Efficacy and Mechanism of Action of Volasertib, a Potent and Selective Inhibitor of Polo-Like Kinases, in Preclinical Models of Acute Myeloid Leukemia

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ABSTRACT
Polo-like kinase 1 (Plk1), a member of the Polo-like kinase family of serine/threonine kinases, is a key regulator of multiple steps in mitosis. Here we report on the pharmacological profile of volasertib, a potent and selective Plk inhibitor, in multiple preclinical models of acute myeloid leukemia (AML) including established cell lines, bone marrow samples from AML patients in short-term culture, and subcutaneous as well as disseminated in vivo models in immune-deficient mice. Our results indicate that volasertib is highly efficacious as a single agent and in combination with established and emerging AML drugs, including the antimitabolite cytarabine, hypomethylating agents (decitabine, azacitidine), and quizartinib, a signal transduction inhibitor targeting FLT3. Collectively, these preclinical data support the use of volasertib as a new therapeutic approach for the treatment of AML patients, and provide a foundation for combination approaches that may further improve and prolong clinical responses.

Introduction
Compounds that interfere with the process of cell division have proven to be effective therapeutic agents in multiple types of cancer. A new generation of antimitotic drugs has recently entered clinical testing, including those targeting Polo-like kinase 1 (Plk1) (Schoffski, 2009; Tsykunova et al., 2012). Genetic screens in yeast and Drosophila had identified key regulators of mitosis, such as the founding member of the Polo-like kinase family (Fenton and Glover, 1993). Since then, five mammalian homologues have been discovered, numbered Plk1 to Plk5, which exhibit largely nonredundant functions and are differentially expressed, and regulated in cells (Zitouni et al., 2014). Whereas Plk2, Plk3, and Plk4 have been implicated in G1-S phase transition and centriole duplication, human Plk5 encodes a truncated protein that lacks part of the kinase domain and is therefore kinase-activity deficient (Strehbhard, 2010). Plk1 is the best characterized member of the Polo-like kinase family and has been shown to control essential steps during mitosis (Petronczki et al., 2008). Plk1 mRNA and protein expression occurs in a cell cycle–dependent manner with peak levels in late G2 and M phase (Golsteyn et al., 1994; Hamanaka et al., 1995). The catalytic activity and subcellular localization of Plk1 are tightly regulated during cell cycle progression (Golsteyn et al., 1995). The enzyme is located at centrosomes and kinetochores during early mitosis, and a fraction translocates to the midzone/midbody late in mitosis. Consistent with its biologic role, Plk1 is highly expressed in proliferating cells and therefore appears to be overexpressed in a broad spectrum of cancers, with high expression levels being correlated with poor prognosis in several cancer types (Takai et al., 2005). Taken together, these observations highlight Plk1 as an attractive molecular target for cancer therapy (Strehbhard and Ullrich, 2006; Christoph and Schuler, 2011; Gjertsen and Schoffski, 2015).

