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Inhibition of PLK1 by capped-dose volasertib exerts substantial efficacy in MDS and sAML while sparing healthy haematopoiesis

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Abstract

Introduction: Targeting the cell cycle machinery represents a rational therapeutic approach in myelodysplastic syndromes (MDS) and secondary acute myeloid leukemia (sAML). Despite substantial response rates, clinical use of the PLK inhibitor volasertib has been hampered by elevated side effects such as neutropenia and infections.

Objectives: The primary objective was to analyse whether a reduced dose of volasertib was able to limit toxic effects on the healthy haematopoiesis while retaining its therapeutic effect.

Methods: Bone marrow mononuclear cells (BMMNCs) of patients with MDS/sAML (n = 73) and healthy controls (n = 28) were treated with volasertib (1 μM to 1 nM) or vehicle control. Short-term viability analysis was performed by flow cytometry after 72 hours. For long-term viability analysis, colony-forming capacity was assessed after 14 days. Protein expression of RIPK3 and MCL-1 was quantified via flow cytometry.

Results: Reduced dose levels of volasertib retained high cell death-inducing efficacy in primary human stem and progenitor cells of MDS/sAML patients without affecting healthy haematopoiesis in vitro. Interestingly, volasertib reduced colony-forming capacity and cell survival independent of clinical stage or mutational status.

Conclusions: Volasertib offers a promising therapeutic approach in patients with adverse prognostic profile. RIPK3 and MCL-1 might be potential biomarkers for sensitivity to volasertib treatment.

KEYWORDS

cell death, myelodysplastic syndromes (MDS), PLK1, secondary acute myeloid leukemia (sAML), volasertib

1 | INTRODUCTION

Myelodysplastic syndromes (MDS) are clonal disorders of the haematopoietic stem cell that are mainly observed in the elder patient.[1] In most cases of MDS bone marrow (BM), cellularity is elevated as well as the proliferative capacity.[2] Due to an increased cell death of the BM compartment, peripheral cytopenia is a common feature of low-risk MDS. However, progression towards higher-risk MDS and secondary AML (sAML) is characterised by an increasing apoptotic resistance.[3,4] High-risk MDS have an unfavourable prognosis and progress to overt AML (Acute Myeloid Leukemia) is less than 1.5 years.[5] Stem cell transplantation is the only effective salvage therapy, for which only a limited number of patients are eligible due to age and comorbidity. The efficacy of further therapeutic strategies is limited in higher-risk MDS,[6] and alternative approaches are urgently needed.[7]

Our previous work has shown that targeting the cell death machinery is an effective therapeutic strategy in higher-risk MDS and sAML.[3,4,8] However, also the aberrant proliferative capacity of the malignant clone(s) may represent a promising target in advanced MDS/secondary AML (sAML). PLK1 inhibition has been shown to cause mitotic block and apoptosis.[9]

Deregulation of PLK expression is known in a number of haematological malignancies including AML.[10,11] Especially PLK1 plays a central role in human cell cycle, and overexpression of PLK1 is associated with a poor outcome in a number of solid cancers.[12-17] To this end, targeting the family of Polo-like kinases (PLK) is auspicious.[10,18] One of the best characterised PLK inhibitors is BI 6727 (volasertib), a low molecular weight compound that exhibits substantial anti-leukaemic activity.[18-20] Here, the IC_{50} value is 0.87 nM for PLK1, 5 nM for PLK2 and 56 nM for PLK3, respectively.[18] The impact of volasertib on primary AML has already been tested in pioneer phase I/II clinical trials.[21-23] Döhner *et al* clearly showed that patients with de novo AML and ineligibility for intensive induction therapy have a significantly prolonged median event-free survival by LDAC (low-dose cytarabine) + volasertib compared with LDAC alone (5.6 vs 2.3 months).[21] However, in the patients treated with volasertib + LDAC an increased frequency of adverse events was detected, especially neutropenic fever and infections. In a smaller, Japanese trial, volasertib mono was applied in patients with AML ineligible for standard induction therapy or with relapsed or refractory disease. A total of 19 patients were treated with three volasertib doses: 350, 400 and 450 mg. The median remission duration of the six patients with complete remission or complete remission with incomplete blood count recovery was 85 days (range 56-358). The most frequently reported adverse events were also febrile neutropenia (78.9%).[23]

Here, we report in vitro data showing the impact of volasertib in a larger cohort of patients with MDS/sAML (n = 73) and healthy controls (n = 28). Given the concern about the elevated haematotoxicity, we aimed to determine whether capping the volasertib dose is a feasible approach for reducing toxic effects on the healthy haematopoiesis while preserving its therapeutic efficacy on the malignant clone. We focused on the impact on colony-forming capacity as a parameter for long-term effects. Further, we investigated the biomarkers to predict sensitivity towards volasertib treatment.

To the best of our knowledge, we show for the first time that volasertib targets malignant stem/progenitor cells of patients with MDS and sAML. Notably, despite a significant reduction in volasertib dosage, efficacy was maintained.

2 | PATIENTS AND METHODS

2.1 | Patient samples

Human Bone Marrow samples were collected as per the institutional guidelines and in concordance with the Declaration of Helsinki. Written informed consent was obtained from each patient. The study was approved by the Local Ethics Committee of the University Hospital of the Technical University Munich (62/16S). Secondary acute myeloid leukaemia (sAML) was defined as ≥20% of blasts in the BM and a history of myelodysplastic syndrome (MDS). All the other MDS or sAML samples were classified as per the International Prognostic Scoring System (IPSS), the revised WHO classification-based Prognostic Scoring System (r-WPSS), and the World Health Organization (WHO) 2016 classification. Samples were obtained when clinically required from patients either before or during the treatment, and irrespective of the therapeutic regimen. Control

samples were obtained from human femoral heads that were discarded after the implantation of total endoprosthesis of the hip joint from 28 haematologically healthy age-matched donors.

2.2 | Cell isolation and culture

Bone marrow mononuclear cells (BMMNCs) were isolated from primary human BM samples via density-gradient centrifugation using the Biocoll Separation Solution (Biochrom AG, Berlin, Germany) as per the manufacturer's instructions. CD34^{+} cells were purified via positive selection using the CD34^{+} MicroBeads kit (Miltenyi Biotec), and a purity of at least 95% was confirmed. BMMNCs were cultured at a density of 5×10^5 cells/mL in serum-free media comprising Iscove's Modified Dulbecco's Medium (IMDM) with L-alanyl-L-glutamine (IMDM GlutaMAX) with 20% BIT 9500 serum substitute (1% [w/v] bovine serum albumin, 10 µg/mL insulin, 200 µg/mL iron-saturated transferrin; STEMCELL Technologies) and enriched with recombinant human stem cell factor (100 ng/mL), FMS-related tyrosine kinase-3 ligand (100 ng/mL), thrombopoietin (10 ng/mL), interleukin-6 (5 ng/mL), interleukin-3 (10 ng/mL; all from R&D Systems, Minneapolis, MN, USA), β-mercaptoethanol (10 µM; Gibco) and low-density lipoproteins (4 µg/mL; Sigma-Aldrich).

