Polo-like Kinase Inhibitor Volasertib Exhibits Antitumor Activity and Synergy with Vincristine in Pediatric Malignancies

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Abstract. Background: Polo-like kinase 1 (PLK1) controls the main cell-cycle checkpoints, suggesting utility of its inhibition for cancer treatment, including of highly proliferative pediatric cancer. This preclinical study explored the selective PLK1 inhibitor volasertib (BI 6727) alone and combined with chemotherapy in pediatric malignancies. Materials and Methods: Inhibition of proliferation was explored in vitro using dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assay. Mice bearing human xenografts were treated with weekly intravenous injections of volasertib. Results: Volasertib inhibited proliferation in all 40 cell lines tested, with a mean half-maximal growth inhibitory concentration of 313 nmol/l (range: 4-5000 nmol/l). Volasertib was highly active against RMS-1 alveolar rhabdomyosarcoma xenografts, resulting in 100% tumor regression. Activity was associated with complete and prolonged G2/M arrest and subsequent apoptotic cell death. Volasertib showed synergistic activity with vincristine but antagonistic effects with etoposide. Conclusion: These findings support the further exploration of volasertib for pediatric malignancies, particularly alveolar rhabdomyosarcoma, and its combination with mitotic spindle poison.

Pediatric cancers exhibit a high growth potential, often with an elevated mitotic index. Specific inhibitors targeting proteins involved in mitosis and microtubule organization are required for further therapeutics. Polo-like kinase 1 (PLK1) is a serine/threonine kinase which plays a central role in the cell cycle and is essential for mitosis and genomic stability (1). It contributes to the regulation of the G2/M checkpoint and mitotic entry (2, 3). During mitosis, PLK1 drives microtubular nucleation and its inhibition causes G2/M arrest (4). PLK1 is overexpressed in many types of cancer and has been reported as a prognostic factor in adult cancer (5-8). PLK1 is reportedly expressed at a high level in hepatoblastoma (9) and neuroblastoma, where high PLK1 expression correlated with poor prognosis (10).

Given its fundamental role in the cell cycle, inhibition of PLK1 has been suggested as a new therapeutic approach in cancer treatment. Antitumor effect in vivo has been observed in several adult cancer types (11-13). PLK1 inhibition using siRNA (14) or the specific inhibitor BI 2536 (10, 15) have shown antitumor effects in pediatric rhabdomyosarcoma and neuroblastoma. Volasertib (BI 6727) is a selective PLK1 inhibitor with superior pharmacokinetic characteristics further developed for clinical use. Volasertib induces cytotoxicity, G2/M arrest and apoptosis in vitro and antitumor effect in vivo in human colonic carcinoma and non-small cell lung cancer models (12). Volasertib as a single agent exhibited potential activity in a large panel of pediatric tumors with no histotype selectivity (16). The phase I trial of volasertib in pediatric malignancies is ongoing (NCT01971476).
This preclinical study evaluated volasertib in vitro and in vivo against various pediatric tumor types. Given its mechanism of action, volasertib would likely be used in combination. A synergistic induction of apoptosis of BI 2536 with vincristine was recently described in neuroblastoma cells (15). We, therefore, explored the combination of volasertib with the mitotic spindle poison vincristine and topoisomerase II inhibitor etoposide in order to evaluate the prospect of clinical development in children with advanced malignancies.

**Materials and Methods**

**Drugs.** Volasertib (BI 6727; kindly supplied by Dr Dorothea Rudolph, Boehringer Ingelheim RCV GmbH & Co KG, Vienna, Austria) was stored as powder protected from light at room temperature. For in vitro experiments, volasertib was dissolved in a stock solution of dimethyl sulfoxide (DMSO) and Roswell Park Memorial Institute (RPMI) 1640 medium to a concentration of 1 mg/ml. For in vivo experiments, powder was resuspended in 0.9% NaCl with 0.1 N HCl to a final concentration of 3 and 4 mg/ml. Vincristine and etoposide were purchased (Teva, Petach Tikva, Israel).