Volasertib (BI 6727; N-((1R,4R)-4-(4-(cyclopropylmethyl)piperazin-1-yl)cyclohexyl)-4-(R)-7-ethyl-8-isopropyl-5-methyl-1-(f)-1,4-thieno[3,2-f][1,2,4]triazolo[4,3-a]1,4-diacepine-6-acetic acid 1,1-dimethyl ester; LDAC, low-dose cytarabine; NPM, nucleophosmin; PBS, phosphate-buffered saline; Plk, polo-like kinase; RT, room temperature; TCTP, translationally controlled tumor protein.)
6-oxo-5,6,7,8-tetrahydropteridin-2-ylamino)-3-methoxybenzamide], the most advanced Plk inhibitor in clinical development to our knowledge, is an ATP-competitive Plk inhibitor derived from a dihydropteridinone lead structure (Rudolph et al., 2009). This compound has replaced its predecessor BI 2536 ([RI]-4-(8-cyclopentyl-7-ethyl-5-methyl-6-oxo-6,7,8-tetrahydropteridin-2-ylamino)-3-methoxy-N-(1-methylpiperidin-4-yl)benzamide] (Steegmaier et al., 2007) in clinical development based on its superior pharmacokinetic profile, i.e., large volume of distribution and long terminal half-life that provides prolonged tumor exposure. Volasertib potently inhibits Plk1 and Plk2 with IC_{50} values of 0.87 and 5 nM, respectively, and shows somewhat lower potency on Plk3 (56 nM) (Rudolph et al., 2009) but does not inhibit Plk4 (>20 μM) (Supplemental Fig. 1). Assays using a panel of more than 50 other kinases failed to identify any inhibitory activity at concentrations up to 10 μM, demonstrating that volasertib is highly selective for the Plk family. Volasertib perturbs centrosome maturation and separation and leads to the formation of a monopolar spindle, resulting in a temporary prometaphase arrest due to activation of the spindle assembly checkpoint. Prolonged arrest finally results in apoptosis. Although we cannot exclude that inhibition of other Plk family members contributes to the efficacy of volasertib, experiments using small interfering RNA indicate that inhibition of Plk1 is sufficient to explain the anti-proliferative and proapoptotic activity of this compound (Liu and Erikson, 2003; Sumara et al., 2004; Lenart et al., 2007; Steegmaier et al., 2007). Volasertib has shown efficacy in multiple solid tumor xenograft models of human cancer at well tolerated doses (Rudolph et al., 2009), and signs of efficacy have been observed in early clinical studies in patients with solid tumors (Janning and Fiedler, 2014). Here we report on the pharmacological profile of volasertib in multiple preclinical models of acute myeloid leukemia (AML) including established cell lines, bone marrow samples from AML patients in short-term culture, and subcutaneous as well as disseminated in vivo models in immune-deficient mice. Considering the high intrinsic genetic instability and heterogeneity of AML, it is generally believed that combination regimens will be required to achieve the desired clinical efficacy. Here we have tested whether volasertib cooperates with established or emerging therapeutic agents when analyzed in these AML models. In the present study, we were able to show in vivo efficacy of volasertib not only as a single agent but also in combination with chemotherapeutic agents, such as cytarabine, decitabine, azacitidine, or investigational targeted agents, such as the FLT3 inhibitor quizartinib.

Materials and Methods

Cell Culture. Tumor cell lines were obtained from American Type Culture Collection (Manassas, VA) or the German Collection of Microorganisms and Cell Culture (Braunschweig, Germany). Cell lines stably expressing luciferase (Luc) and green fluorescent protein (GFP) were generated using retroviral transduction of pLPNIG (Demajo et al., 2014) followed by drug selection (100 μg/ml geneticin). For single-cell term culture, and subcutaneous as well as disseminated in vivo models in immune-deficient mice. Considering the high intrinsic genetic instability and heterogeneity of AML, it is generally believed that combination regimens will be required to achieve the desired clinical efficacy. Here we have tested whether volasertib cooperates with established or emerging therapeutic agents when analyzed in these AML models. In the present study, we were able to show in vivo efficacy of volasertib not only as a single agent but also in combination with chemotherapeutic agents, such as cytarabine, decitabine, azacitidine, or investigational targeted agents, such as the FLT3 inhibitor quizartinib.

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Live-Cell Microscopy and Cell Fate Analysis. Automated wide-field fluorescence microscopy was performed on a Molecular Devices (Sunnyvale, CA) ImageXpress Micro XL screening microscope equipped with reflection-based laser autofocus and a 10×0.5 numerical aperture S Fluor dry objective (Nikon, Tokyo, Japan). Time-lapse images were taken every 4.6 or 8 minutes. Total movie duration was 61 hours. Mitotic entry statistics and cell fate analysis were performed using the CellCognition software (www.cellcognition.org). Automated cell detection, tracking over time, and morphology classification by supervised machine learning were conducted as described (Neumann et al., 2006).

Assays with Primary Bone Marrow Samples from AML Patients. Whole bone marrow samples were plated into 96-well plates containing volasertib. Blasts were characterized according to their light scatter properties and stained with antibodies to the following markers: CD11b, CD13, CD14, CD34, CD45, CD64, CD117, and HLA-DR. AML progenitor cells were incubated for 96 hours in RPMI 1640 medium (20% fetal calf serum, 2% HEPES, 1% L-glutamine) containing a human cytokine cocktail of 100 ng/ml stem cell factor (StemCell, Vancouver, BC, Canada), 50 ng/ml interleukin-3 (StemCell), 40 ng/ml of interleukin-6 (Milenyi Biotec, San Diego, CA), 200 ng/ml granulocyte macrophage colony-stimulating factor (Peprotech, Rocky Hill, NJ), 200 ng/ml granulocyte colony-stimulating factor (Peprotech), 4 U/ml erythropoietin (StemCell), 0.94 g/l human transferrin, and 5×10^{-2} mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). To identify live leukemic cells, two antibodies that unequivocally identify the pathologic cell population in the patient sample were selected in combination with annexin-V. EC_{50} values were extrapolated from the dose-response curve fit. Studies were performed at Vivia Biotech, Madrid, Spain.