2.3 | Inhibitors

Volasertib (BI 6727; Boehringer Ingelheim) and GSK461364A (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) and used in final concentrations from 1 µM to 1 nM. DMSO was used at 0.001% as vehicle control.

2.4 | Colony formation assay

Haematopoietic progenitors were assessed following treatment with volasertib at final concentrations from 1 µM to 1 nM or DMSO (0.001%) for 72 h in cytokine-supplemented, serum-free culture. Thereafter, 1×10^4 BMMNCs were plated in duplicates in methylcellulose medium supplemented with an optimal cytokine mix as per the manufacturer's protocols (MethoCult H4435 enriched; STEMCELL Technologies). Number of erythroid progenitor colonies (Burst-forming units-erythroid or colony-forming units for the granulocytic-macrophagic lineage, and multi-potential granulocytic-erythroid-macrophagic-megakaryocytic lineage) were assessed after 10-14 days. Transmitted light photographs were taken on a Keyence BIOREVO BZ-900 microscope.

2.5 | Flow cytometry

BMMNCs were stained with Annexin V-FITC in Annexin V staining solution (0.1 M HEPES/NaOH, pH 7.4, 1.4 M NaCl 0.9%, 25 mM

$CaCl_2$), followed by staining with fluorescently labelled antibodies against CD34 (clone 4H11). For intracellular staining, cells were stained against CD34, followed by fixation in 2% paraformaldehyde, permeabilisation using perm/wash buffer (BD Bioscience, and subsequent staining with fluorescently labelled antibodies against MCL-1 (clone D2W9E; Cell Signaling) or respective isotype controls (clone DA1E; Cell Signaling). Dead cells were excluded by Fixable Viability Dye staining. If not otherwise stated, reagents and antibodies were purchased from eBioscience. Flow analysis was performed on a BD FACS Canto II (BD Bioscience) and data were analysed using FlowJo software (TreeStar Inc).

2.6 | Immunohistochemistry

BM biopsies from MDS or sAML patients and healthy controls were stained for Ki-67 (clone MIB-1, ImmunoLogic, 1:500) (brown), and the positivity rate was determined.

2.7 | Gene expression analysis

Using Affymetrix HG U133 Plus 2.0 gene expression profiles for PLK1, PLK2 and PLK3 for bulk BM were obtained from 164 MDS patient samples and from 69 healthy controls (GEO: GSE15061); for CD34^{+} BM cells, they obtained from 183 MDS patient samples and from 17 healthy controls (GEO: GSE19429). The data were collected from Gene Expression Omnibus (GEO). For probe set annotation, custom chip definition files (CDFs) were used based on GeneAnnot version 2.0, synchronised with GeneCards version 3.04. These CDFs decrease the total number of probe sets (one probe set per gene) and potentially increase the specificity of the analyses by the elimination of cross-hybridising probes (probes are restricted by sequence specificity). With the Robust Multichip Average (RMA) method, data were normalised, as already published.[24]

2.8 | Statistical analyses

For the comparison of two samples from different individuals, the unpaired Student's *t* test was used to detect significant differences between the treated samples and the controls. All the reported *P*-values are two-sided, with a significance level of 0.05 and have not been adjusted for multiple testing. One-way ANOVA and post hoc pairwise comparison were performed for dose titration analyses. Statistical analyses were performed using GraphPad Prism version 6.0 g (GraphPad Software, Inc).

3 | RESULTS

Sustaining a proliferative capacity is one of the hallmarks of cancer.[25] Targeting the proliferative capacity of the malignant clone is

TABLE 1 Clinical annotations of primary MDS and sAML patient samples

No.	Age	Sex	WHO	Karyotype	Severe anaemia	Number of cytopaenias	WPSS	IPSS	IPSS-R	high-risk mutation
1	83	m	SLD	46,XY	Absent	1	Very low	Low	Low	None
2	42	f	MLD	46,XX	Present	1	Int.	Low	Int.	None
3	71	m	MLD	46,XY	Absent	1	Low	Low	Very Low	n. a.
4	66	m	MLD	46,XY	Present	2	Int.	Low	Low	None
5	80	m	MLD	45,X,-Y	Absent	3	Low	Int.-1	Int.	n. a.
6	74	m	MLD	46,XY	Present	3	Int.	Int.-1	Low	ASXL, RUNX1
7	69	m	MLD	46,XY,del(5q)	Absent	1	Low	Low	Low	RUNX1
8	84	m	MLD	46,XY	Present	3	Int.	Int.-1	Low	ASXL, EZH2
9	80	f	MLD	46,XX,del(5q)	Present	3	Low	Int.-1	Int.	n. a.
10	43	f	MLD	46,XX	Absent	1	Low	Low	Very low	None
11	83	m	MLD	46,XY,del(9) (p21q33)	Absent		Int.	Int.-1	Low	n. a.
12	81	m	MLD	46,XY	Present	2	Int.	Int.-1	Int.	ASXL, EZH2
13	86	f	MLD	46,XX	Absent	1	Low	Low	Low	None
14	68	m	MLD	46,XY	Present	2	Int.	Int.-1	Int.	n. a.
15	74	f	MLD	46,XX	Absent	1	Low	Low	Very low	None
16	65	f	MLD	46,XX	Present	3	Int.	Int.-1	High	None
17	86	f	MLD	46,XX del(5q);47,XX,+21	Absent	1	Low	Low	Int.	None
18	82	m	MLD	46,XY	Present	2	Low	Int.-1	Low	ASXL, EZH2
19	74	m	MLD	46,XY	Present	3	Int.	Int.-1	Int.	None
20	75	f	MLD	46,XX, del(5)(q13q34)	Present	1	Int.	Low	Low	None
21	74	f	MLD (tMDS)	46,XX,del(5) (q22q33), r(7)(p11q11), del(12) (p11p13)	Present	3	High	Int.-2	High	TP53
22	53	f	MLD (tMDS)	47,XX,+19	Absent	1	Int.	Low	Int.	ASXL
23	77	m	MLD	45,X,-Y	Absent	3	Low	Int.-1	Low	TP53
24	68	m	MLD	46,XY	Absent	3	Low	Int.-1	Very low	None
25	67	m	MLD	46,XY	Absent	3	Low	Int.-1	Very low	None
26	76	f	RS-SLD	46,XX	Absent	2	Low	Low	Low	ASXL
27	73	f	RS-SLD	46,XX	Present	1	Low	Low	Low	None
28	78	m	RS-SLD	47,XY + 8	Present	2	Low	Int.-1	Int.	n. a.
29	77	m	RS-MLD	46,XY	Present	2	Int.	Low	Low	ASXL