**Cell lines.** The Innovative Therapies for Children with Cancer (ITCC) cell line panel (http://www.itcc-consortium.org/targeted-drug-evaluation.php) comprised 40 cell lines of six pediatric tumor types: five medulloblastoma (DAOY, D283 MED, D341 MED, MED-MB-8A, UW228.2), and seven each of Ewing sarcoma [A673, EW 7, ORS, POE, RD-ES, SIM(EW27), STA-ET-1], acute lymphoblastic leukemia (ALL) (HbP-ALL, KOPN-8, MOLT-16, REH, SEM, SUP-B15, UOC-B6), neuroblastoma (IMR-32, NGP, SH-SY5Y, SJ-NB-6, SJ-NB-8, SK-N-AS, SK-N-BE), osteosarcoma (HOS, IOR-OS-9, IOR-OS-14, IOR-OS-18, MG-63, SAOS-2, U-2OS), and rhabdomyosarcoma (A204, RD, RH-18, RH-30, RH-41, RMS-1, RMS-YM) (17). In addition, IGR-N91 neuroblastoma, SF188 high-grade glioma, and RES186 pilocytic astrocytoma cell lines were used. SUP-B15 was maintained in McCoy's 5A medium containing 20% fetal calf serum (FCS); SF188 and RES1 in Dulbecco's minimum essential medium Glutamax containing 10% FCS; all other cell lines were cultured in RPMI-1640 containing 10% FCS at 37°C and 5% CO₂ (all Life Technologies, Saint Aubin, France). Cells were tested to be free of mycoplasma.

**Dimethylthiazol carboxymethoxyphenyl sulphophenyl tetrazolium (MTS) cell-proliferation assay.** Cells were seeded at 5,000 or 25,000 per well in 96-well plates. After 24 hours, volasertib was added to final concentrations of 5, 10, 50, 100, 500, 1000, 5000, 10000, 50000 and 100000 nmol/l and cells further incubated for 72 h. Cell viability was determined using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS assay; Promega, Mannheim, Germany). Each concentration and time point was tested in quadruplet and all experiments in triplicates. Means and standard deviations were calculated from quadruple optical density (OD) measurements. Viability of treated cells was compared to that of untreated cells and to that prior to drug exposure, and drug concentrations which inhibited cell growth by 50% (GI₅₀) and reduced viability by 50% (LC₅₀) were calculated as described previously (18).

Combination studies had been performed using vincristine or etoposide with volasertib at various concentration simultaneously or not. Combination effects were estimated by combination indices calculated by the Chou and Talalay method using CalcuSyn software (Biosoft, Cambridge, UK) (19). The combination index (CI) values for synergism are 0 to <0.9, for additivity between >0.9 and 1.1, for antagonism >1.1 to infinity.

**Western blot analysis.** Total lysates of adherent and supernatant cells were separated electrophoretically and immunodetected as previously described (17) using monoclonal mouse antibody to human β-actin (AC-15, diluted 1:1000; Sigma-Aldrich, St Quentin-Fallavier, France), and rabbit polyclonal antibodies to human Poly [ADP-ribose] polymerase 1 (PARP1) (Ab-2, 1:600; Calbiochem, Darmstadt, Germany) and caspase 3 (1:600, Cell Signaling Technology, Ozyme, St Quentin-en-Yvelines, France).

**Cell-cycle analysis by flow cytometry.** Cells were treated in exponential growth phase with volasertib at 10, 30 and 100 nM, harvested at 2, 6, 18, 24, 48 and 72 h, stained with propidium iodide buffer and DNA content was measured using a FACS calibur (BD Biosciences, Erembodegem, Belgium) (20).

**In vitro imaging.** Cells were seeded in a 35 mm Ibidi petri μ-dish (Biovalley, Marne la Vallée, France) and incubated after 24 hours with volasertib at 100 nM. Cell cytoplasm was stained with 1 μmol carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR, USA) for 1 minute and cell DNA was stained with 25 nM SYTO® 59 (Molecular Probes) for 1 minute and cell DNA was stained with 25 nM SYTO® 59 (Molecular Probes). Video was acquired, starting immediately after volasertib addition, with a FluoView FV10i confocal microscope (Olympus, Hamburg, Germany) at 492 (CFSE-green) and 622 nm (SYTO® 59 - red) over 10 hours; one image per hour was captured.

