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 AML including established cell lines, bone marrow samples of 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 anti-metabolite 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 anti-mitotic drugs has recently entered clinical testing, including those targeting Polo-like kinase 1 (Plk1) (Tsykunova, et al., 2012; Schoffski, 2009). 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, 5 mammalian homologs have been discovered, numbered Plk1 to Plk5, which exhibit largely non-redundant functions and are differently expressed, localized and regulated in cells (Zitouni, et al., 2014). While 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 (Strebhardt, 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 biological role, Plk1 is highly expressed in proliferating cells and therefore appears to be over-expressed 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 (Gjertsen and Schoffski, 2014; Christoph and Schuler, 2011; Strebhardt and Ullrich, 2006).

Volasertib (BI 6727), to our knowledge the most advanced Plk inhibitor in clinical development, is an ATP-competitive Plk inhibitor derived from a dihydropteridinone lead structure (Rudolph, et al., 2009). This compound has replaced its predecessor BI 2536
(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\textsubscript{50} values of 0.87 nM and 5 nM, respectively, and shows somewhat lower potency on Plk3 (56 nM) (Rudolph, et al., 2009) but does not inhibit Plk4 (> 20 \mu M) (Supplemental Figure 1). Assays using a panel of more than 50 other kinases failed to identify any inhibitory activity at concentrations up to 10 \mu M, 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 siRNA indicate that inhibition of Plk1 is sufficient to explain the anti-proliferative and pro-apoptotic activity of this compound (Liu and Erikson, 2003; Steegmaier, et al., 2007; Lenart, et al., 2007; Sumara, et al., 2004). 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 AML including established cell lines, bone marrow samples of AML patients in short-term culture and subcutaneous as well as disseminated \textit{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 \textit{in vivo} efficacy of volasertib 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.
Material and Methods

Cell culture

Tumor cell lines were obtained from ATCC or the German Collection of Microorganisms and Cell Culture. Cell lines stably transfected with a GFP-containing vector (LMN-Luc2-IHRES-GFP) (Demajo, et al., 2013) were incubated in media containing 100 μg/ml geneticin. For single cell live-cell microscopy experiments, HeLa ‘Kyoto’ cells stably expressing H2B-mCherry were used (Neumann, et al., 2006; Dick and Gerlich, 2013) and were grown in DMEM (10% FBS and 1% penicillin-streptomycin) without phenol red and riboflavin to reduce autofluorescence. Volasertib was added 1 h prior to time-lapse recordings.

Live-cell microscopy and cell fate analysis

Automated wide-field fluorescence microscopy was performed on a Molecular Devices ImageXpressMicro XL screening microscope equipped with reflection-based laser autofocus and a 10x 0.5 N.A. S Fluor dry objective (Nikon). Time-lapse images were taken each 4.6 or 8 min. Total movie duration was 61 h. Mitotic entry statistics and cell fate analysis was performed using the CellCognition software (www.cellcognition.org). Automated cell detection, tracking over time, and morphology classification by supervised machine learning was 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, HLA-DR. AML progenitor cells were incubated for 96 h in RPMI 1640 medium (20% FCS, 2% HEPES, 1% L-glutamine) containing a human cytokine cocktail of 100 ng/ml stem cell factor
(StemCell), 50 ng/ml IL-3 (StemCell), 40 ng/ml of IL-6 (Miltenyi Biotech), 200 ng/ml granulocyte macrophage colony-stimulating factor (Peprotech), 200 ng/ml granulocyte colony-stimulating factor (Peprotech), 4 U/ml erythropoietin (EPO, StemCell), 0.94 g/L human transferrin and 5x10^{-5} mmol/L 2-mercaptoethanol (Sigma). To identify live leukemic cells, two antibodies that unequivocally identify the pathological 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 (BioRad). Staining was performed with the corresponding antibodies recognizing phospho-Thr199 NPM, total NPM, phospho-Ser46 TCTP, total TCTP, β-actin (Cell Signaling), phospho-Ser10 histone H3 (Millipore) and total histone H3 (Thermo Fisher Scientific). The ECL system (GE Healthcare) was used for detection.

**FACS analysis**

MV-4-11 cells were incubated for 24, 48 and 72 h with 0.1% DMSO or volasertib at various concentrations. To determine DNA content, cell suspensions were fixed in 80% ethanol, treated for 5 min with 0.25% Triton X-100 in PBS and incubated with 0.1% RNAse and 10 µg/ml propidium iodide (PI) in PBS for 20 min at RT. Cell-cycle profiles were determined by flow cytometry (FACSCanto™, BD Biosciences).