(Continues)

TABLE 1 (Continued)

No.	Age	Sex	WHO	Karyotype	Severe anaemia	Number of cytopaenias	WPSS	IPSS	IPSS-R	high-risk mutation
30	86	m	RS-MLD	46,XY,der(18)t(9;18)	Present	3	High	Int.-2	High	ASXL
31	77	f	RS-MLD	46,XX	Present	2	Int.	Int.-1	Int.	None
32	74	f	RS-MLD	46,XX	Present	2	Int.	Int.-1	Int.	n. a.
33	73	m	EB-1	46,XY; 47,XY + 8	Present	3	Very high	Int.-2	Very high	ASXL, EZH2
34	69	m	EB-1	45,XY,-7	Absent	2	Int.	Int.-2	High	None
35	70	m	EB-1	47,XY,+8	Absent	3	High	Int.-2	High	ASXL, RUNX1
36	67	m	EB-1	47,XY,+8	Absent	3	High	High	High	ASXL, RUNX1
37	66	m	EB-1	47,XY,del(20q)	Present	3	High	Int.-1	Int.	None
38	80	m	EB-1	46,XY	Absent	2	Int.	Int.-1	High	n. a.
39	75	m	EB-1	46,XY	Present	3	High	Int.-1	High	ASXL, RUNX1
40	79	f	EB-1	46,XX,del(5q), del(3)(q21),+8	Absent	3	High	Int.-2	High	n. a.
41	66	m	EB-1	46,XY	Present	3	High	Int.-1	High	ASXL
42	74	f	EB-1	46,XX	Absent	2	Int.	Int.-1	Int.	None
43	60	m	EB-1	complex (3 aberrations)	Absent	3	High	Int.-2	High	TP53
44	63	m	EB-1 (tMDS)	46,XY, t(4;19)	Absent	3	High	High	Very high	ASXL
45	71	m	EB-1	complex (>3 aberrations)	Absent	3	Very high	Int.-2	Very high	None
46	71	m	EB-1	complex (>3 aberrations)	Present	3	Very high	Int.-2	Very high	None
47	72	m	EB-1	complex (>3 aberrations)	Absent	2	Very high	Int.-2	Very high	None
48	78	m	EB-2	46,XY	Present	3	High	High	High	n. a.
49	74	m	EB-2	45,XY,del(11q),-Y,del(5q)	Present	3	Very high	High	High	n. a.
50	73	f	EB-2	complex (>3 aberrations)	Present	2	Very high	Int.-2	Very high	EZH2
51	45	m	EB-2	complex (>3 aberrations)	Present	2	Very high	High	Very high	TP53
52	41	m	EB-2	47,XY,+8	Present	3	Very high	High	Very high	n. a.
53	69	m	EB-2	46,XY	Absent	3	High	Int.-2	High	None
54	62	m	EB-2	46/47,XY, inv(11),+14,+8	Absent	3	Very high	High	Very high	None

(Continues)

TABLE 1 (Continued)

No.	Age	Sex	WHO	Karyotype	Severe anaemia	Number of cytopaenias	WPSS	IPSS	IPSS-R	high-risk mutation
55	62	m	EB-2	46/47, XY, inv(11),+14,+8	Absent	3	Very high	High	Very high	None
56	71	m	EB-2	46,XY	Absent	3	High	Int.-2	Int.	ASXL, RUNX1
57	75	m	EB-2	45,X,-Y,del(11)(q14q25)	Absent	3	High	High	High	None
58	81	m	sAML	46,XY, t(2;8)(p10;q10)	Present	2	n.a.	n.a.	n.a.	ASXL
59	65	f	sAML	complex (>3 aberrations)	Present	3	n.a.	n.a.	n.a.	TP53
60	81	m	sAML	46,XY, t(2;8)(p10;q10)	Present	3	n.a.	n.a.	n.a.	ASXL
61	85	f	sAML	complex (>3 aberrations)	Present	3	n.a.	n.a.	n.a.	TP53
62	77	m	sAML	46,XY	Absent	3	n.a.	n.a.	n.a.	ASXL, RUNX1
63	81	f	sAML	46,XX	Absent	2	n.a.	n.a.	n.a.	None
64	76	m	sAML	complex (>3 aberrations)	Present	3	n.a.	n.a.	n.a.	None
65	71	m	sAML	46,XY,+1, der(1;22)(q10;q10)	Absent	2	n.a.	n.a.	n.a.	ASXL
66	64	f	sAML	46,XX,inv(12)(p13q21)	n.k.	n.k.	n.a.	n.a.	n.a.	None
67	70	f	sAML	46,XX	Absent	3	n.a.	n.a.	n.a.	None
68	70	m	sAML	46,XY	Present	3	n.a.	n.a.	n.a.	None
69	66	m	sAML	46,XY, del(7)(q21q36), del(20){q11q13}	Absent	3	n.a.	n.a.	n.a.	None
70	46	m	sAML	complex (>3 aberrations)	Present	3	n.a.	n.a.	n.a.	None
71	75	f	sAML	46,XX	Absent	2	n.a.	n.a.	n.a.	None
72	58	f	sAML (tMDS)	46,XX,del(5q), del(16q)	Absent	3	n.a.	n.a.	n.a.	n.a.

Note: Age, sex defined as male (m) or female (f), WHO category 2016 defined as MDS with single-lineage dysplasia (MDS-SLD), MDS with single-lineage dysplasia and ring sideroblasts (MDS-RS-SLD), MDS with multilineage dysplasia (MDS-MLD), MDS with multilineage dysplasia and ring sideroblasts (MDS-RS-MLD), MDS with blast excess <10% (MDS EB-1), MDS with blast excess >10% (MDS EB-2), karyotype, presence or absence of severe anaemia (defined as haemoglobin <8 g/dL in females and <9 g/dL in males). MDS risk groups were determined as per the International Prognostic Scoring System (IPSS), the revised World Health Organization (WHO)-based prognostic scoring system (WPSS), and the revised International Prognostic Scoring System (IPSS-R). Adverse prognostic mutations are indicated if applicable. Patients with secondary acute myeloid leukaemia (sAML) were defined as per the WHO category (>20% blast cells) and a history of MDS. Not applicable (n.a.) denotes sAML wherein risk scores were not calculated.

also a promising strategy in myeloid malignancies. Pharmacological inhibitors of central members of the PLK family are commercially available.[9] Volasertib is one of the best characterised PLK1 inhibitors already used in phase III clinical trials.[21,23]

In the following experimental approaches, we show the cell death-inducing efficacy of volasertib in an enlarged cohort of MDS/sAML patient samples (n = 72) compared with healthy, age-matched controls (n = 28) (Tables 1 and 2). First, we analysed the therapeutic impact of PLK1 inhibition in MDS and sAML using primary human stem and progenitor cells extracted from the BM and cultivated ex vivo as described previously.[3] Second, we focused on investigating the cytotoxic side effects on healthy haematopoiesis, due to infectious complications seen in clinical testing.[21-23] Third, we asked whether potential selection biomarkers can be identified as a basis to preselect patients for volasertib treatment.