Western Blot Analysis. Protein extracts (15 μg) from volasertib- or nocodazole-treated MV-4;11 cells were separated by SDS-PAGE. Immunoblotting was carried out by TransBlot Turbo Transfer System (Bio-Rad, Hercules, CA). Staining was performed with the corresponding antibodies recognizing phospho-Thr199 nucleophosmin (NPM) (total NPM, phospho-Ser46 translationally controlled tumor protein (TCTP), total TCTP, β-actin (Cell Signaling Technology, Danvers, MA), phospho-Ser10 histone H3 (Millipore, Billerica, MA), and total histone H3 (Thermo Fisher Scientific, Waltham, MA). The enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ) was used for detection.

Fluorescence-Activated Cell Sorter Analysis. MV-4;11 cells were incubated for 24, 48, and 72 hours with 0.1% dimethylsulfoxide or volasertib at various concentrations. To determine DNA content, cell suspensions were fixed in 80% ethanol, treated for 5 minutes with 0.25% Triton X-100 in phosphate-buffered saline (PBS), and incubated with 0.1% RNase and 10 μg/ml propidium iodide in PBS for 20 minutes at room temperature (RT). Cell cycle profiles were determined by flow cytometry (FACSscan; BD Biosciences, San Jose, CA). Inucyte Assay. MV-4;11 cells were plated (1×10^6/well) in 96-well plates (BD BioCoat poly-n-lysine–coated black/clear bottom plate). For apoptosis detection, Cell Player Kinetic caspase-3/7 Reagent (Essen BioScience, Ann Arbor, MI) was added and diluted in medium to a final concentration of 1.25 μM. Cell confluence and green fluorescence as a measure of caspase-3/7 activation were monitored for 36 hours. Images were taken every 3 hours.

Fast Activated Cell-Based Enzyme-Linked Immunosorbent Assay. MOLP-8 cells were plated (6×10^6/well) in 96-well plates (Millipore Multiscrreen). Serial dilutions of compound were added for 4 hours at 37°C. Cells were fixed (4% paraformaldehyde in PBS), washed (0.1% Triton X-100 in PBS), and incubated with quenching buffer (hydrogen peroxide, final concentration 1.2% v/v). After another wash [Triton/bovine serum albumin (BSA)] and incubation in blocking buffer (5% BSA in PBS with 0.1% Triton X-100), cells were incubated with the first antibody (anti-cMyc, #9402, 1:1000; Cell Signaling Technology) overnight at 4°C and with a secondary antibody (goat anti-rabbit horseradish peroxidase, #PO448, 1:750; Dako, Glostrup, Denmark) for 1 hour at RT. Peroxidase staining was performed and measured at optical density 450 nm with a SpectraMax absorbance reader ( Molecular Devices). EC_{50} values were calculated for the inhibition of cMyc protein expression.
BRD4 AlphaLISA Assay. AlphaLISA Glutathione Acceptor Beads and AlphaScreen Steptavidin Donor Beads (PerkinElmer, Waltham, MA) were premixed at a concentration of 15 μg/ml each in assay buffer (50 mM HEPES, pH 7.3, 25 mM NaCl, 2 mM diithiothreitol, 0.1% BSA, 0.05% Tween 20). Binding partner mixes were premixed in assay buffer at equimolar concentrations (GST-BRD4-BD1 (GenScript); acetyl-histone H4 (Lys5, 8, 12, 16) peptide, biotin conjugate (Millipore) at 15 nM each; GST-BRD4-BD2 (GenScript); acetyl-histone H4 (Lys5, 8, 12, 16) peptide, biotin conjugate (Millipore) at 150 nM each; GST-BRD2-BD1 (GenScript); acetyl-histone H4 (Lys5, 8, 12, 16) peptide, biotin conjugate (Millipore) at 30 nM each). The assay was performed on a fully automated robotic system in the dark. Test compounds, binding partner mix, and bead mix were added on a Proxiplate-384 PLUS (PerkinElmer). After incubation at RT for 60 minutes, the signal was measured in a PerkinElmer Envision HTS Multilabel Reader using the AlphaScreen specs from PerkinElmer. IC50 values were calculated using a four parametric logistic model.

