Thermal proteome profiling identifies PIP4K2A and ZADH2 as off-targets of Polo-like kinase 1 inhibitor volasertib

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Abstract
Polo-like kinase 1 (PLK1) is an important cell cycle kinase and an attractive target for anticancer treatments. An ATP-competitive small molecular PLK1 inhibitor, volasertib, has reached phase III in clinical trials in patients with refractory acute myeloid leukemia as a combination treatment with cytarabine. However, severe side effects limited its use. The origin of the side effects is unclear and might be due to insufficient specificity of the drug. Thus, identifying potential off-targets to volasertib is important for future clinical trials and for the development of more specific drugs. In this study, we used thermal proteome profiling (TPP) to identify proteome-wide targets of volasertib. Apart from PLK1 and proteins regulated by PLK1, we identified about 200 potential volasertib off-targets. Comparison of this result with the mass-spectrometry analysis of volasertib-treated cells showed that phosphatidylinositol phosphate and prostaglandin metabolism pathways are affected by volasertib. We confirmed that PIP4K2A and ZADH2—marker proteins for these pathways—are, indeed, stabilized by volasertib. PIP4K2A, however, was not affected by another PLK1 inhibitor onvansertib, suggesting that PIP4K2A is a true off-target of volasertib. Inhibition of these proteins is known to impact both the immune response and fatty acid metabolism and could explain some of the side effects seen in volasertib-treated patients.

Keywords
acute leukemia, CETSA, PIP4K2A, Polo-like kinase 1, thermal proteome profiling, volasertib, ZADH2
**1 | INTRODUCTION**

Pediatric acute leukemia account for approximately 40% of malignancies in children. Considerable progress has been made in the leukemia treatment that allows to rescue about 80% of all patients. As a result of the therapy, the survivors often live with life-long debilitating conditions due to non-specific effects of the drugs on healthy tissues. Therefore, a search for new selective drugs for pediatric leukemia is constantly ongoing.

Polo-like kinase 1 (PLK1) has been extensively explored as a target for anticancer drug development. It belongs to the PLK family, a group of serine-threonine kinases, involved in several stages of cell cycle regulation. In humans, it consists of five family members—PLK1, PLK2, PLK3, PLK4, and PLK5—each with distinct functions in mitosis regulation. PLK1 is the well-studied member due to its essential role in cell division. It regulates mitotic entry, G2M checkpoint progression, spindle assembly, DNA replication, and cytokinesis among other functions. PLK1 expression is abnormally high in cancer cells of different origins, and it was found to be essential for cancer cell survival. This prompted the development of drugs targeting PLK1 to be used as anticancer agents. Volasertib is an ATP-competitive PLK1 inhibitor that has reached as far as phase III in clinical trials of refractory acute myeloid leukemia (AML) as a combination treatment with cytarabine. The study was, however, terminated due to a number of serious hematological and immunological side effects in the patients who were administered volasertib in combination with cytarabine. Despite that, clinical investigations of volasertib have continued and it was even granted rare pediatric disease and orphan drug designations in 2020. This means that volasertib will be fast-tracked into clinical trials of refractory acute myeloid leukemia (AML) as a combination treatment with cytarabine. The study was, however, terminated due to a number of serious hematological and immunological side effects in the patients who were administered volasertib in combination with cytarabine. Despite that, clinical investigations of volasertib have continued and it was even granted rare pediatric disease and orphan drug designations in 2020. This means that volasertib will be fast-tracked into clinical use in diseases with no effective treatments or in patients that do not respond to available drugs. Thus, the side effects of volasertib need to be addressed.

Upon discovery, it was determined that volasertib, apart from PLK1, is active against PLK2 and PLK3. It was also in vitro screened against a panel of kinases and was not found to affect any in this assay. Thus, there is currently no mechanistic explanation for the side effects observed in patients. Cellular thermal shift assay (CETSA) and thermal proteome profiling (TPP) have recently emerged as powerful tools to detect targets of small molecules on a whole proteome level. Upon binding to a target, the small molecule affects the thermal stability and the aggregation temperature ($T_{agg}$) of the target protein, and this change in the temperature can be detected by western blotting or mass spectrometry. In this study, we investigated the target landscape of volasertib in pediatric leukemia using TPP and quantitative mass spectrometry proteomics (qMS). Our data show that besides cell cycle regulation, volasertib also affects other pathways, such as fatty acid metabolism and the immune response. We confirmed that volasertib stabilizes at least two proteins, namely, zinc-binding alcohol dehydrogenase domain-containing protein 2 (ZADH2) and phosphatidylinositol-5-phosphate 4-kinase type 2 alpha (PIP4K2A). Importantly, the volasertib-induced inhibition of these proteins could potentially explain some of the side effects seen in volasertib-treated leukemia patients.