Primary bone marrow mononuclear cells (BMMNCs) were kept in growth-factors-enriched medium as described previously[3] and treated with volasertib for up to 72 hours. As a short-term

TABLE 2 Distribution of the age of the MDS and sAML patients and healthy controls

Category	Number	Median age	Male	Female
All	100	71	68	32
Healthy	28	64	20	8
MDS/sAML	72	73	48	24
MDS	57	73	40	17
WPSS				
Very low	1	83	1	0
Low	15	76	8	7
int.	17	74	10	7
High	14	71	12	2
Very high	10	71	9	1
IPSS				
Low	14	74	5	9
int.-1	21	75	16	5
int.-2	13	71	10	3
High	9	63	9	0
IPSS-R				
Very low	5	68	3	2
Low	13	77	10	3
int.	14	74	7	7
High	15	74	12	3
Very high	10	67	9	1
sAML	15	71	8	7

Note: The number of analysed bone marrows in each category with the mean age and sex distribution. Myelodysplastic syndromes (MDS) and secondary acute myeloid leukaemia (sAML) were subdivided as per the MDS risk groups of the International Prognostic Scoring System (IPSS), the revised World Health Organization (WHO)-based prognostic scoring system (WPSS), and revised International Prognostic Scoring System (IPSS-R). Patients with sAML were defined as per the WHO category (>20% blast cells) and a history of MDS.

outcome, viability was analysed after 24, 48 and 72 hours by flow cytometry using Annexin V and 7-amino-actinomycin D (7-AAD) staining. Only dividing cells should be susceptible to PLK1 inhibition—however, no further growth stimulation or synchronisation was performed to exclude experimental bias. In first, clinical trials with volasertib dose escalation cohorts were analysed for pharmacokinetics. Here, the peak plasma concentration of patients using 350 mg of volasertib (at day 1 and 8) reached 600 ng/mL. Due to its relatively high protein binding, in vitro data of volasertib cannot be completely matched to in vivo concentrations. However, 600 ng/mL in vitro corresponds to an in vitro concentration of 1 μM.[20] Therefore, we analysed the impact of volasertib on primary MDS and sAML samples in vitro at the extrapolated standard dosage of 1 μM first.

As shown in Figure 1, the cell death-inducing efficacy of volasertib treatment was time-dependent with 72 hours as an optimal read-out time point (Figure 1A). Here, volasertib effectively induced cell death—defined as viability <60% relative to soluble control—in 22 out of 43 samples (51.2%) (Figure 1B). However, the effect on viability varied strongly across samples (Figure 1B). To understand the differential effects, we classified the samples according to World Health Organization (WHO) categories, specifically: early-stage MDS (defined as MDS with single-lineage dysplasia and ring sideroblasts [MDS-RS-SLD], MDS with multilineage dysplasia [MDS-MLD] and MDS with multilineage dysplasia and ring sideroblasts [MDS-RS-MLD]), advanced stage MDS (defined as MDS with blast excess <10% [MDS EB-1] and MDS with blast excess >10% [MDS EB-2]), and sAML. Notably, sensitivity to the treatment was completely independent from both the WHO classification system (Figure 1B) and other prognostic variables like the mutational status (data not shown). In line with this finding, the proliferative capacity determined by immunohistochemistry was nearly identical in n = 8 patients with MDS [RS-] MLD, n = 6 patients with MDS EB-1, n = 9 patients with MDS EB-2, and n = 8 patients with sAML (Supplementary Figure S1). PLK1 inhibition has been shown to cause apoptosis after inducing the mitotic block.[9] We therefore concluded that further aberrations, for example in the apoptotic machinery might have some impact on the treatment response. Due to small samples sizes, only in a very limited number of samples (n = 4) sensitivity of CD34$^+$ stem/progenitor cells to volasertib was correlated with values of central BCL2 family proteins. However, a statistically significant correlation was detected between the viability after treatment and the MCL-1 values for the 72-h read-out (Pearson's ρ = 0.9707; P = .293) (Figure 1C). MCL-1 is part of the BCL-2 family of proteins and an anti-apoptotic protein. As shown previously, MCL-1 is closely connected to PLK1 efficacy.[26] As expected, high levels of MCL-1 were related to volasertib treatment resistance (Figure 1C).

To also investigate the effect of volasertib on the progenitor cell level, the colony-forming capacity was analysed in a random cohort of poor, intermediate and good responders (Figure 1D). To this end, 1 × 10^4 cells were transferred into growth-factors-enriched methylcellulose-based media after 72 hours of pretreatment with volasertib. Colonies were scored after 10-14 days. Exceptionally, the colony-forming capacity was significantly reduced in all samples. Thus, volasertib

showed a significant effect at the progenitor cell level, even in individuals classified as "low responder" in the short-term analysis (Figure 1D).

To further elucidate a broader effect of volasertib on haematopoiesis, we analysed its impact on healthy, age-matched BM cells. As healthy controls, we utilised BM mononuclear cells (BMMNC) isolated from femoral heads of elderly patients (mean age: 64.4 years) (Table 2) undergoing surgical hip replacement. Final concentrations of volasertib ranged from 1 nM to 1 μM. Concerning the cytotoxic effects on healthy haematopoiesis, we identified a clear cut-off dose for volasertib of 10 nM (Figure 1E-G). Concentrations up to 10 nM (1-10 nM) had no significant impact on the viability of healthy control cells, neither in the CD34$^+$ population (Figure 1E) nor in the bulk of BMMNCs (Figure 1F). The colony-forming capacity was also not affected by a low-dose regimen from 1 to 10 nM (Figure 1G). However at concentrations of 50 nM or higher, volasertib significantly reduced the CD34$^+$ compartment after 72 hours as well as the colony-forming capacity of the healthy, age-matched controls (Figure 1E, G).