**Gene-expression profiling.** RNA expression of HG-U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, CA, USA) were normalized with the MAS5.0 algorithm within the GCOS program of Affymetrix (Affymetrix, Inc.). Samples of pediatric malignancies and cell lines were compared to those of adult cancer and normal tissues within the R2 database.

**Experimental in vivo design.** Experiments were carried out under the conditions established by the European Community (Directive 86/609/CCE). Antitumor activity was evaluated against advanced-stage subcutaneous RMS-1 rhabdomyosarcoma or SJ-NB-6 neuroblastoma xenograft tumors in female SPF-Swiss athymic mice as described previously (21). Volasertib was administered intravenously at 30 and 40 mg/kg once weekly for 4 or 5 weeks; controls received 0.9% NaCl and 0.1 N HCl vehicle. Statistical significance was determined using the two-tailed non-parametric Mann–Whitney or Kruskal–Wallis test and Prism® software version 3.00 (GraphPad Software, Inc., La Jolla, CA, USA).

**Results**

**PLK1 mRNA is expressed in pediatric primary malignancies and cell lines.** Gene-expression profiling showed PLK1 expression in all pediatric primary tumor samples and cell lines tested (Figure 1). Compared to normal tissues, higher levels of PLK1 expression were observed in Ewing sarcomas, medulloblastomas, neuroblastomas and rhabdomyosarcomas, while expression of acute lymphoblastic leukemias and...
osteosarcomas was within the range of normal tissues. All pediatric tumor cell lines were characterized by high PLK1 expression.

Volasertib exhibits significant cytotoxicity against pediatric cell lines in vitro. We evaluated volasertib against a large panel of different pediatric tumor cell lines and cell viability was reduced in all 40 cell lines tested (Figure 2; Table I). The calculated mean GI50s per cell line ranged from 3.56 nmol/l to 5045 nmol/l. The RMS-1 and RH-41 rhabdomyosarcoma, POE Ewing sarcoma, Med-Meb-8A medulloblastoma, IMR-32 neuroblastoma, and Hpb-ALL leukemia were most sensitive to volasertib, with GI50s <10 nmol/l. Thirty-seven out of 40 cell lines tested exhibited GI50 values <1210 nmol/l (6/7 Ewing sarcoma, 7/7 leukemia, 5/5 medulloblastoma, 7/7 neuroblastoma, 5/7 osteosarcoma, and 7/7 rhabdomyosarcoma) which corresponds to the maximum plasma concentration of volasertib at the recommended dose in the adult phase I study (300 mg) (22).

The LC50s determined for volasertib on the cell line panel ranged from 7.9 nmol/l to 24200 nmol/l with overall a mean LC50 of 2750 nmol/l and a median LC50 of 47.5 nmol/l. RMS-1 rhabdomyosarcoma, Med-Meb-8A medulloblastoma, and Hpb-ALL leukemia were most sensitive to volasertib with LC50s <10 nmol/l. LC50s below 1240 nmol/l were observed for 27 out of 40 cell lines (5/7 Ewing sarcoma, 7/7 leukemia, 4/5 medulloblastoma, 7/7 neuroblastoma, 3/7 rhabdomyosarcoma, and 1/7 osteosarcomas).

Thus, volasertib exhibits significant antiproliferative activity at concentrations in the nanomolar range in various pediatric cell lines independently of the tumor type.

Volasertib cytotoxicity is mediated by arrest in G2/M and prophase followed by apoptotic cell death. To explore the mechanism by which volasertib exhibits its activity, we treated RMS-1 and RD rhabdomyosarcoma, SJ-NB-6, IMR-32 and IGR-N91 neuroblastoma, SF188 and RES186 glioma with 10 and 100 nM volasertib and first performed cell-cycle analysis using flow cytometry. In the most sensitive RMS-1 cells (Figure 3A), volasertib resulted in dose-dependent G2/M arrest starting at 6 h and being complete at 24 h. The effects were less pronounced at 10 nM and cell-cycle arrest was overcome at 72 hours. However, at 100 nM volasertib arrested 72% cells in G2/M which remained persistent up to 72 h. Of note, 14% of cells were found in >4N at 24 h.