**2 | MATERIALS AND METHODS**

**2.1 | Cell lines and treatments**

Cell lines obtained from ATCC. Culture media RPMI-1640 was supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/mL of streptomycin, and 100 U/mL of penicillin (all from Nordic Biolabs, Stockholm, Sweden). Cell lines 697, CCRF-CEM, Jurkat, HL60, and THP1 were cultured in HyClone RPMI-1640. SupB15 and MHH-CALL2—in RPMI-1640 Gibco with HEPES (Thermo Scientific, Life Technologies Europe, BV). Volasertib and onvansertib were purchased from SMS-gruppen (Rungsted, Denmark).

**2.2 | CETSA in cells**

Cells were split 24 hours before the experiment in a density 200 000 cells/mL. Volasertib, onvansertib, and DMSO were added to a final concentration of 10 or 20 μM. Cells were incubated for 80 minutes at 37°C and 5% CO₂.

After that, the cells were spun down and resuspended in PBS, supplemented with protease cOmplete and phosphatase PhosSTOP inhibitors (Roche), then aliquoted to 0.2 mL tubes. Further, samples were heated to 37-57°C in a thermal cycler Life Touch (Bioer Technology, Hangzhou, PR of China) or 48°C-63°C in the CFX C1000™ Real-Time System Touch™ Thermal Cycler (Bio-Rad) for 3 minutes, followed by 3 minutes cooling at RT and immediate snap-freezing in liquid nitrogen. The cells were then lysed by repeated freeze-thawing and centrifuged at 15 000g for 45 minutes at 4°C. The supernatants were transferred to new tubes.

**2.3 | Isothermal dose-response (ITDR) in cells**

Cells were split 24 hours before the experiment. Volasertib was added to final concentrations of 20 000-0.0128 nM. Cells were incubated for 80 minutes at 37°C and 5% CO₂. After centrifugation, the pellet was washed in PBS and cells were resuspended in PBS with protease inhibitor cOmplete and phosphatase PhosSTOP inhibitors (Roche) and aliquoted to 0.2 mL tubes. All tubes were heated up to 46°C (for PLK1) in a thermal cycler Life Touch (Bioer Technology, Hangzhou,
PR of China) or to 57°C (PIP4K2A) or 60°C (ZADH2) in the CFX C1000™ Real-Time System Touch™ Thermal Cycler (Bio-Rad) for 3 minutes, followed by 3 minutes cooling at RT and immediate snap-freezing in liquid nitrogen. The cells were lysed by 3× freeze-thawing and centrifuged at 15,000g for 45 minutes at 4°C. The supernatant was transferred to new tubes.

2.4 CETSA and ITDR in lysate

Cells were split 24 hours before the harvest. About 10^7 cells per sample were collected, washed in PBS, and resuspended in 100 µL of PBS with protease cOmplete and phosphatase PhosSTOP inhibitors (Roche) and aliquoted to 0.2 mL tubes, then freeze-thawed three times. Volasertib was added to the lysates in indicated concentrations for 10 minutes RT and then the samples were heated for 3 minutes in thermal cycler either to multiple temperatures (for CETSA) or to one temperature (for ITDR). After that, cell debris was separated by centrifugation at 15,000g for 45 minutes at 4°C.

2.5 Western blotting

Western blotting was performed as described previously.12 The following primary antibodies were used: β-actin (#A5441, Sigma-Aldrich), GAPDH (#ab8245, Abcam), PLK1 (T210) (#4513), PIP4K2A (D83C1 Rabbit mAb #5527), all from Cell Signaling Technology, ZADH2 (#PA5-54418) from Thermo Fisher Scientific. Secondary antibodies were purchased from LI-COR IRDye: goat anti-mouse 800CW (926-32210) and goat anti-rabbit 800CW (926-32211) (Lincoln, Nebraska, USA). Quantification was performed using Image J and normalized to β-actin or GAPDH.