These findings are in line with the results obtained with an alternative PLK1 inhibitor, GSK 461 364 (Supplementary Figure S2). Again, the pool of CD34$^+$ stem/progenitor cells was also more sensitive to PLK1 inhibition than BM bulk. Low dosages spared healthy hematopoiesis while commonly used dosages of 20 to 40 nM GSK 461 364 nearly eradicated healthy hematopoiesis.
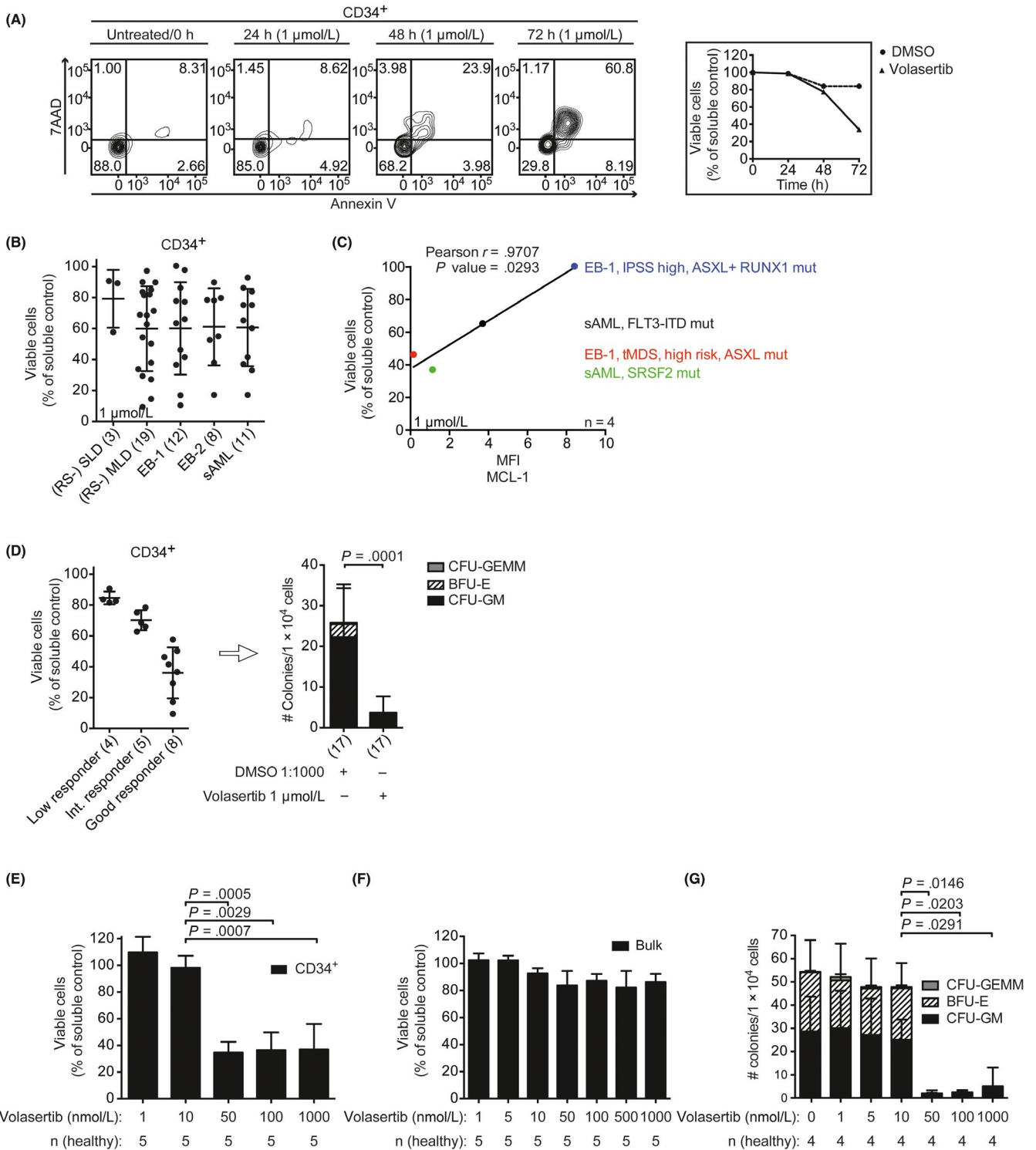
To further elucidate these findings, we evaluated open-access gene expression data (2). Expression levels of PLK1, PLK2 and PLK3

were analysed in bulk BM (Figure 2A) and in the CD34$^+$ stem/progenitor cell compartment (Figure 2B). Data were available also for the different MDS subtypes including MDS-SLD, MDS-RS-SLD, MDS EB-1 and MDS EB-2. Interestingly, *PLK1* had a slightly higher expression in MDS than in healthy and non-leukaemic controls while healthy progenitors showed a higher expression level of *PLK3* (2). A moderately higher expression of PLK1 might be suggestive for a slightly higher proliferative potential in MDS. The IC$_{50}$ of volasertib on PLK2 is 5 nM and that for PLK3 is 56 nM, whereas the IC$_{50}$ on PLK1 is 0.87 nM. We therefore conclude that a higher dosage of volasertib might also affect other PLK family members, being especially relevant for healthy hematopoiesis.

Next, we tested the therapeutic benefit of capped-dose volasertib on primary malignant MDS and sAML cells. Analysis of cell death induction in the short-term analysis (up to 72 hours) revealed that low-dose volasertib (10 nM) significantly reduced the CD34$^+$ population derived from patients with MDS or sAML compared with healthy controls (Figure 3A). Thus, despite dose reductions the therapeutic benefit on the malignant clone in MDS and sAML was retained. To understand differential effects of capped-dose volasertib, we classified the analysed samples according to WHO categories. We identified a comparable cytotoxic effect on samples across all clinical stages of MDS, suggesting that treatment response was independent of clinical staging (Figure 3B) or other prognostic variables (data not shown). Yet, we found that some patient samples responded exceptionally well to the treatment

