzomib; Rx lines = regimen lines with induction, high dose chemotherapy with autologous stem cell transplant and maintenance counted as one line; TTNT = time to next treatment; aza 30, 40, 50 = azacytidine 30, 40, 50 mg/m2; BIW = twice a week; QW = once a week; Y = yes; N = no; SD = stable disease; MR = minor response; PR = partial response; VGPR = very good partial response; * PID18 Reached PR after > 7 months, therefore received extended azacytidine, lenalidomide and dexamethasone; after 16.75 months, treatment was administered closer to home and prednisone was substituted for dexamethasone due to better tolerance, given off study but PID18 followed with PI monthly. Response duration includes treatment continuation off-study. ** PID9 Received azacitidine, lenalidomide and dexamethasone according to study protocol but off study/off site after 4 months due to distance from Rx center but was followed by PI monthly. PFS duration includes off study/off site treatment period.

aza contributed to clinical benefit, at least in some pts. Participant ID (PID) 32 had never reached a response or disease control longer than 13 months prior to study entry, including while on treatment with lenalidomide and dexamethasone, and reached MR lasting 32 months on study. PID18 never reached response lasting longer than 11 months despite treatment with bortezomib, lenalidomide and dexamethasone (VRd), high-dose chemotherapy with autologous stem cell transplant and carfilzomib. With study treatment (using continuous azacytidine beyond 6 months as suggested per protocol for response below PR at that time point), PID18 reached PR (at 7 months) with disease control lasting over 2 years before deciding for allogeneic stem cell transplant in the context of subprogression level increase in monoclonal proteins. PID102 progressed on bortezomib, lenalidomide, and prednisone immediately prior to study entry and reached MR with disease control for 7.8 months on study.

Myeloma DNA methylation and gene expression analyses in a subset of patients

CD138 purified bone marrow cells for global array-based DNA methylation analysis at screening could be obtained from 11 pts and on day 28 in 8 of them. The array interrogates over 480,000 CpGs and based on methylation signals assigns each CpG an average methylation beta value between 0 and 1 with 0 for completely unmethylated and 1 for completely methylated alleles. Clustering DNA methylation profiles based on Pearson correlation of methylation patterns revealed that myeloma samples of this study clustered closer together with normal plasma cells (NPCs) than with human myeloma cell lines (MM1-S and MM1-R) and were most distinct from unsorted normal bone marrow (data not shown). The methylation array can detect a difference in beta value of 0.2 with 99% confidence [49]. In accordance with the threshold for differentially methylated sites used by Agirre et al. [2], we required a difference in beta value of at least 0.25 to call a CpG site differentially methylated between samples. Compared to normal plasma cells, myeloma cells had a median of 128,040 CpGs hypomethylated (range 7866-165,998) and a median of 12,700 CpGs hypermethylated (range 3066-26,316) for an overall median number of differentially methylated CpGs of 146,752 (range 93,646-178,257). GpG islands represented a median of 40% (range 24-56%) of hypermethylated CpGs and 8% (range 6-10%) of hypomethylated CpGs. Treatment reduced to number of CpGs that were differentially methylated (DM) in NPCs by > 0.5% in 6 and by over 5% (9-68%) in 3 of 8 pts with on-treatment samples while DM CpGs increased by 4 and 16% in 2 pts. Marked decrease in the number of DM CpGs in participant 18 (MM-18) was associated with achievement of PR and approximately 2 year TTP. Participants 9 (sPCL-9) and 102 (MM-102) also achieved responses (PR and MR, respectively) and TTP > 6 months associated with less DM CpGs on day 28 than at screening. However, despite reduction in DM CpGs with treatment, participant 103 (MM-103) did not achieve disease control while participant 1 (MM-1) achieved a short-lived MR despite increase in DM CpGs (Fig. 3A). Interestingly, treatment caused predominant hypomethylation in MM-1 and MM-103 while the proportion of CpGs that became more methylated with treatment, possibly by induction of differentiation pathways was greater in participants 9, 18, and 102 who achieved TTP > 6 months (Fig. 3C). To assess whether treatment caused plasma cell specific changes in methylation, we also compared myeloma samples to unpurified normal bone marrow. The 2 myeloma samples (MM-18 and MM-102) with most profound treatment-induced reduction in DM CpGs compared to NPCs, by 68 and 23%, respectively, increased the number of DM CpGs compared to NLBM by 22 and 11%, respectively, on day 28 suggesting plasma cell specific epigenetic reprogramming may have occurred (Fig. 3B). The majority of methylation changes on treatment consisted of increase in methylation (Fig. 3C) but decreases in methylation more commonly affected CpG islands (Fig. 3D) suggesting DNA demethylation at discrete hypermethylated sites has led to partial correction of aberrant hypomethylation. Concurrent gene expression profiling demonstrated significant increase in immunoglobulin gene expression on treatment in 3 samples, including MM-18 and MM-102 (Suppl. Table 2), generally supporting the hypothesis that azacytidine may induce differentiation although responsible pathways could not be identified. Genes directly involved in DNA methylation were not increased in expression. Other observations included a ratio of increased to decreased genes in favor of increased in pts who achieved at least MR and increase in expression of inflammation associated genes in 4 pts, including 3 of 4 pts who achieved at least MR (supplemental table 1).