PLK4 Kinase Assay. The assay was performed at Life Technologies (Carlsbad, CA) using the LantaScreen Eu kinase binding assay system. In brief, binding of an Alexa Fluor conjugate or tracer to a kinase is detected by addition of a Eu-labeled anti-tag antibody. Binding of the tracer and antibody to a kinase results in a high degree of fluorescence resonance energy transfer, whereas displacement of the tracer with a kinase inhibitor results in loss of fluorescence resonance energy transfer. For the PLK4 kinase assay, tracer 236 at a tracer concentration of 1 nM was used. Routinely, test compounds were screened in 1% dimethylsulfoxide (final concentration), and 10-point titrations with 3-fold serial dilution compounds were conducted using 20 μM as a start concentration. The incubation time was 1 hour. Staurosporine was reported to interfere with PLK4 at an IC50 of 1.82 nM and served as a positive control for this assay.

Viability Assays. For this assay, 1 × 106 cells/well were plated in 96-well flat-bottom microtiter plates and incubated overnight at 37°C in a CO2 incubator. Test compound was added at various concentrations for 72 hours. After 6-hour incubation with Alamar Blue solution (Invitrogen, Carlsbad, CA) at 37°C, fluorescence was measured (Envision MultimodeReader; PerkinElmer) using an excitation wavelength of 531 nm and emission at 595 nm.

Data Analysis. Data were fitted by iterative calculation using a sigmoidal curve analysis program (Prism version 3.0; Graph Pad, La Jolla, CA) with variable hill slope. EC50 values were calculated. Three-dimensional (3D) assays in matrigel were performed as described (Li et al., 2008).

Efficacy Studies in Mice. Female CIEA-NOG mice (NOD.Cg-PrkdcscidLystbg-J/Crl; Charles River, Koln, Germany). This study was performed at Experimental Pharmacology & Oncology Berlin-Buch GmbH, Berlin, Germany. Volasertib, cytarabine, decitabine, and azacitidine, respectively, were formulated in hydrochloric acid (0.1 N), diluted with 0.9% NaCl, and injected intravenously or intraperitoneally. Quizzartib was dissolved in 22% 2-hydroxypropyl-β-cyclodextrin and administered p.o. An administration volume of 10 ml/kg body weight was used.

Results

Volasertib Inhibits Proliferation of AML Cell Lines and Patient-Derived AML Blasts. To test the activity of volasertib in a broad range of preclinical models, we initially selected a panel of eight established AML cell lines representing a spectrum of genetic aberrations (Table 1). Volasertib showed efficacy in two-dimensional (2D) and 3D assays, respectively, using Alamar Blue staining as a measure of viability, with EC50 values in the range of 16–170 nM in the 2D assay format and 19–40 nM in the 3D Matrigel assay format (Table 1). Moreover, bone marrow samples from 14 AML patients were also tested in 2D assays followed by flow cytometry using annexin-V to detect live versus apoptotic leukemic cells (Table 2); these primary patient samples were exposed to volasertib in the presence of a cytokine cocktail to stimulate blast proliferation. Volasertib inhibited proliferation of 13/14 samples with EC50 values below 100 nM (range, 8–99 nM) irrespective of risk category or mutational status, except for one sample, which was less sensitive (377 nM).

Volasertib Mechanism of Action. The AML cell line MV-4-11 was used to study the effect of volasertib on cell cycle progression. Propidium iodide staining and flow cytometry of cells treated with volasertib showed a time- and dose-dependent increase in the G2/M peak followed by an increase in the sub-G1 peak at 48 and 72 hours, indicative of cell death (Fig. 1, A and B). Similar results were obtained in an independent study using Kasumi-1 cells (Supplemental Fig. 2). To discriminate between an arrest in G2 phase or M phase of the cell cycle, several markers were assessed by Western blot analysis (Fig. 1C). Increased phosphorylation of histone H3 (Ser10) and NPM (Thr199) clearly demonstrates mitotic arrest of
inhibition of mitotic entry and the incidence of mitotic failures (Fig. 2D) and individual cell fates (Fig. 2, E).