FIGURE 1 Capping the volasertib dose protects healthy haematopoiesis against cytotoxic adverse effects. (A) Viability of the bone marrow mononuclear cells (BMMNC) measured with flow cytometry using Annexin V and 7-AAD and gating on CD34$^+$ cells from one individual representative patient who was diagnosed with secondary acute myeloid leukaemia (sAML) after treatment with volasertib (1 μM) or with vehicle control (DMSO). (B) Viable BMMNC of the MDS or sAML patients with WHO-based classification as MDS with single-lineage dysplasia (MDS-SLD), MDS with single-lineage dysplasia and ring sideroblasts (MDS-RS-SLD), MDS with multilineage dysplasia (MDS-MLD), MDS with multilineage dysplasia and ring sideroblasts (MDS-RS-MLD), MDS with blast excess <10% (MDS EB-1) and MDS with blast excess >10% (MDS EB-2) were treated for 72 hours with volasertib (1 μM) or vehicle control (DMSO) and gated on CD34$^+$ cells. (C) Viable BMMNC of 2 patients with WHO-based classification as MDS with blast excess <10% (EB-1) and 2 patients with sAML were treated for 72 hours with volasertib (1 μM) or vehicle control (DMSO). Viability was measured by flow cytometry after staining with Annexin V and 7-AAD and after gating on CD34$^+$ cells. Each circle describes the ratio between viable cells after inhibitor treatment and vehicle treatment (DMSO). To quantify protein expression of MCL-1, the mean fluorescence intensity (MFI) of the stained antibody was divided by the MFI of the isotype control. The correlation between MFI ratio and viability after treatment with volasertib was calculated by the Pearson correlation coefficient, the *P* value is indicated. (D) The analysed cohort was subdivided according to the response rates to the 72-h viability assay. Low responders had ≥ 80% viable cells after treatment with 1 μM volasertib 72 h compared with the soluble control, intermediate responders had a viability of <80% and ≥ 60% and good responder had a viability of <60%. BMMNCs (1 × 10^4) were plated in growth-factors-enriched methylcellulose following 72 hours of treatment with volasertib (1 μM). Numbers of the colony-forming units (CFU) of multi-potential granulocytic-erythroid-macrophagic-megakaryocytic lineage (CFU-GEMM), CFU-granulocytic-macrophagic lineage (CFU-GM), burst-forming units-erythroid (BFU-E) and total number of colonies were scored after 10-14 days for the indicated patient samples. The figure presents the mean and SD values of the total number of the single colony types as stacked bar chart for the indicated treatment. The cohort comprised of 10 low-risk MDS and 7 high-risk MDS or sAML samples. Pairwise comparison was performed, and the *P* value is indicated. (E, F) BMMNC from healthy, elderly donors were treated for 72 h with volasertib at the indicated concentrations. The viability of purified CD34$^+$ cells (D) and bulk bone marrow cells (E) (n = 5) was measured using flow cytometry with Annexin V and 7-AAD staining. The figure shows the ratio between the viable cells after 72-h inhibitor or vehicle treatment (DMSO) with the mean and standard deviation (SD) values. One-way analysis of variance and post hoc pairwise comparison were performed. One-way ANOVA resulted in a *P* value of *P* < .0001 for the CD34$^+$ compartment (D) and 0.0002 for bulk BMMNC (E). (G) BMMNC (1 × 10^4) was plated in growth-factors-enriched methylcellulose following 72 h of volasertib treatment at the indicated concentrations. Numbers of colony-forming units (CFU) of multi-potential granulocytic-erythroid-macrophagic-megakaryocytic lineage (CFU-GEMM), CFU-granulocytic-macrophagic lineage (CFU-GM), burst-forming units-erythroid (BFU-E) and total number of colonies were scored after 14 days for the indicated samples. The figure presents the mean and SD values of the total number of the single colony types as stacked bar chart for the indicated treatment. One-way ANOVA and post hoc pairwise comparison were performed. One-way ANOVA resulted in *P* < .0001

(A) CD34+

Untreated/0 h | 24 h (1 µmol/L) | 48 h (1 µmol/L) | 72 h (1 µmol/L)

(B) CD34+

(C) Pearson r = .9707, P value = .0293
- EB-1, IPSS high, ASXL+ RUNX1 mut
- sAML, FLT3-ITD mut
- EB-1, tMDS, high risk, ASXL mut
- sAML, SRSF2 mut
1 µmol/L   n = 4

(D) CD34+
P = .0001
- CFU-GEMM
- BFU-E
- CFU-GM

DMSO 1:1000   +   −
Volasertib 1 µmol/L   −   +

(E)
P = .0005
P = .0029
P = .0007
CD34+
Volasertib (nmol/L): 1 10 50 100 1000
n (healthy): 5 5 5 5 5

(F) Bulk
Volasertib (nmol/L): 1 5 10 50 100 500 1000
n (healthy): 5 5 5 5 5 5 5

(G)
P = .0146
P = .0203
P = .0291
- CFU-GEMM
- BFU-E
- CFU-GM
Volasertib (nmol/L): 0 1 5 10 50 100 1000
n (healthy): 4 4 4 4 4 4 4

whereas others showed a relative resistance (3,3) despite a comparable proliferative capacity determined by immunohistochemistry as discussed above.

Analysing the longer-term kinetics of volasertib treatment in a random subset of 6 patients, we found a significant decrease in the colony-forming capacity in all samples independent of the short-term outcome (3C-E). Of note, considerable reduction in the volasertib concentration showed a significant decrease in the colony-forming capacity compared with DMSO (Figure 3C) and

healthy control cells (Figure 3D) independent of the short-term outcome (Figure 3E).

Further, volasertib did not only reduce the absolute number of colonies as a long-term effect on proliferation, but also decreased the colony size (Figure 3F). We therefore conclude that especially the progenitor cell compartment is affected.