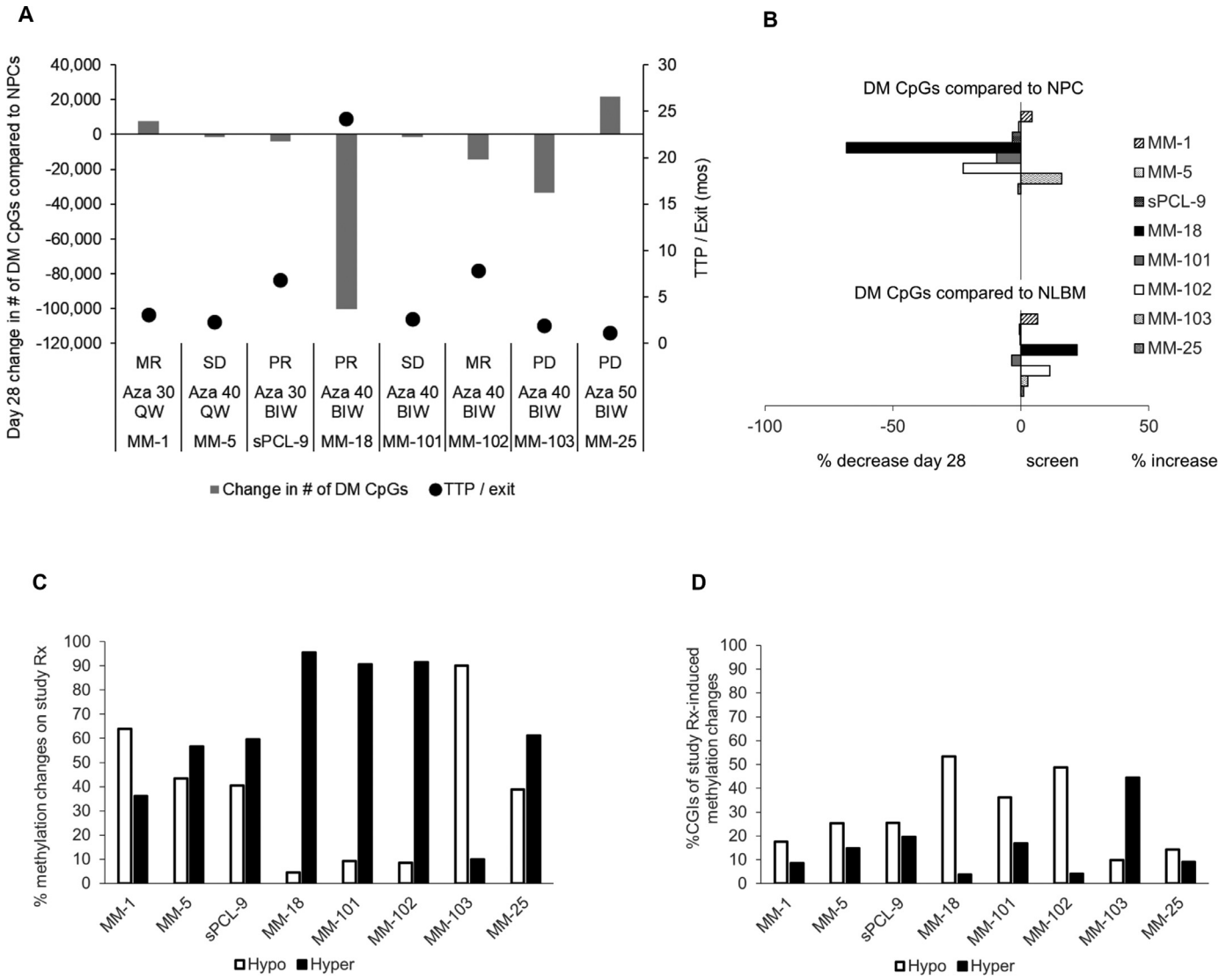


Fig. 3. Global DNA methylation profiles and treatment-induced changes. (A) Relationship between the change in the number of differentially methylated CpGs compared to normal plasma cells on day 28 and best overall response as well as time to progression or study exit in participants treated at indicated dose levels. (B) Treatment-induced changes in the number of differentially methylated CpG dinucleotides compared to normal plasma cells and unsorted normal bone marrow cells displayed as precent change from baseline at screening. In most patients, treatment reduced the number of differentially methylated CpG dinucleotides compared to normal plasma cells while increasing the number of differentially methylated CpGs compared to normal unsorted bone marrow. (C) The majority of DNA methylation changes on treatment consisted of increase in methylation in 6 of 8 patients with paired screening and on treatment samples. (D) Study treatment induced reduction in methylation more commonly affected CpG islands than treatment induced increase in methylation in 7 of 8 patients. Abbreviations: Aza = azacytidine; BIW = twice a week; CGI = CpG island; DM = differentially methylated; hyper = increased in methylation on treatment; Hypo = decreased in methylation on treatment; MM = multiple myeloma; MR = minor response; NLBM = unsorted normal bone marrow; NPC(s) = normal plasma cell(s); PD = progressive disease; PR = partial response; QW = once a week; Rx = treatment; SD = stable disease; sPCL = secondary plasma cell leukemia; TTP = time to progression; 30, 40, 50 = 30, 40, 50 mg/m^2.

Discussion