In contrast in neuroblastoma cells, G2/M arrest was incomplete for IMR-32 (Figure 3B) and SJ-NB-6, and the cell-cycle distribution was returned to baseline at 72 hours. However, at 100 nM volasertib arrested 72% cells in G2/M which remained persistent up to 72 h. Of note, 14% of cells were found in >4N at 24 h.

In the least sensitive RES186 low-grade glioma (GI50 >100 nM) (Figure 3C), we observed an accumulation of tetraploid (4N) cells at 24 h to 100 nM volasertib. At 72 h, it was impossible to distinguish further the different cell populations, however, a few >4N cells remained.
Using video imaging on unsynchronized cells in order to follow cell mitosis under treatment, we found that volasertib-treated SF188 cells stopped in prophase and detached from plates whereas control cells proceeded through mitosis (Figure 3D). Further western blot analysis for key regulators of apoptotic cell death showed PARP1 cleavage in both sensitive SF188 and RMS-1 cell lines at 24 and 48 hours, while caspase 3 cleavage was detected in SF188 cells at 24 hours and less in RMS-1 (Figure 3E).

Thus, complete and persistent G2/M arrest in prophase followed by apoptotic cell death was associated with cytotoxic activity of volasertib. Overcoming the G2/M checkpoint and >4N cells may represent an escape mechanism from PLK1-inhibiting treatment.

**Antitumor activity in vivo of volasertib against RMS-1 and SJ-NB-6 xenografts.** We then evaluated volasertib in vivo against RMS-1 rhabdomyosarcoma and SJ-NB-6 neuroblastoma that exhibited GI50s of 5 and 20 nM respectively (Table II, Figure 4). Animals bearing RMS-1 tumors of 74 to 266 mm3 were randomly assigned to treatment groups 19 days after xenograft transplantation. Volasertib administrations at 30 and 40 mg/kg once weekly were chosen based on the tolerance and activity in prior experiments performed at Boehringer Ingelheim (12). Animals of both treatment groups gained body weight and no toxicity was noted. RMS-1 xenografts were highly sensitive to volasertib in a dose-dependent fashion. Volasertib at 30 mg/kg resulted in one partial and two complete responses out of nine tumors and significant median tumor growth delay of 36 days (p<0.001; Kruskal–Wallis), 40 mg/kg resulted in 100% tumor regression with two partial and seven complete responses out of nine tumors and a median tumor growth delay of 39 days (p<0.001). In contrast, SJ-NB-6 neuroblastoma tumors of 73 to 345 mm3 treated from 21 days
after transplantation showed no tumor regression or growth delay to volasertib.

Thus, volasertib exhibited a significant antitumor activity against rhabdomyosarcoma xenografts.

**Cytotoxicity of volasertib in combination with cytotoxic agents.** In order to determine the potential therapeutic use of volasertib in clinical pediatric protocols, we performed combination studies on RMS-1 cells with cytotoxic agents, an inhibitor of topoisomerase II and a vinca alkaloid, both also interfering in the cell cycle. The concentration for inhibition of 50% of proliferation was determined for each drug alone as 12 nM, 0.5 nM and 500 nM respectively for volasertib, vincristine and etoposide. According to the Chou and Talalay method, RMS-1 cells were treated with volasertib (at 6, 9, 13.5, 20.25 nM) and vincristine (at 0.26, 0.4, 0.5 nM) or etoposide (at 10, 50, 500 nM) which are fractions or multiples of the GI50 concentration for each drug.

Vincristine and volasertib administered simultaneously showed synergistic cytotoxicity, with a CI of <0.9 at fractions...
Figure 3. Continued.

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of ca. 0.7 and 1.0 of the GI_{50}s (Figure 5A and B). Cell viability was significantly reduced with the combination at 9 nM (p<0.0001) and 13.5 nM of volasertib (p<0.05). Conversely, the combination of volasertib and etoposide was antagonistic, with CIs of >1 (Figure 5C and 5D). Inhibition of cell viability by volasertib alone was always higher than the combinations at 13.5 nM (p<0.0001) and 20.25 nM of volasertib (p<0.0001).