Summary statistics of mitotic entry indicated that volasertib arrests cells in early mitosis. In addition, assays measuring the displacement of an acetylated histone epigenetic marks (Ciceri et al., 2014). To investigate the pharmacological relevance of this interaction, we performed assays measuring the displacement of an acetylated histone H4–derived peptide from the bromodomains 1 or 2 of BRD4 (Fig. 3, A and B). IC50 values for volasertib in these two assays were 300 and 770 nM, respectively, confirming bromodomain inhibition by volasertib in a biochemical binding assay. In MOLP-8 cells, expression of cMyc, a BRD4 target gene, was downregulated by volasertib with an EC50 of 730 nM (Fig. 3C), consistent with the potency observed in the biochemical assays. In contrast, viability of these cells was inhibited with an EC50 of 9 nM (Fig. 3D), i.e., at 80-fold lower concentration, consistent with the potency of volasertib as a Plk1 inhibitor. Data for the bona fide bromodomain and extraternal family of proteins that acts as a reader of myelodysplasia-related changes.

To assess the phenotype of volasertib-treated cells in more detail, we performed long-term live-cell imaging experiments. As AML cells growing in suspension are not suitable for this assay, we used HeLa cells engineered to express a fusion protein of histone 2b with the fluorescent protein mCherry as a chromatin marker. Volasertib reduced the rate of entry into prometaphase followed by cell death or mitotic exit without chromosome segregation. Summary statistics of mitotic entry rates (Fig. 2D) and individual cell fates (Fig. 2, E–G) show that inhibition of mitotic entry and the incidence of mitotic failures correlates with increasing concentrations of volasertib. Hence, volasertib inhibits proliferation by reducing the rate of prometaphase entry and by perturbing mitosis in early prometaphase.

Recently published data suggest that volasertib binds to the first bromodomain of BRD4, a member of the bromodomain and extramteral family of proteins that acts as a reader of epigenetic marks (Ciceri et al., 2014). To investigate the pharmacological relevance of this interaction, we performed assays measuring the displacement of an acetylated histone H4–derived peptide from the bromodomains 1 or 2 of BRD4 (Fig. 3, A and B). IC50 values for volasertib in these two assays were 300 and 770 nM, respectively, confirming bromodomain inhibition by volasertib in a biochemical binding assay. In MOLP-8 cells, expression of cMyc, a BRD4 target gene, was downregulated by volasertib with an EC50 of 730 nM (Fig. 3C), consistent with the potency observed in the biochemical assays. In contrast, viability of these cells was inhibited with an EC50 of 9 nM (Fig. 3D), i.e., at 80-fold lower concentration, consistent with the potency of volasertib as a Plk1 inhibitor. Data for the bona fide bromodomain and extraternal family inhibitor JQ1 (6-[4-chlorophenyl]-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine-6-acetic acid 1,1-dimethylethyl ester) (Filippakopoulos et al., 2010) in these four assays are shown as supplemental data (Supplemental Fig. 3).

Efficacy in Mouse Models of AML. Initial studies to test the efficacy of volasertib in vivo used immune-deficient nude mice subcutaneously injected with MOLM-13 AML cells.

MV-4-11 cells treated with volasertib. In line with this, treatment with nocodazole, a microtubule-stabilizing agent that arrests cells in mitosis, leads to a similar pattern of marker modulation. However, increased levels of phospho-TCTP (Ser46), another marker of mitosis and a Plk1 substrate (Cucchi et al., 2010) in these four assays are shown.

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<th>Sample</th>
<th>Previous MDS/MPS</th>
<th>Karyotype ISCN 2005</th>
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FAB, French-American-British; ISCN, International System of Cytogenetic Nomenclature; ITD, internal tandem duplication; MDS, Myelodysplastic syndrome; MPS, Myeloproliferative syndrome; nd, not determined; neg, negative; pos, positive.
Intravenous treatment was initiated when tumors had reached a volume of approximately 150 mm³. Efficacy was dose-dependent with treated over control (T/C) values of 20 and 53%, respectively, at doses of 20 or 40 mg/kg once a week (Fig. 4A). All dose levels were well tolerated as neither body weight loss nor adverse signs were observed (Fig. 4B). Of note, 40 mg/kg