We aimed to develop a DNA methylation inhibiting azacytidine regimen that is tolerated in combination with lenalidomide and dexamethasone in relapsed or refractory myeloma patients to reverse this mutation-like common final path of diverse epigenetic mechanisms of treatment resistance. The study demonstrated tolerability of azacytidine at 50 mg/m^2 SC twice a week together with GFR adjusted lenalidomide and weekly dexamethasone in extensively pretreated predominantly treatment refractory myeloma patients. In addition, it yielded the impression that aza can help overcome the len refractory state but at a low rate. Association of low plasma CDA levels with clinical benefit suggests that drug exposure may have been a limiting factor. Mechanisms of treatment success may include induction of differentiation with restoration of a methylome that moves toward normal plasma cells through limited demethylation and more extensive secondary methylation of sites that are hypomethylated in myeloma.

The safety of lenalidomide and dexamethasone was not substantially affected by addition of azacytidine up to 50 mg/m^2 twice a week but febrile neutropenia (10% across dose levels and 9% at the top dose) and G3-4 neutropenia (38% across dose levels and 52% at the top dose) appeared higher than in the pivotal study for lenalidomide and dexamethasone in relapsed or refractory myeloma in the US by Weber et al. [50], which used a higher cumulative dexamethasone dose of 40 mg daily, 4 days on, 4 days off and enrolled a less extensively pretreated MM population (febrile neutropenia 3.2%; G-3-4 neutropenia 41.2%) and higher than in the study which established the lower cumulative

dexamethasone dose of 40 mg once a week as standard of care in newly diagnosed myeloma pts where no febrile neutropenia was reported and G3-4 neutropenia found in 20% [51]. Injection site reactions were all of G1-2 and observed in 26% of pts treated with aza 50 mg/m^2 twice a week (Suppl. Table 1).