**Incucyte assay**

MV-4-11 cells were plated (1x10^4/well) in 96-well plates (BD BioCoat poly-d-lysine coated black/clear bottom plate). For apoptosis detection, Cell Player Kinetic Caspase 3/7 Reagent (Essen BioScience) 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 h. Images were taken every 3 hours.

**FACE ELISA**

Molp-8 cells were plated (5x10^4/well) in 96-well plates (Millipore Multiscreen). Serial dilutions of compound were added for 4 h at 37°C. Cells were fixed (4% PFA in PBS), washed (0.1% TritonX-100 in PBS) and incubated with quenching buffer (hydrogen peroxide, final conc. 1.2%w/vol). After another wash (Triton/BSA) and incubation in blocking buffer (3% BSA in PBS with 0.1% TritonX-100), cells were incubated with the 1st antibody (anti cMyc, Cell Signaling #9402, 1:1000) overnight at 4°C and with a secondary antibody (goat anti rabbit HRP, Dako #P0448, 1:750) for 1 hour at RT. Peroxidase staining was performed and measured at OD 450 nm with a SpectraMax absorbance reader. EC_{50} values were calculated for the inhibition of cMyc protein expression.

**BRD4 Alpha LISA assay**

AlphaLISA Glutathione Acceptor Beads and AlphaScreen Streptavidin Donor Beads (PerkinElmer) were pre-mixed at a concentration of 15 µg/ml each in assay buffer (50 mM HEPES pH 7.3, 25 mM NaCl, 2 mM DTT, 0.1% BSA, 0.05% Tween 20). Binding partner mixes were pre-mixed in assay buffer at equimolar concentrations (GST-BRD4-BD1 (Genescript); Acetyl-histone H4 (Lys5, 8, 12, 16) peptide, biotin conjugate (Millipore) @ 15 nM each; GST-BRD4-BD2 (Genescript); Acetyl-Histone H4 (Lys5, 8, 12, 16) peptide, biotin conjugate (Millipore) @ 150 nM each; GST-BRD2-BD1 (Genescript); Acetyl-Histone H4 (Lys5, 8, 12, 16) peptide, biotin conjugate (Millipore) @ 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 4 parametric logistic model.

**PLK4 kinase assay**

The assay was performed at Life Technologies 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 FRET, whereas displacement of the tracer with a kinase inhibitor results in loss of FRET. For the PLK4 kinase assay, tracer 236 at a tracer concentration of 1 nM was used. Routinely, test compounds are screened in 1% DMSO (final concentration), 10 point titrations with 3-fold serial compound dilutions were conducted using 20 µM as a start concentration. The incubation time was 1 h. Staurosporine is reported to interfere with PLK4 at an IC₅₀ of 1.82 nM and serves as a positive control for this assay.

**Viability assays**

1x10⁵ 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) 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) with variable hill slope. EC₅₀ values were calculated. 3D assays in matrigel were performed as described (Li, et al., 2008).

**Efficacy studies in mice**
Female CIEA-NOG mice (NOD.Cg-Prkdc<sup>scid</sup>IL2rg<sup>tm1Sug</sup>/JicTac) (Taconic, Denmark) were engrafted i.v. with 1x10<sup>7</sup> MOLM-13 or MV-4-11 AML cells, respectively. Cells stably transfected with a luciferase-expression vector (LMN-Luc2-IHRES-GFP) (Demajo, et al., 2013) to allow monitoring of tumor load by imaging approaches were kindly provided by Johannes Zuber from the Institute of Molecular Pathology, Vienna, Austria. Tumor load was measured based on bioluminescence imaging using the IVIS Lumina III from Perkin Elmer. 150 mg/kg firefly D-luciferin (Perkin Elmer) was administered i.p. 10 min before measurements. Luciferase signal intensity was quantified using the Living Imaging software (Perkin Elmer). The following scoring system was used: score 0 no clinical signs, score 1 tail or hind limb weakness, score 2 tail and hind limb weakness. Animals were sacrificed based on severity criteria including appearance of paralysis score ≤ 2 and/or body weight loss exceeding 20%.