Fig. 1. Volasertib arrests AML cells in mitosis, inhibits proliferation, and induces apoptosis. (A and B) Exponentially growing MV-4-11 cells were treated for 24, 48, and 72 hours, respectively, with vehicle, 20, or 200 nM volasertib. Cells were harvested, stained with propidium iodide, and DNA content was measured by flow cytometry. A representative experiment of two independent studies is shown. (A) Dose-dependent increase in the G2/M peak at 24 hours. (B) Time-dependent increase in the sub-G1 peak indicative of apoptotic cells. (C) Western blot analysis of extracts derived from MV-4-11 cells treated with volasertib at various concentrations or with 100 and 300 nM nucodazole, a microtubule stabilizing agent that arrests cells in mitosis, for 1 or 24 hours. An increase in phospho-Ser10 histone H3 and phospho-Thr199 NPM indicates arrest in mitosis, whereas a decrease in phospho-Ser46 TCTP, a Plk1 substrate, indicates inhibition of Plk1 activity. (D) Live-cell imaging of MV-4-11 cells over 36 hours shows complete inhibition of cell proliferation (left) and an increase in caspase-3/7–stained apoptotic cells (right) induced by 50 nM volasertib. A representative study of n = 30 studies is shown. DMSO, dimethylsulfoxide.
once weekly had previously been defined as the maximum tolerated dose.

Although subcutaneous xenograft models are widely used for compound profiling, we also attempted to assess the efficacy of volasertib in AML models which more closely reflect the clinical situation. To this end, $10^7$ MOLM-13 or MV-4-11 cells, stably transduced with a luciferase-expression vector to allow assessment of tumor load by bioluminescence imaging, were injected into the tail vein of CIEA-NOG mice. Treatment with volasertib at 20 or 40 mg/kg i.v. once weekly was initiated 3 (MOLM-13 model) or 8 (MV-4-11 model) days after cell injection. The animals were closely monitored for clinical signs and were sacrificed when the disease burden exceeded a prespecified grade as a surrogate endpoint for survival. In the MOLM-13 model, the median survival of the vehicle-treated control animals was 15 days. Median survival
of animals treated with 20 or 40 mg/kg once a week was 37 and 48 days, respectively, corresponding to an increase in life span by about 150 and 220% (Fig. 4C). Imaging data generated on day 14 (Fig. 3D) demonstrate the significantly reduced disease burden observed in volasertib-treated mice. In the MV-4-11 study, median survival of vehicle-treated control animals was 33 days; survival of animals treated with 20 mg/kg once weekly was 57 days (73% increase), and for mice treated at 40 mg/kg, the survival endpoint was not reached (85% increase) (data not shown).

Volasertib in Combination with Established and Investigational Therapeutic Agents. The antimetabolite cytarabine is a standard-of-care agent in the treatment of AML patients. A combination of volasertib with cytarabine was therefore tested in a patient-derived subcutaneous xenograft model, AML-6252. Tumor-bearing Scid-beige mice were treated intravenously for 2 weeks with vehicle (once weekly), volasertib given as a single agent (20 or 40 mg/kg once weekly), cytarabine (100 mg/kg i.p. twice a week every third or fourth day), or volasertib in combination with cytarabine. Combination therapy at both dose levels showed improved efficacy compared with either single agent and was well tolerated (Fig. 5, A and B). Interestingly, the efficacy of the cytarabine combination with 20 mg/kg volasertib was comparable to single-agent activity of volasertib at 40 mg/kg. Addition of cytarabine to 40 mg/kg volasertib, corresponding to the maximum tolerated dose in mice, further improved efficacy (Fig. 5A).

The hypomethylating agents decitabine and azacitidine were tested in combination with volasertib in the subcutaneous MV-4-11 xenograft model. Mice were treated intravenously once a week with either vehicle or 10 or 20 mg/kg volasertib as a single agent or in combination with 1.25 mg/kg decitabine, administered intraperitoneally on 2 consecutive days. Combination therapy at both dose levels of volasertib showed improved efficacy and was well tolerated (data not shown). Similar results were obtained when MV-4-11 tumor-bearing mice were treated intravenously once a week with a combination of volasertib (20 mg/kg) and azacitidine (40 mg/kg). Combination therapy showed improved efficacy compared with single-agent treatment (Fig. 5C). Of note, even though single-agent azacitidine resulted in a median body weight loss of up to 8%, tolerability did not deteriorate further when volasertib was added to this treatment regimen (Fig. 5D).