2.6 qMS Protein extraction, digestion, and labeling

The cell pellets from triplicate experiments were dissolved in 500 µL of lysis buffer (4% SDS, 50 mM HEPES pH 7.6, 1 mM DTT), heated to 95°C, and sonicated. The total protein amount was estimated (Bio-Rad DC). Protein (200 µg) digestion (LysC and trypsin, sequencing grade modified, Pierce) was performed using SP3-protocol.13 Seventy micrograms of peptides from each sample was labeled with isobaric TMTpro™-tags (Thermo Fisher Scientific). Before labeling, samples were pH adjusted using TEAB pH 8.5. Labeled peptide samples were pooled and cleaned by solid-phase extraction (SPE strata-X-C, Phenomenex) and dried in a Savant SpeedVac™ vacuum centrifuge (Thermo Fisher Scientific). Five hundred micrograms of pooled peptides was prefractonated using high-resolution isoelectric focusing (HiRIEF) as previously described14 using the peptide IEF-IPG (isoelectric focusing by immobilized pH gradient) in the pH range as previously described with minor modifications.15 All reagents and media were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. Jurkat cells were cultured to a density of 1.9 x 10^6 cells/mL and 97% cell viability (NucleoCounter® NC-200™, ChemoMetec). Cells were distributed into T25 flasks to a density of 12 x 10^6 cells/mL in 10 mL of medium. They were incubated with 20 µM volasertib or DMSO at a final DMSO concentration of 0.2% for 1.5 hours at 37°C and 5% CO2. Cells were pelleted at 400 g and RT for 3 minutes and washed 2x with Hank’s balanced salt solution (Thermo Fisher Scientific). Cells were resuspended to a density of 100 x 10^6 cells/mL and distributed as 100 µL of aliquots into 0.2-mL PCR tubes. One of each of the compounds and DMSO-containing tubes was heated in parallel for 3 minutes to the respective temperatures (37°C, 41°C, 44°C, 47°C, 50°C, 53°C, 56°C, 59°C, 63°C, 67°C), followed by a 3 minutes incubation at RT. Afterward, cells were flash-frozen in liquid nitrogen. Cells were thawed at 25°C and lysed by this freeze-thawing cycle repeated for another two times. Cell debris and precipitated proteins were removed by centrifugation at 21,000g and 4°C for 40 minutes. Supernatants were transferred to new tubes and protein concentrations were determined (DC protein assay, Bio-Rad, Hercules, CA). Equal volumes of each condition that correspond to 140 µg protein in the 37°C sample were transferred to new tubes and subjected to the following digestion. First, the samples were diluted to contain 50 mM TEAB, 0.1% SDS, and 5mM TCEP. Reduction was performed at 65°C for 30 minutes. The samples were then cooled down to RT and alkylated with 15 mM of chloroacetamide for 30 minutes. The proteins were digested overnight with 1-40 Lys-C (Wako Chemicals GmbH, Neuss, Germany) to protein-ratio 1:25 enzyme to protein (Waltham, MA) at a 1-25 enzyme to protein ratio. The digested peptides were labeled by 10-plex TMT-tags (TMT10, Thermo Fisher Scientific, Waltham, MA, USA) using 0.6 mg of the respective label for each sample. Of note, the labeling efficiency was determined by LC-MS/MS before pooling of the samples. For the sample clean-up step, a solid phase extraction (SPE strata-X-C, Phenomenex, Torrance, CA, USA) was performed and purified samples were dried in a vacuum centrifuge. An aliquot of 10 µg was suspended in LC mobile phase A (3% acetonitrile in 0.1% formic acid) and 1 µg

2.7 Thermal proteome profiling (TPP)

Thermal proteome profiling was performed over a temperature range as previously described with minor modifications.15
was injected on the LC-MS/MS system. Labeled peptide extracts were combined to a single sample per experiment (volasertib, DMSO) and pre-fractionated by means of high pH reversed-phase chromatography (XBridge BEH C18 (2.1 mm × 250 mm) 300A, 3.5 μm, Waters, Milford, MA, USA) using a 63 minutes gradient from 3% to 80% acetonitrile in 20 mM ammonia. Collected fractions were pooled into 14 samples, dried using a Savant SpeedVac™ vacuum centrifuge (Thermo Fisher Scientific) and subjected to an LC-MS/MS measurement.