Concludingly, sole inhibition of PLK1 showed a significant and selective cytotoxic effect on bone marrow samples from patients with MDS/sAML without relevant effects on healthy controls.
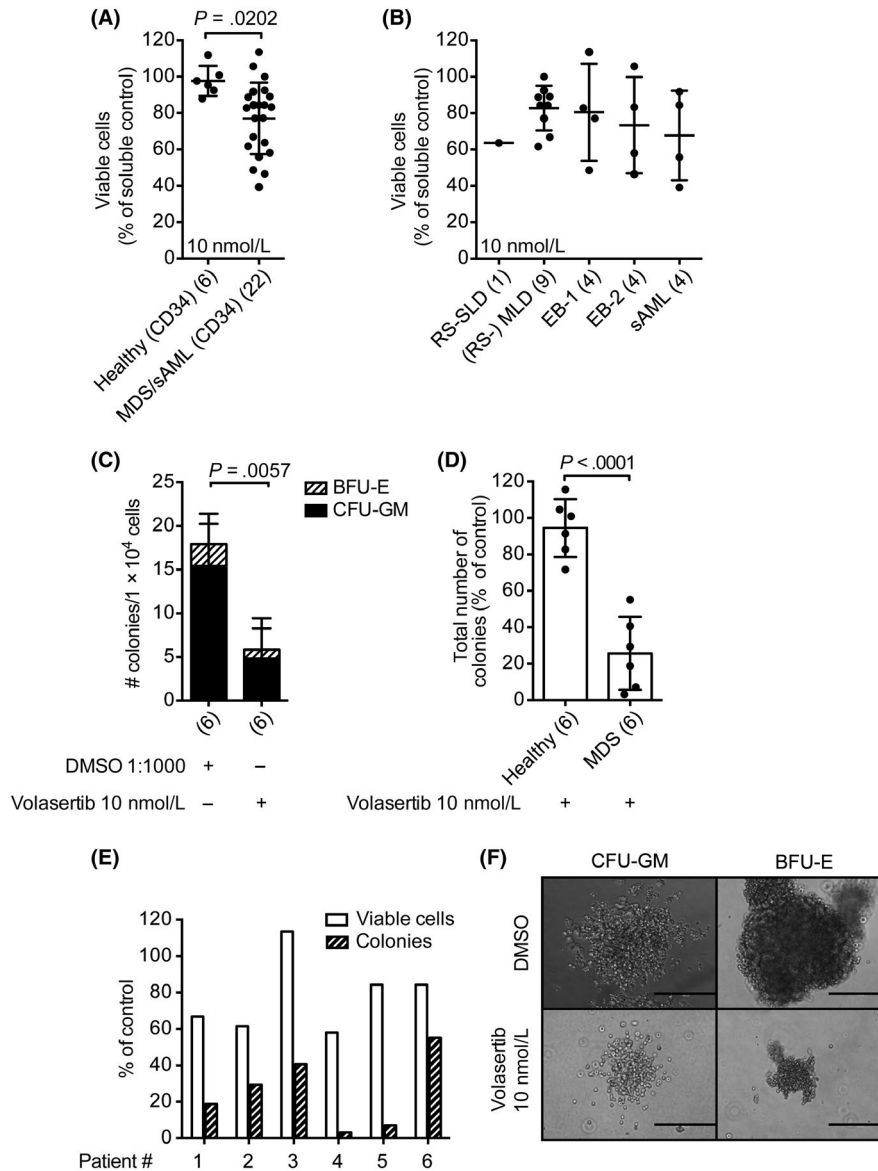
FIGURE 2 Gene expression of critical Polo-like kinase (PLK) family members in primary human samples. All panels: Gene expression was measured in primary human myelodysplastic syndromes (MDS) bone marrow (BM) samples and healthy controls using the Human Genome U133 Plus 2.0. Array. The mean expression of healthy controls is indicated by a red line. One-way analysis of variance (ANOVA) and post hoc pairwise comparisons were performed, wherever applicable. (A-C) Gene expression was measured in 164 primary human MDS bulk BM samples and 69 non-leukaemic controls (GEO: GSE15061). Non-leukaemic controls comprised healthy BM and non-leukaemic diseases including megaloblastic anaemia, haemolysis, iron deficiency and idiopathic thrombocytopenic purpura. (A) Gene expression of PLK1 in the bulk of primary human MDS BM samples (n = 164) and non-leukaemic controls (n = 69) (genecard probe ID: GC16P023690_at). PLK1 is upregulated in MDS BM bulk when compared to healthy controls (P = .0011). (B) Gene expression of PLK2 in the bulk of primary human MDS BM samples (n = 164) and non-leukaemic controls (n = 69) (genecard probe ID: GC05M057749_at). (C) Gene expression of PLK3 in the bulk of primary human MDS BM samples (n = 164) and non-leukaemic controls (n = 69) (genecard probe ID: GC01P045265_at). PLK3 is upregulated in healthy controls (P < .0001). (D-F) Gene expression was measured in 183 primary human CD34^{+} MDS BM samples (GEO: GSE19429). MDS were categorised into distinct MDS subtypes including MDS with single-lineage dysplasia (MDS-SLD, n = 55), MDS with single-lineage dysplasia and ring sideroblasts (MDS-RS-SLD, n = 48), MDS with blast excess <10% (EB-1, n = 37), MDS with blast excess >10% (EB-2, n = 43) and 117 healthy controls. (D) Gene expression of PLK-1 in primary CD34^{+} human MDS BM samples (n = 183) and healthy controls (n = 17) (genecard probe ID: GC16P023690_at). PLK1 is upregulated in early MDS (MDS-SLD and MDS-RS-SLD) as compared to that in healthy controls (P = .0353 and P < .0001, respectively). One-way ANOVA showed P < .0001. (E) Gene expression of PLK2 in primary CD34^{+} human MDS BM samples (n = 183) and healthy controls (n = 17) (genecard probe ID: GC05M057749_at). One-way ANOVA showed P = .2117. (F) Gene expression of PLK3 in primary CD34^{+} human MDS BM samples (n = 183) and healthy controls (n = 17) (genecard probe ID: GC01P045265_at). PLK3 is upregulated in MDS with single-lineage dysplasia and ring sideroblasts (RS-SLD) and MDS with blast excess <10% (EB-1) compared to that in healthy controls (P = .0203 and P = .0391). One-way ANOVA showed P = .0052
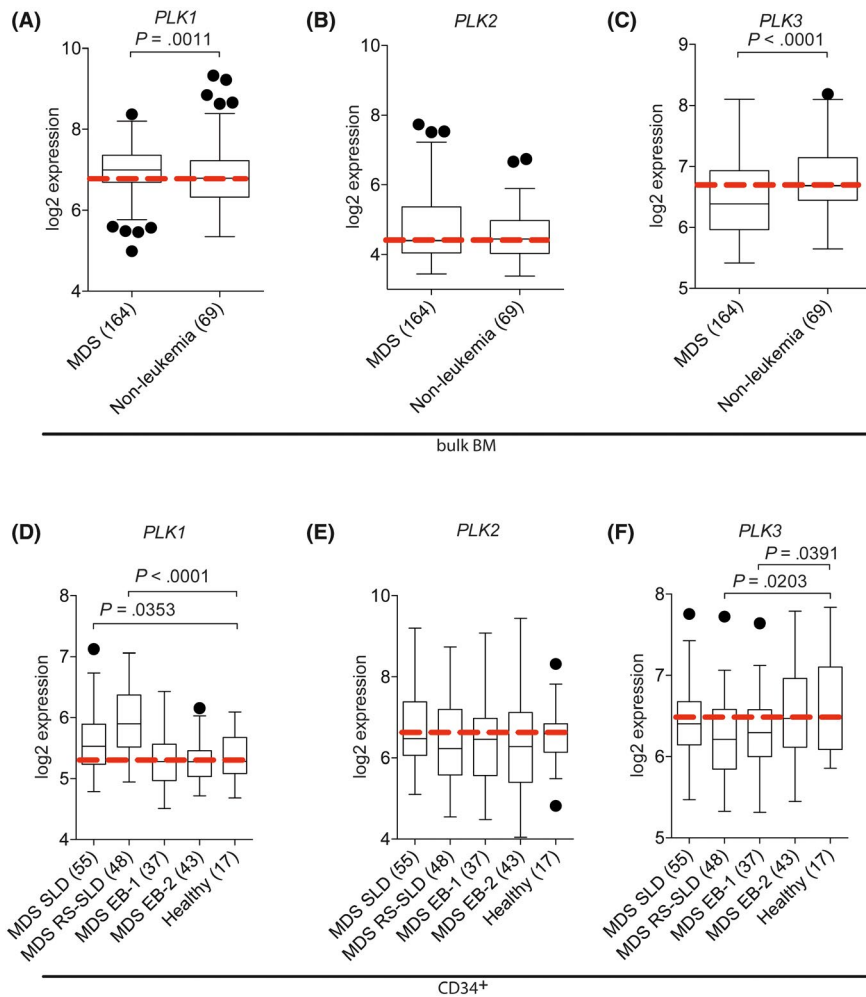
FIGURE 3 Volasertib shows constant high efficacy in MDS and sAML in spite of dose modification. (A) Viable BMMNC of 6 healthy, age-matched controls and 22 patients with MDS or sAML were treated for 72 hours with volasertib (10 nM) or vehicle control (DMSO) and gated on CD34$^+$ cells. Unpaired t test was performed, and the P value is indicated. (B) Viable BMMNC of the MDS or sAML patients with WHO-based classification as MDS with single-lineage dysplasia and ring sideroblasts (MDS-RS-SLD), MDS with multilineage dysplasia (MDS-MLD), MDS with multilineage dysplasia and ring sideroblasts (MDS-RS-MLD), MDS with blast excess <10% (EB-1) and MDS with blast excess >10% (EB-2) were treated for 72 h with volasertib (10 nM) or vehicle control (DMSO) and gated on CD34$^+$ cells. (C) BMMNC (1 × 10^4) was plated in growth-factors-enriched methylcellulose following 72 h of treatment with volasertib (10 nM) or vehicle control (DMSO). Numbers of CFU-GM, BFU-E and the total number of colonies were scored after 14 days for the indicated patient samples. Shown are the mean and the SD values of the total number of the single colony types as a stacked bar chart for the indicated treatment. The cohort comprised 4 low-risk MDS and 2 high-risk MDS or sAML patient samples. Low risk is defined as IPSS low or intermediate-1 and high risk as IPSS intermediate-2 or high. No difference was seen between the IPSS risk groups. The significance level applies to the total number of all colony types. Pairwise comparison was performed, and the obtained P value is indicated. (D) The reduction of colony numbers in percentage of soluble controls is shown in 6 healthy patients and 6 patients with MDS after pretreatment with volasertib at a concentration of 10 nM. (E) In 6 patients with MDS or sAML, the short-term and longer-term effects of treatment with volasertib (10 nM) for 72 hours are shown in parallel. The short-term effect is defined as a percentage of the viable cells measured using flow cytometry after 72 h of treatment. Longer-term effect is defined as the ratio of the total numbers of colonies (treated cells/soluble control) detected in methylcellulose after 14 days of culture following the initial treatment phase with volasertib in liquid culture for 3 days. Patients were classified as per the WHO classification system. Patients 1, 4, 5 and 6 had MDS-MLD, Patient 2 had MDS EB-2, and Patient 3 had MDS EB-1. (F) Representative pictures of CFU-GM and BFU-E colonies of BM of one high-risk MDS patient pretreated with DMSO (1:1000) and volasertib (10 nM), respectively. Colonies pretreated with volasertib (10 nM) showed a smaller size compared to those treated with DMSO (1:1000). The scale bar is 200 μm [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