The potential of FLT3 inhibition as a therapeutic option for AML patients is currently being evaluated in clinical studies. We tested the combination of volasertib with the
FLT3 inhibitor quizartinib in the subcutaneous MV-4-11 xenograft model characterized by an FLT3 internal tandem repeat mutation (Fig. 5, E and F). Animals were treated for 2 weeks with either 20 mg/kg volasertib i.v. once weekly, with a daily oral dose of quizartinib (5 or 10 mg/kg), or a combination of these agents. At the end of the treatment period, efficacy of
Fig. 5. Efficacy and tolerability of volasertib in combination with cytarabine, azacitidine, or quizartinib in human subcutaneous xenograft models of AML. (A and B) AML-6252 tumor-bearing Scid-beige mice were treated for 2 weeks with vehicle once weekly (open squares), volasertib once a week at 20 and 40 mg/kg i.v., respectively (black triangles and black bar), cytarabine twice a week every third or fourth day at 100 mg/kg i.p. (black squares), a combination of cytarabine and 20 mg/kg volasertib (black diamonds), or a combination of cytarabine and 40 mg/kg volasertib (black circles). The bar at the top of (A) indicates the treatment period. Mean tumor volumes of eight animals per treatment group with error bars depicting S.D. (A) and body weight in grams with error bars depicting S.D. (B) are shown. (C and D) MV-4-11 tumor-bearing nude mice were treated for 3 weeks with vehicle (open squares), volasertib once a week at 20 mg/kg i.v. (black triangles), azacitidine once a week at 40 mg/kg i.v. (black squares), or a combination of both (black diamonds). Mean tumor volumes of eight animals per treatment group with error bars depicting S.D. (C) and body weight changes in percentage of initial weight with error bars depicting S.D. (D) are shown. The bar at the top of (C) indicates the treatment period. (E and F) MV-4-11 tumor-bearing nude mice were treated for two cycles with vehicle (open squares), volasertib once a week at 20 mg/kg i.v. (black triangles), a daily dose of quizartinib at 5 mg/kg (black squares) or 10 mg/kg (gray squares), or a combination of volasertib and quizartinib at 5 and 10 mg/kg (gray or black diamonds). Mean tumor volumes of eight animals per treatment group with error bars depicting S.D. (E) and body weight changes in percentage of initial weight with error bars depicting S.D. (F) are shown. The bar at the top of (E) indicates the treatment period.
single-agent quizartinib was comparable to that observed with the drug combination. However, during a post-treatment observation period, tumors in the quizartinib groups started to regrow 45–60 days after termination of treatment, whereas combination with volasertib resulted in long-term efficacy without evidence for tumor regrowth until the last day of the study, 72 days after termination of treatment.

Discussion

In recent years, advances in our knowledge of the regulation of cell division have resulted in the identification of several potential mitosis-specific targets for therapeutic intervention, including Plk1. Plk1 is expressed in dividing cells in a cell cycle–dependent manner, with low expression during the G0/G1 phase, a rise in S phase, and peak levels in M phase (Hamanaka et al., 1995). Plk1 has previously been found to be aberrantly expressed in AML cells compared with normal bone marrow mononuclear cells (Ikezoe et al., 2009; Renner et al., 2009), and has often been correlated with poor prognosis, disease stage, histologic grade, metastatic potential, and survival (Takai et al., 2005). These observations have prompted research into the potential therapeutic application of Plk1 inhibitors in AML. In a previous communication (Rudolph et al., 2009), we have described the chemical structure, target binding mode, pharmacokinetics in rodents, and an initial pharmacological profile of volasertib, focusing on cell lines and in vivo models derived from solid tumors. In this report, we demonstrate that treatment of AML cells with volasertib leads to potent and selective inhibition of Plk1, as specified by inhibition of phospho-TCTP, a Plk1 substrate, and subsequent mitotic arrest followed by a strong induction of apoptosis. In our analysis of AML cell lines as well as patient samples in viability assays, we found that the large majority of these were highly sensitive to volasertib, irrespective of karyotype and mutational status. A recent publication has claimed that volasertib may have an additional inhibitory activity and could be a dual kinase-bromodomain inhibitor based on data showing that volasertib binds to the bromodomain of BRD4 (Ciceri et al., 2014). In AML, the gene encoding BRD4 was identified by an epigenetically focused systematic in vivo RNA interference screen as highly essential for promoting proliferation and blocking differentiation (Zuber et al., 2011). When using a BRD4 inhibitory compound, antileukemic effects across genomic AML subtypes were precisely recapitated and found to largely depend on blocking the oncogenic transcription factor MYC (Zuber et al., 2011). Therefore, a potential inhibitory effect of BRD4 exerted by volasertib is of particular interest in light of the emerging role of BRD4 as a cancer target and ongoing attempts to discover and develop BRD4 inhibitors (Papavassiliou and Papavassiliou, 2014). Although our data clearly confirm that volasertib interferes with the binding of an acetylated peptide derived from histone H4 to the bromodomains 1 and 2 of BRD4 in biochemical assays, our data also show that BRD4-dependent target gene modulation (MYC levels) occurs at markedly (80-fold) higher concentrations than the Plk1-dependent antiproliferative effect observed with volasertib in vitro. Considering the pharmacokinetics of volasertib in patients with peak plasma levels of about 1 μM (Schoffski et al., 2012), we consider it unlikely that BRD4 inhibition contributes to its efficacy. Ultimately, further studies are needed to address the contribution of BRD4-dependent blockade of target genes, such as MYC to volasertib’s efficacy in AML patients.