In our study, the response rate (≥ PR) and CBRR (≥ MR) for pts with len and/or pom refractory disease was 15 and 26%, respectively, for 27 pts who received twice a week aza with lenalidomide and dexamethasone and 6 of 7 pts who achieved at least MR with twice a week aza achieved TTP > 6 months compared to a median TTP of 2.7 months in the entire study. Insufficient information exists on the efficacy of re-treatment with lenalidomide and dexamethasone in lenalidomide refractory myeloma to draw definite conclusions on the contribution of aza to observed clinical benefit. In a Czech retrospective study of 41 pts who were refractory to their last line of treatment but for whom the len refractory state was not reported, response to initial len treatment was 68.6% and to retreatment 14.2% [52]. Another retrospective study of retreatment with IMiD compounds from the Mayo Clinic included 4 pts who progressed on len-based induction and were subsequently retreated with 1 pt achieving PR [53]. In a study that retrospectively analyzed response to re-treatment with IMiD compounds after daratumumab in IMiD refractory pts, a sensitization by daratumumab was postulated. In this study, 8 pts received lenalidomide with dexamethasone as re-treatment and 4 achieved at least PR for an ORR of 50% [54]. In our study, no pts had received prior daratumumab but considering that response to re-treatment with lenalidomide and dexamethasone may be in the range we observed, we performed a detailed review of prior treatment histories and identified 3 len refractory pts who achieved superior duration of disease control than with any prior regimen or response immediately following progression on a lenalidomide and bortezomib containing regimen. In these 3 pts the clinical context therefore strongly suggested contribution of aza to clinical benefit.

The observation that plasma levels of the aza inactivating enzyme CDA inversely correlated with achieving at least MR and TTP > 6 months further suggests that aza exposure contributed to observed benefit despite low overall response. High plasma CDA activity decreases aza-nucleoside half-life and inversely correlates with outcome of patients with MDS treated with azacitidine or decitabine as well [46]. Combination of decitabine with inhibitors of CDA such as tetrahydrouridine [55-57] or cedazuridine [58-60] allows oral administration and may limit risk of relapse in the liver, a high CDA expressing organ [61]. Increasing exposure to epigenetically effective aza nucleoside levels while avoiding high peak levels is being pursued for the tetrahydrouridine decitabine combination with promising initial results in sickle cell anemia [56] while PK equivalence has been the main goal for the development of cedazuridine with decitabine and resulted FDA approval in MDS July 7, 2020 [62]. An oral formulation for azacytidine (CC-486) without CDA inhibitor was pursued by Celgene which yields approximately 10-fold lower peak concentrations at 300 mg po than SC azacytidine at 75 mg/m^2 [42,63], but reaches the range (around 0.5 μM) where epigenetic effects would be expected based on in vitro experiments [43]. Accordingly, DNA demethylation was confirmed in the human dose exploration PK/PD study [63]. On September 1, 2020 The FDA approved CC-486 (Onureg) at a dose of 300 mg po daily for the first 14 days of a 28-day cycle for maintenance treatment in pts with AML in CR or CR with incomplete blood count recovery who are unable to complete intensive curative therapy [64] based on approximately 10 months longer survival and doubling of PFS compared to placebo in a phase 3 study [65]. Optimizing aza exposure through use of CDA inhibitors or regimens that allow reaching epigenetically effective levels more often may improve efficacy of aza in myeloma. Interestingly, a dose finding study of CC-486 in combination with lenalidomide and dexamethasone by Kalff et al. [66] in len refractory myeloma patients arrived at a dose of 100 mg daily, 3 times lower than the one used for AML but given for the first 21 days of a 28 day cycle, and reached an ORR and CBRR of 44 and 78% in 9 response-evaluable pts treated at this dose. The low number of pts treated at this dose and the lack of pharmacodynamic assays to confirm an effect on the DNA methylome in this study make additional research necessary but generally support a frequent low dose exposure concept. Our results have also suggested that the frequency of aza administration is more important than the dose within a range of 30 to 50 mg/m^2 (Fig. 1) although the weekly administration was tested in only 6 pts. Additionally, relatively low doses of decitabine (5 mg/m^2 IV daily over 5 days) have shown promising results in combination with twice a week SC bortezomib and dexamethasone in pts with relapsed or refractory myeloma with ORR 87% in 46 pts and 75% in 8 bortezomib refractory pts reported from a Chinese phase 2 study [67].