Since cell sensitivity to these agents may depend on the cell cycle, we further performed combination cell viability tests with different scheduling of both agents. A delay of 24 h
between drug addition in medium was chosen based on our observation that cell-cycle arrest in G2/M phase of RMS-1 cells was effective after 24 h of volasertib exposure. For scheduling combination test with volasertib and vincristine, lower concentrations of 6 and 0.26 nM, respectively, were chosen based on the high cytotoxicity of the simultaneous combination at GI50s of both agents. Adding vincristine before or after volasertib (Figure 4E) resulted in decreased cytotoxicity as compared to simultaneous administration of both agents. In contrast, the combination of etoposide added 24 hours after volasertib was more efficient in cell cytotoxicity than the reverse or simultaneous combination of both agents (Figure 4F). Nevertheless, all etoposide combinations significantly abrogated the single-agent activity of volasertib alone.

Thus, synergistic cytotoxicity of volasertib and vincristine was observed at submaximal concentrations of both agents and when administered simultaneously, whereas the combination with etoposide was strongly antagonistic and reduced the effects of volasertib.

**Discussion**

This study shows the preclinical activity of the newly developed PLK1 inhibitor volasertib in a wide panel of pediatric malignancies independently of tumor histology. We confirmed that complete and prolonged G2/M arrest in prophase and subsequent induction of apoptotic cell death is also the main mechanism of action for the cytotoxicity of this class of agents towards pediatric cell lines. Moreover, we demonstrated that synergistic activity is achieved with spindle cell mitotic poisons when combined simultaneously with PLK1 inhibition.

In the large R2 database, *PLK1* mRNA expression in pediatric tumor samples was comparable to that in tumors from adult and higher expression levels were observed in pediatric
tumor cell lines compared to primary tumor samples. Although PLK1 expression has been associated with poor outcome in some diseases including neuroblastoma (5, 6, 23), expression levels have not been strictly linked to the inhibition of PLK1 activity. In this regard, ALL and osteosarcoma cell lines were found to have the lowest PLK1 expression levels compared to the other pediatric tumor types which were not reflected by lower sensitivity in vitro in the ALL samples. Overall within the 40 cell lines tested, GI50s and LC50s ranged over one log-scale. The phase I trial in adult patients found a maximum plasma concentration of 1210 nM at the recommended phase II dose of 300 mg volasertib given as an intravenous infusion (24). Therefore, we considered most of our cell lines as being sensitive to volasertib. Nevertheless, those considered resistant were mostly found among the osteosarcoma cell lines. These results were consistent with in vitro activity of volasertib in previous pediatric studies (16, 25).

In our mechanistic studies, we showed that complete, prolonged G2/M arrest in prophase followed by apoptotic cell death (sub-G1 cells) is the main mechanism of cytotoxicity to volasertib. Our findings are consistent with the data in adult cell lines with volasertib (12) and the

![Figure 5. Volasertib combination with vincristine and etoposide. The percentage cell viability of RMS-1 cells treated with volasertib in combination with (A) vincristine (VCR) or (C) etoposide (VP16) at 72 h is shown. Combination index (CI) calculated by the Chou and Talalay method shows synergism (<0.9) for volasertib and vincristine at concentrations around the 50% growth-inhibitory concentration (B) but antagonism (>1.1) with etoposide (D). Scheduled combination cell-viability assays show that addition of vincristine 24 h before or after volasertib reduces the combination effects as compared to simultaneous administration (E). Adding etoposide 24 h after volasertib leads to more efficient cytotoxicity than the reverse scheduling but all combinations result in reduced cytotoxicity as compared to volasertib alone (F). *p<0.05; **p<0.001; ***p<0.0001.](image-url)
preceding PLK1 inhibitor BI 2536 (25, 26), suggesting that PLK1 depletion or inhibition leads to a poor formation of bipolar spindle (4) and apoptosis (15, 27). We found a dose-dependent effect, and overcoming G2/M arrest as observed in neuroblastoma cells seemed to be one mechanism of escape. A second mechanism of escape appeared to be an accumulation of tetraploid and >4N cells. This was noted only in a limited number of cells in two different sensitive cell lines but reflected the main cell DNA distribution pattern in the resistant RES186 low-grade glioma cell line. Cells began a new cycle without ending mitosis. Hyperploidy had been described as being induced by mitotic spindle poison as an escape mechanism (28).