To establish subcutaneous AML models, female BomTac:NMRI-Foxn1<sup>nu</sup> mice (Taconic, Denmark) were injected with 5x10<sup>6</sup> MOLM-13 cells (DMSZ ACC-544), mixed 1:1 with Matrigel™ (BD Biosciences). To establish the AML-6252 patient-derived xenograft model, tumor fragments were transplanted subcutaneously into female Scid-beige mice (CB17.Cg-Prkdc<sup>scid</sup>Lyst<sup>bg-J</sup>/Crl) (Charles River, Germany). This study was performed at Experimental Pharmacology & Oncology Berlin-Buch GmbH (EPO), Berlin, Germany. Volasertib, cytarabine, decitabine and azacitidine, respectively, were formulated in hydrochloric acid (0.1 N), diluted with 0.9% NaCl and injected i.v. or i.p.. Quizartinib was dissolved in 22% HP-CD and administered p.o.. An administration volume of 10 ml per 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 8 established AML cell lines representing a spectrum of genetic aberrations (Table 1). Volasertib showed efficacy in 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 vs. 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 h, indicative of cell death (Figure 1A-B). Similar results were obtained in an independent study using Kasumi-1 cells (Supplemental Figure 2). To discriminate between an arrest in G2 phase or M phase of the cell cycle, several markers were assessed by Western blot analysis (Figure 1C). Increased phosphorylation of histone H3 (Ser10) and nucleophosmin (NPM; Thr199) clearly demonstrates mitotic arrest of 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 (translationally controlled tumor protein; Ser46), another marker of mitosis and a Plk1 substrate (Cucchi, et al., 2010), were observed after treatment with nocodazole, while treatment with volasertib resulted in a decrease in
phospho-TCTP to undetectable levels within 1 h. These data indicate that volasertib arrests cells in early mitosis. In additional assays, live-cell imaging of MV-4-11 cells was performed over 36 h to measure cell density, and a caspase 3/7 live cell fluorescent stain was used to detect apoptotic cells (Figure 1D). The results confirm that volasertib inhibits proliferation and induces apoptosis.

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 in these cells and strongly interfered with mitotic progression (Figure 2). In contrast to untreated control cells, most cells that entered mitosis in the presence of volasertib did not progress to anaphase, but remained in a prolonged prometaphase followed by cell death or mitotic exit without chromosome segregation. A summary statistics of mitotic entry rates (Figure 2D) and individual cell fates (Figure 2E-G) shows 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 extra-terminal (BET) 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 (Figure 3 A-B). IC\textsubscript{50} 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 c-myc, a BRD4 target gene, was down-regulated by volasertib with an EC\textsubscript{50} of 730 nM (Figure 3C), consistent with the potency observed in biochemical assays. In contrast, viability of these cells was inhibited with an EC\textsubscript{50} of 9 nM (Figure 3D), i.e. at 80-fold lower
concentration, consistent with the potency of volasertib as a Plk1 inhibitor. Data for the bona
fide BET family inhibitor JQ1 (Filippakopoulos, et al., 2010) in these four assays are shown
as supplemental data (Supplemental Figure 3).

**Efficacy in mouse models of AML**

Initial studies to test the efficacy of volasertib *in vivo* were using immune-deficient
cube mice subcutaneously injected with MOLM-13 AML cells. Intravenous treatment was
initiated when tumors had reached a volume of approximately 150 mm³. Efficacy was dose-
dependent with T/C values of 20% and 53%, respectively, at doses of 20 or 40 mg/kg once a
week (Figure 4A). All dose levels were well tolerated as neither body weight loss nor adverse
signs were observed (Figure 4B). Of note, 40 mg/kg once weekly had previously been
defined as the MTD.

While 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, 10⁷ MOLM-13 or MV-4-11 cells, stably transfected 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 mg/kg or
40 mg/kg once weekly i.v. was initiated three (MOLM-13 model) or eight (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 pre-specified 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 mg/kg or 40 mg/kg once a
week was 37 days and 48 days, respectively, corresponding to an increase in lifespan by
about 150% and 220% (Figure 4C). Imaging data generated on day 14 (Figure 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 (>61 days, >85% increase) (data not shown).

**Volasertib in combination with established and investigational therapeutic agents**

The anti-metabolite 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 i.v. for two weeks either with vehicle (once weekly), with volasertib given as a single agent (20 mg/kg or 40 mg/kg once weekly), with cytarabine (100 mg/kg i.p. twice a week every third or fourth day) or with volasertib in combination with cytarabine. Combination therapy at both dose levels showed improved efficacy compared to either single agent and was well tolerated (Figure 5A-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 MTD in mice, further improved efficacy (Figure 5A).