Considering the high intrinsic genetic instability and heterogeneity of AML cells, it is generally believed that combination regimens will be required to achieve the desired clinical efficacy. Therefore, it was of considerable interest to learn whether volasertib would cooperate with established or emerging therapeutic agents when analyzed in AML models. In the present study, we were able to show in vivo efficacy of volasertib not only as a single agent but also in combination with chemotherapeutic agents, such as cytarabine, decitabine, azacitidine, or investigational targeted agents, such as the FLT3 inhibitor quizartinib. Remarkably, in all combinations, efficacy of standard agents is improved, and this is not offset by toxicity. Further studies will be required to elucidate the detailed molecular mechanisms underlying these combinatorial effects.

Volasertib is the first Plk inhibitor that has advanced to late-stage clinical development. Early clinical studies in patients with solid tumors had indicated that the compound has therapeutic potential and is well tolerated (Gjertsen and Schoffski, 2015), with mainly hematologic toxicities and the notable absence of neuropathies, and thus may be particularly beneficial for patients unable to tolerate aggressive chemotherapy. Importantly, recent clinical data in AML patients showed that volasertib combined with low-dose cytarabine (LDAC) was associated with higher response rates and improved event-free survival than LDAC alone (Dohner et al., 2014). On this basis, volasertib in combination with LDAC is currently being evaluated in a phase III clinical study in patients with previously untreated AML who are ineligible for intensive therapy (NCT01721876; POLO-AML-2). Collectively, our preclinical results support the concept that volasertib is a novel therapeutic option for patients diagnosed with AML and shows potential for combination with a range of existing and emerging AML drugs. Future clinical testing will determine the potential of volasertib—when applied in a range of combination settings—to cause durable clinical responses across a broad range of AML genomic subtypes.

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References


Demajo S, Uribezabal J, Gutiérrez A, Ballare C, Capdevila S, Roth M, Zuber J, Martin-Caballero J, and Di Groce L (2014) ZRF1 controls the retinoic acid pathway


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Efficacy and mechanism of action of volasertib, a potent and selective inhibitor of Polo-like kinases, in preclinical models of acute myeloid leukemia

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Supplemental data

**Supplement Figure 1** Volasertib does not inhibit Plk4 kinase activity in a Lanthascreen EU kinase binding assay

**Supplement Figure 2** Volasertib arrests AML cells in mitosis, inhibits proliferation and induces apoptosis.
(A-B) Exponentially growing Kasumi-1 cells were treated for 24, 48 and 72 h, respectively with vehicle, 20 or 200 nM volasertib. Cells were harvested, stained with propidium iodide and DNA content was measured by flow cytometry. (A) Dose-dependent increase in the G2/M peak at 24 h. (B) Time-dependent increase in the subG1 peak indicative of apoptotic cells.

Supplemental Figure 3 The BET family inhibitor JQ1 displaces a Histone H4-derived peptide from the bromodomains 1 and 2 of BRD4 in a biochemical assay and downregulates myc protein levels in MOLP-8 cells in line with its activity on MOLP-8 cells in a viability assay.

(A-B) JQ1 activity was measured in a BRD4 Alpha Lisa assay that detects the displacement of an acetylated histone H4-derived peptide from the bromodomains 1 (A) or the bromodomain 2 (B) of BRD4. IC<sub>50</sub> values are indicated by vertical lines. (C) Dose-response curve of MOLP-8 cells in a 3-day 2D Alamar Blue assay using various concentrations of
volasertib. The EC$_{50}$ value is indicated by a vertical line. (D) FACE ELISA to determine the effect of volasertib on myc protein levels in MOLP-8 cells. The EC$_{50}$ value is indicated by a vertical line.