### 2.8 LC-MS/MS runs

Online LC-MS was performed as previously described using a Dionex UltiMate™ 3000 RSLCnano System coupled with a Q-Exactive-HF mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Each of the samples was dissolved in 20 μl of solvent A and 10 μl were injected. Samples were trapped on a C18 guard-desalting column (Acclaim PepMap 100, 75 μm × 2 cm, nanoViper, C18, 5 μm, 100 Å, Thermo Fisher Scientific, Waltham, MA, USA), and separated on a 50 cm long C18 column (Easy spray PepMap RSLC, C18, 2 μm, 100 Å, 75 μm × 50 cm, Thermo Fisher Scientific). The nanocapillary solvent A was 95% water, 5% DMSO, 0.1% FA; and solvent B was 5% water, 5% DMSO, 95% acetonitrile, 0.1% FA. At a constant flow of 0.25 μL/min, a curved gradient from 3% to 8% B (in 2 minutes, curve of 4) and from 8% to 45% B (in 148 minutes, curve of 5) was used followed by a steep increase to 99% B in 2 minutes. FTMS master scans were performed in a mass range of 3000-1500 m/z applying a resolution of 60 000 (mass range 300-1500 m/z), followed by data-dependent MS/MS (35 000 resolution) on the top five ions using higher energy collision dissociation at 30% normalized collision energy. Precursors were isolated with a 2 m/z window and 0.5 m/z isolation offset. Automatic gain control targets were 1e6 for MS1 and 1e5 for MS2. Maximum injection times were 100 ms for MS1 and 100 ms for MS2. The entire duty cycle lasted ~2.5 seconds. Dynamic exclusion was used with 30 seconds duration. Precursors with unassigned charge state or charge state 1 were excluded. An underfill ratio of 1% was used.

### 2.9 TPP MS data processing

Analyses of the acquired MS TPP data for the identification of the drug targets were performed as previously described. For processing, quantification and normalization IsobarQuant (1.1.0) package was used. Peptide and protein identification were performed using Mascot 2.4 (Matrix Science) search engine. The searches were performed against a human database from UniProt including known contaminants and the reversed protein sequences. Search parameters included trypsin as a digestion enzyme, three allowed missed cleavages, a precursor tolerance of 10 ppm at 0.02 Da for MS/MS tolerance. As fixed modifications, carbamidomethylation on cysteines and TMT10plex on lysine were included. Variable modifications were acetylation on protein N termini, oxidation of methionine, and TMT10plex on peptide N-termini. The determination of melting curves and significant Tagg shifts from the quantitative protein data of the raw output data from IsobarQuant was then performed with the “TPP” package.

### 2.10 qMS data processing

Orbitrap raw MS/MS files were converted to mzML format using msConvert from the ProteoWizard tool suite. Spectra were then searched using MSGF+ (v10072) and Percolator (v2.08) where search results from eight subsequent fractions were grouped for Percolator target/decoy analysis. All searches were performed against the human protein subset of Ensembl 99 in the Galaxy platform. MSGF+ settings included precursor mass tolerance of 10 ppm, fully tryptic peptides, maximum peptide length of 50 amino acids, and a maximum charge of 6. Fixed modifications were TMT-16plex on lysine and peptide N-termini, and carbamidomethylation on cysteine residues, a variable modification was used for oxidation on methionine residues. Quantification of TMT-16plex reporter ions was performed using OpenMS project’s IsobaricAnalyzer (v2.0). PSMs found at 1% FDR (false discovery rate) were used to infer gene identities. Protein quantification by TMT16-plex reporter ions was calculated using TMT PSM ratios to the entire sample set (all 16 TMT channels) and normalized to the sample median. The median PSM TMT reporter ratio from peptides unique to a gene symbol was used for quantification. Protein FDRs were calculated using the picked-FDR method using gene symbols as protein groups and limited to 1% FDR.

### 3 RESULTS

#### 3.1 Volasertib decreases Tagg of PLK1 in CETSA

To evaluate whether CETSA-based assays are suitable to study the target engagement of volasertib, we first assessed its effects on the primary target PLK1 using CETSA followed by Western blotting for PLK1. Jurkat cells were treated with DMSO or 10 μM of volasertib for 80 minutes to achieve maximal ligand saturation. Cell viability remained unchanged by the treatment (measured by CellTiter-Glo, not shown). We then heated the cells in a temperature range between 37°C (no aggregation) and 55°C (full protein aggregation is expected) and...
analyzed the non-aggregated fraction using western blotting (Figure 1A). We observed that volasertib generally destabilized the PLK1 protein as the amount of PLK1 in the soluble fraction of volasertib-treated cells was lower compared to DMSO-treated cells (Figure 1A,C). Moreover, the decrease in T\textsubscript{agg} of PLK1 by volasertib was confirmed in several other pediatric leukemia cell lines (Supplementary Figure 1A-D). We then performed an isothermal dose-response (ITDR) analysis by treating the cells with volasertib in a concentration range of 0-10 000 nM at a temperature of 46°C (when about 50% of the PLK1 protein is aggregated) and determined that volasertib-induced destabilization of the PLK1 protein was dose-dependent (Figure 1B,D). Taken together, we established that volasertib binds to PLK1 and reduces its stability in a dose-dependent manner. We also concluded that CETSA can be used to study the targets of volasertib.