The hypomethylating agents decitabine and azacitidine were tested in combination with volasertib in the subcutaneous MV-4-11 xenograft model. Mice were treated i.v. once a week with either vehicle or 10 mg/kg or 20 mg/kg volasertib as a single agent or in combination with 1.25 mg/kg decitabine, administered i.p. 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 i.v. once a week with a combination of 20 mg/kg volasertib (20 mg/kg) and azacitidine (40 mg/kg). Combination therapy showed improved efficacy compared to single-agent treatment (Figure 5C). Of note, even though single-agent azacitidine resulted in a median body weight loss up to 8%, tolerability did not deteriorate further when volasertib was added to this treatment regimen (Figure 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 (Figure 5E-F). Animals were treated for 2 weeks either with 20 mg/kg volasertib i.v. once weekly, with a daily oral dose of quizartinib (5 or 10 mg/kg) or with a combination of these agents. At the end of the treatment period, efficacy of 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 (Renner, et al., 2009; Ikezoe, 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 p-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, the large majority of those 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 RNAi screen as highly essential for promoting proliferation and blocking differentiation (Zuber, et al., 2011). When using a BRD4 inhibitory compound, anti-leukemic effects across genomic AML subtypes were precisely recapitulated 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). While 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 anti-proliferative 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 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, 2014), with mainly hematological 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 low-dose cytarabine (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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Authorship Contributions

Participated in research design: Adolf, Gerlich, Garin-Chesa, Kraut, Moll and Rudolph

Conducted experiments: Albrecht, Blaukopf, Geiselmann, Impagnatiello and Tontsch-Grunt

Performed data analysis: Gerlich, Impagnatiello, Rudolph, Savarese, Sommer, Tontsch-Grunt and Wernitznig

Wrote or contributed to the writing of the manuscript: Adolf, Gerlich, Garin-Chesa, Kraut, Moll, Reschke and Rudolph
References


Footnotes

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Figure legends

**Figure 1** Volasertib arrests AML cells in mitosis, inhibits proliferation and induces apoptosis

(A-B) Exponentially growing MV-4-11 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 representative experiment of two independent studies is shown. (A) Dose-dependent increase in the G2/M peak at 24 h (B) Time-dependent increase in the subG1 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 nocodazole, a microtubule stabilizing agent that arrests cells in mitosis, for 1 or 24 h. An increase in phospho-Ser10 Histone H3 and phospho-Thr199 NPM indicates arrest in mitosis, while 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 h 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.

**Figure 2** Volasertib suppresses entry into prometaphase and leads to prolonged mitosis and mitotic cell death.

(A-G) Single-cell long-term live-cell microscopy of HeLa cells stably expressing a chromatin marker (histone 2B fused to mCherry). Cell fate was determined by automated cell detection, tracking over time and morphology classification by supervised machine learning. Representative image frames of cells with different fates are shown. Cell fate trajectories are color-coded as indicated. (A) An untreated control interphase cell entered mitosis to generate two daughter cells, of which one entered another mitosis at 1528 min. (B) A cell treated with 25 nM volasertib entered a prolonged mitosis (8 - 1960 min) followed by cell death with
compact chromatin morphology (1992 - 3664 min). (C) A cell treated with 25 nM volasertib entered a prolonged mitosis (8 - 1936 min) and then progressed to interphase with a multi-lobed nucleus, resulting from chromosome segregation failure. (D) Fraction of interphase cells entering prometaphase within the first 12 h after addition of increasing concentrations of volasertib. Error bars indicate standard deviation for n = 3 independent biological replicates. (E-G) Mitotic cell fate trajectories at different concentrations (5 and 25 nM) of volasertib as illustrated in panels (A-C) for all cells entering mitosis within the first 12 h of the experiment. Blue trajectories indicate the duration of mitosis for individual cells that proceed to interphase (t = 0 min at prometaphase onset); red trajectories indicate the duration of mitosis for individual cells that die in mitosis (t = 0 min at prometaphase onset). (E) Untreated control cells. More than 90 % of the cells exit mitosis within 30 min after prometaphase onset. (F) Treatment with 5 nM volasertib induces mitotic cell death after prolonged prometaphase in about 30 % of the cells. Most other cells exit mitosis with substantial time delay. (G) Treatment with 50 nM volasertib substantially reduces the number of cells entering mitosis. Those cells entering mitosis either die in mitosis or exit mitosis after substantially prolonged prometaphase. Time-lapse: 4.6 or 8 min. Scale bars: 10 μm.

Figure 3 Volasertib acts as a BRD4 inhibitor in biochemical assays. It inhibits viability of MOLP-8 cells in the low nM range but shows only submicromolar activity on a BRD4 PD biomarker (myc protein levels) in a cellular assay using MOLP-8 cells.