Since some cells are able to overcome this prometaphase arrest, it appears intriguing to add other spindle assembly inhibitors. We found that treatment of volasertib combined with the vinca alkaloid vincristine had a strong synergistic effect on cytotoxicity. As shown for the RMS-1 rhabdomyosarcoma cell line, synergy was observed on one hand at 'sub-maximal' concentrations of both agents, i.e. doses around the GI50, and on the other hand when drugs were given simultaneously rather than successively with time delay. Our data confirm and extend those reported by the group of Fulda in neuroblastoma cell lines with the previous PLK1 inhibitor BI 2536 (15) and in rhabdomyosarcoma cells with volasertib (27). Combination with vinca alkaloids induced apoptosis in vitro and suppressed tumor growth in vivo. The main underlying mechanisms of the synergism of the combination was found to be the mitotic arrest and activation of both caspase-dependent and caspase-independent apoptosis through down-regulation of induced myeloid leukemia cell 1 (MCL1), B-cell lymphoma 2 (BCL2) and B-cell lymphoma-extra large (BCL-XL) inactivation, BCL2-associated X protein (BAX)/BCL2 homologous antagonist/killer (BAK) activation and reactive oxygen species production (27). Given the main activity of volasertib during prophase and the mechanisms of vinca alkaloid as mitotic spindle poison, we have shown the importance of simultaneous administration to maximize the combined effects.

In contrast, the combination of volasertib with etoposide was highly antagonistic and resulted in abrogation of cytotoxic effects of volasertib. Etoposide inhibits topoisomerase II and induces G2/M arrest before mitotic entry (29). As the main mechanism of cytotoxicity of volasertib is a pro-metaphase arrest, etoposide-mediated cell-cycle arrest before mitotic entry could by-pass the action of volasertib on mitosis and abrogate or delay its cytotoxicity. This hypothesis is supported by our findings that administration in vitro of etoposide after volasertib resulted in less antagonistic effects on volasertib cytotoxicity than the reverse or simultaneous administration. Drug scheduling might also be important for the combination with doxorubicin and paclitaxel, which failed to show synergistic effects in rhabdomyosarcoma cells (27). Doxorubicin synchronizes cells in S phase, whereas paclitaxel stabilizes the mitotic spindle in metaphase, both events that occur after the effects in prophase mediated by volasertib. Our results highlight the importance of exploring various scheduling for drug combinations with anti-mitotics and mechanistic studies before using new association in clinical study.

Finally, in vivo experiments showed high sensitivity of RMS-1 alveolar rhabdomyosarcoma, with 100% tumor responses to 40 mg/kg volasertib, whereas SJ-NB-6 neuroblastoma failed to respond to volasertib despite similar in vitro sensitivity. Recently a novel, distinct mode of action for PLK1 was discovered (30). PLK1 is a kinase involved in the phosphorylation and activation of paired box 3-forkhead box O1 (PAX3–FOXO1), the hallmark of alveolar rhabdomyosarcoma, and required for expression of several direct transcriptional targets (31). This additional role of PLK1 in PAX3–FOXO1 fusion-positive rhabdomyosarcoma may underly the sensitivity observed in RMS-1 cells and our findings in RMS-1 tumors further support this new hypothesis.

PLK1 inhibition using volasertib represents a promising novel therapeutic for pediatric cancer. Its cytotoxic activity against a wide range of tumor types mediated by prometaphase arrest and induction of apoptosis, synergistic activity with other mitotic spindle poisons, and the particular sensitivity in vivo of PAX3–FOXO1 fusion-positive rhabdomyosarcoma motivate further clinical evaluation of volasertib in combination with other agents. Our results further highlight the major importance of the current preclinical combination study for evaluation of new therapeutics.

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References