Inhibition of PLK-1 is a promising approach in neoplasms with a high proliferative capacity. In our report, the PLK-1 inhibitor volasertib showed high efficacy on the malignant stem/progenitor cell compartment of patients with sAML and MDS. Interestingly, volasertib at a concentration of 10 nM not only lowered colony numbers in our methylcellulose assays, but also reduced the colony size. We therefore conclude that prominently the progenitor cell compartment is targeted by PLK1 inhibition. However, further clinical trials and long-term follow-ups will be

needed to confirm a substantial efficacy on the pool of leukaemia-initiating stem cells by PLK1 inhibition. Interestingly, the efficacy of PLK1 inhibition was not limited by stage or risk group and even patients of the higher-risk groups/ sAML or with known adverse mutational profile showed sensitivity towards treatment. We therefore argue that PLK1 inhibition might be a promising approach particularly in patients with an unfavourable prognosis.

However, viability was also dramatically decreased in the healthy controls. A significant reduction in the volasertib concentration (10 nM) retained the cytotoxic effect, but considerably lowered the toxic effects on healthy $CD34^+$ cells and the bulk of BM in vitro. As shown by analysis of open-access data, inhibition of further PLK family members might be a relevant side effect of a higher volasertib dosage.

Furthermore, it has to be discussed whether combination treatment might be a promising therapeutic strategy. In our in vitro setting, high levels of anti-apoptotic MCL-1 were associated with resistance to volasertib treatment. Overcoming apoptotic resistance might therefore be a substantial addendum to PLK1 inhibition. In his recent work, Liccardi *et al*[27] showed a mitosis-specific interaction between PLK1 and RIPK3 (Receptor-interacting serine/threonine-protein kinase 3). RIPK3 is a central player in necroptosis, another form of regulated cell death. Interestingly, analysis of a broader cohort of myeloid malignancies including chronic myeloid leukemia and de novo AML showed a clear correlation between the sensitivity towards low-dose volasertib treatment and RIPK3 levels (Supplementary Figure S3). A proven interaction between RIPK3 and central members of the BCL-2 family including MCL-1 strengthen this hypothesis.[28-30]

In summary, our data strengthen the scientific rationale of a therapeutic approach with volasertib in patients with MDS/sAML. The low-dose regimen shows a selective efficacy on malignant cells mainly in the longer-term outcomes. The current dosing schedule exerts a considerable toxicity on the non-malignant hematopoiesis, thus aggravating cytopenia and associated infectious complications.[31-34] To achieve durable responses, toxic side effects should be minimised in a cohort of elderly and frail patients. Further clinical trials will be needed to test the impact of our work on clinical reality. Yet, based on our in vitro findings, avoiding higher plasma concentrations of volasertib may have a critical impact. However, we conclude that using lower doses of volasertib (alone or in combination) represents a promising therapeutic strategy in patients with MDS and sAML.

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

AUTHOR CONTRIBUTIONS
SJ conceived and supervised the project, analysed the data and wrote the manuscript. JK performed the experiment and analysed the data. RTH, VD, LB, TO, RM and JSH performed the experiments. CMT, MCK, BS, PMP, DH, FB and AH provided primary samples and clinical data and gave conceptual advice. WK, TH, FB, CP and KSG gave conceptual advice. ICW gave conceptual advice and contributed to writing of the manuscript. UH supervised the project and analysed the data. PJJ conceived and supervised the project.

ETHICAL APPROVAL
Human BM samples were collected according to the institutional guidelines and in concordance with the Declaration of Helsinki. Written informed consent was obtained from each patient. The investigation was approved by the local ethics committee of the University Hospital of the Technical University in Munich (Vote #62/16S). Written informed consent was obtained from each patient according to the institutional guidelines.

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DATA AVAILABILITY STATEMENT
Further information regarding Material and Methods and Clinical characteristics of MDS/sAML patients contributing samples are provided in the Supplementary data section.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.