Preclinical and translational studies have demonstrated that aza nucleoside treatment can induce differentiation in cancer cells when used at nontoxic doses [28,29]. In multiple myeloma, differentiation would mean an increase of DNA methylation, since the majority of differential methylation in myeloma compared to normal plasma cells consists of hypomethylation [2,10,30,31]. We confirmed this pattern with approximately 10 times more hypomethylated than hypermethylated CpGs in myeloma cells at diagnosis compared to normal plasma cells. Among the 8 pts with on-treatment purified myeloma cells, 2 had clinically convincing aza contribution to benefit and underwent DNA methylome changes that brought their myeloma cells closer to NPCs, predominantly by increase in DNA methylation but with decrease in methylation at discrete CpGs that were more commonly located in CpG islands (Fig. 3) and net increase in gene expression including of immunoglobulin genes (Suppl. Table 2). While this suggests aza may have induced differentiation we could not identify significant changes in potential pathways which may be due limited sensitivity of the methods we used. Emergence of de-differentiated clones is a well described mechanism for resistance to the proteasome inhibitor bortezomib [32] and may result in resistance to other drugs that target the plasma cell program, including IMiD compounds and BCMA targeting approaches. Defining whether and how DNMT inhibitors can induce differentiation in myeloma may therefore have broad implications for overcoming treatment resistance and facilitate the design of pharmacodynamic assays that guide their development.

With the availability of clinically evaluable CDA inhibitors, selecting pts for treatment based on CDA expression should not be required despite our observation that low CDA associates with benefit but measurement of achieved suppression of CDA activity on treatment may help refine their dosing. Alternatively, frequent low-level exposure, as in the study by Kalff et al. [66] may obviate the need for CDA inhibition. Measuring aza nucleoside incorporation [68] and/or DNMT1 depletion in myeloma cells [69] may help in dose and schedule optimization for myeloma and combination with well tolerated epigenetic agents that target epigenetic modifiers with documented impact in myeloma, such as the EZH2 inhibitor tazemetostat [33] may help achieve optimized epigenetic regimens. Reversal of global hypomethylation may be a seemingly counterintuitive but potentially valuable epigenetic endpoint in future studies and sensitive methods such as single cell RNA sequencing may help identify responsible mechanisms and guide patient selection if optimized epigenetic regimens still require it.

Conflict of interest statement

Frederic J. Reu: Celgene research support, Bristol-Myers Squibb stock

Yap Chew: Zymo Research employment

Beth M Faiman: Celgene / Bristol-Myers Squibb Speaker's Bureau

No relevant conflicts of interest: Jack Khouri, Dale Grabowski, Reda Z Mahfouz, Shahper N Khan, Wei Wei, Robert Dean, Christy Samaras, Hillard Lazarus, Erica L. Campagnaro, Ehsan Malek, Janice Reed, Mary Ann Karam, Kimberly Hamilton, Matt Kalaycio, Hien Liu, Ronald Sobecks, Jason Valent, Sherry Fada, Babal K Jha, Yogen Saunthararajah, Mohammed Orloff.

Authors contribution

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Dale Grabowski: Investigation, Data Curation, Project Administration.

Reda Z Mahfouz: Investigation, Methodology, Data Curation, Supervision.

Shahper N Khan: Methodology, Investigation, Data Curation.

Wei Fairman: Formal Analysis, Visualization.

Robert Dean, Christy Samaras, Hillard Lazarus, Erica L Campagnaro, Ehsan Malek, Janice Reed, Mary Ann Karam, Kimberly Hamilton, Matt Kalaycio, Hien Liu, Ronald Sobecks and Jason Valent: Investigation, Resources.

Sherry Fada: Data Curation, Project Administration.

Babal K Jha: Supervision.

Yogen Saunthararajah: Conceptualization, Methodology, Supervision.

Yap Chew: Investigation.

Mohammed Orloff: Investigation.

Frederic J Reu: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Writing – Review and Editing, Visualization, Supervision, Project Administration, Funding Acquisition.

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