(A-B) Volasertib 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\textsubscript{50} 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\textsubscript{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\textsubscript{50} value is indicated by a vertical line.
Figure 4 Efficacy and tolerability of volasertib in xenograft models of human AML

(A-B) Nude mice bearing established subcutaneous MOLM-13 tumors were treated i.v. with either vehicle (open squares) or with volasertib once a week at 20 mg/kg (black triangles) or 40 mg/kg (black diamonds). Mean tumor volumes with error bars depicting S.D. of 8 animals per treatment group (A) and mean body weight changes in % of initial weight with error bars depicting S.D. (B) are shown. The bar at the top of (A) indicates the treatment period. (C-D) CIEA-NOG mice were injected i.v. with 1x10^7 MOLM-13 AML cells. Starting on day 3 after cell injection, mice were treated once a week i.v. with vehicle or with 20 mg/kg or 40 mg/kg volasertib. (C) The Kaplan-Meier curve shows prolonged survival of animals treated with volasertib (vehicle = dotted line, 20 mg/kg = black line, 40 mg/kg grey line) (D) Bioluminescence imaging on day 14 – left: vehicle control, middle: volasertib 40 mg/kg, right: volasertib 20 mg/kg.

Figure 5 Efficacy and tolerability of volasertib in combination with cytarabine, azacitidine or quizartinib in human s.c. xenograft models of AML

(A-B) AML-6252 tumor bearing Scid-beige mice were treated for 2 weeks either with vehicle once weekly (open squares), with volasertib once a week i.v. at 20 and 40 mg/kg, respectively (black triangles and black bar), with cytarabine twice a week every third or fourth day i.p. at 100 mg/kg (black squares), with 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 8 animals per treatment group with error bars depicting S.D. (A) and body weight in gram with error bars depicting S.D. (B) are shown. (C-D) MV-4-11 tumor bearing nude mice were treated for 3 weeks with either vehicle (open squares), with volasertib once a week i.v. at 20 mg/kg (black triangles), with azacitidine once a week i.v. at 40 mg/kg (black squares) or with a combination of both (black diamonds). Mean tumor volumes of 8 animals per treatment group with error bars depicting S.D. (C) and body weight changes in % of initial weight with
error bars depicting S.D. (D) are shown. The bar at the top of (C) indicates the treatment period. (E-F) MV-4-11 tumor bearing nude mice were treated for 2 cycles either with vehicle (open squares), with volasertib once a week i.v. at 20 mg/kg (black triangles), with a daily dose of quizartinib at 5 mg/kg (black squares) or 10 mg/kg (grey squares) or with a combination of volasertib and quizartinib at 5 and 10 mg/kg (grey or black diamonds). Mean tumor volumes of 8 animals per treatment group with error bars depicting S.D. (E) and body weight changes in % of initial weight with error bars depicting S.D. (F) are shown. The bar at the top of (E) indicates the treatment period.
<table>
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<th>Cell line</th>
<th>Mutations</th>
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<th>Number of experiments</th>
<th>EC_{50} +/- S.D. [nM]</th>
<th>Number of experiments</th>
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<td>170 +/- 51 n = 3</td>
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<td>2D assay</td>
<td>40 +/- 7 n = 4</td>
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Data are expressed as mean EC_{50} values +/- S.D. EC_{50} values in 2D assays were measured after 72 hours; EC_{50} values in 3D assays were measured after 6 days.
Table 2

EC_{50} values determined in 2D assays with leukemic cells derived from bone marrow cells of 1st line AML patients (n = 14) treated *in vitro* with various concentrations of volasertib (0.5 nM – 10 μM).

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<th>Sample</th>
<th>Previous MDS/MPS</th>
<th>Karyotype ISCN</th>
<th>FAB</th>
<th>Cytogenetic classification</th>
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EC\textsubscript{50} values were measured after 96 hours. Results from a single experiment with 14 different samples, each measured in triplicates, is shown in table 2.
Figure 1

A. MV4-11 20/200nM volasertib 24h
Dose-dependent increase of G2/M peak

B. MV4-11 200nM volasertib
Time-dependent increase of apoptosis

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<td>G2/M [%]</td>
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C. Time-course Western Blot

D. Confluence and Intensity over time
Mitosis (followed by interphase)

Mitosis (followed by cell death)

Cells entering prometaphase within 12 h (%)

Volasertib concentration (nM)

Cell fate trajectories

Time (min)

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**Figure 3**

(A) BRD4_BD1

- IC₅₀: 300 nM

(B) BRD4_BD2

- IC₅₀: 770 nM

(C) MOLP-8

- EC₅₀: 9 nM

(D) MYC

- EC₅₀: 730 nM
Figure 4

A) Tumor Volume [mm³]

B) Mean weight Change [%]

C) Percent survival

D) Images of mice treated with different compounds.
Figure 5

A

B

C

D

E

F

Mean tumor volume [mm³]

Mean body weight [g]

Mean tumor volume [mm³]

Mean weight change [%]

Mean tumor volume [mm³]

Mean weight change [%]