3.2 | Thermal proteome profiling of volasertib targets in acute leukemia

To study the proteins that volasertib binds to across the proteome, Jurkat cells were treated with 10 μM of volasertib or DMSO for 80 minutes and analyzed by TPP (each treatment in duplicates). In total, we identified 6995 proteins (Supplementary Data 1, Figure 2A). Assessment of the melting curves for PLK1 (Figure 2B) confirmed the results we observed in the CETSA experiments, namely that volasertib decreased the T\textsubscript{agg} of PLK1.

Proteins that had four melting curves (two replicates for each treatment) and a minimum of two quantified unique peptides were selected for further analysis. The significance threshold (adjusted p-value) was calculated using a nonparametric analysis of response curves (NPARC)\textsuperscript{10,17} which was set to P ≤ 0.001. These criteria were fulfilled for 241 proteins (Supplementary Data 1. Figure 2A). We then used STRING to exclude the proteins that had previously described interactions with PLK1 to focus on true off-targets of volasertib. Pathway analysis indicated enrichment for lipid metabolism regulators (eg, phosphatidylinositol pathway, PIP4K2A, and PIP4K2C) and the prostaglandin synthesis pathway (eg, prostaglandin reductases ZADH2 and PTGR2). In the TPP experiment, volasertib treatment increased the T\textsubscript{agg} of these proteins (Figures 2C,D, S2A,B, Table 1).

3.3 | Validation of ZADH2 as an off-target of volasertib

We first set out to validate the TPP finding that volasertib binds to ZADH2. For this, we used a B-cell acute
lymphoblastic leukemia (B-ALL) cell line MHH-CALL-2 that had a high endogenous expression of ZADH2 (data not shown). CETSA analysis showed that ZADH2 started to aggregate at 55.5°C in DMSO-treated samples, but not in the volasertib-treated cells (Figure 3A). ZADH2 was observed in volasertib-treated cells up to 61°C in contrast to the control treatment where it was not detectable. Volasertib-mediated stabilization of ZADH2 was dose-dependent as evident from the ITDR analysis performed at 60°C. ZADH2 protein gradually accumulated with the increase in volasertib concentration (Figure 3B). These results corroborated the TPP experiment where volasertib increased the T$_{agg}$ of ZADH2 in MHH-CALL-2 cells.

3.4 | Volasertib but not onvansertib binds to PIP4K2A

According to the TPP data, the T$_{agg}$ of PIP4K2A increased in cells treated with volasertib. The CETSA results in Jurkat cells treated with volasertib confirmed that the drug, indeed, increased the T$_{agg}$ of PIP4K2A as detected in the temperature range of 53.9°C to 57.7°C (Figure 4A). PIP4K2A accumulation was also detected in volasertib-treated THP1 cells (Figure S2C). Similarly, when lysates from the Jurkat cell line were treated with volasertib, PIP4K2A protein accumulated at 55.5°C in comparison to DMSO-treated cells (Figure 4B). Higher doses of volasertib led to the accumulation of a higher amount of PIP4K2A soluble fraction when the drug was applied to cell lysates (Figure 4C). Together, these results strengthen the hypothesis that volasertib directly binds to PIP4K2A.

To investigate whether the observed effect of volasertib on PIP4K2A was attributable to PLK1 inhibition or to volasertib itself, we analyzed another small molecule ATP-competitive PLK1 inhibitor, onvansertib. When Jurkat and THP1 cells were with 10 μM of onvansertib and subjected to CETSA, the stability of PLK1 decreased between 51 and 53.9°C, similarly to the effect observed after volasertib treatment (Figures 4D, S2D). However, onvansertib did not change the amount of the soluble PIP4K2A protein compared to the DMSO-treated samples at any of the temperatures in either of the cell lines (Figures 4D, S2D). Thus, the T$_{agg}$ of PIP4K2A is not altered...
by onvansertib, indicating that PIP4K2A is an off-target of volasertib.

### 3.5 Changes in the proteome of leukemic cells induced by 24 hours treatment with volasertib

According to previous studies, and our own data, 10-100 nM of volasertib inhibits mitotic entry and cell proliferation in cancer cell lines. The maximum measured the concentration of volasertib and its metabolite CD10899 in plasma of patients treated with volasertib is 926 nM. Thus, to study the effects of volasertib on leukemia cells in a more physiological setting, we chose to treat Jurkat cells with 25 and 500 nM of volasertib for 24 hours and then performed qMS analysis of the whole proteome.

In total, we identified and quantified 9077 proteins for all conditions (1% FDR, median sequence coverage—35% for each protein) (Figure 5A, Supplementary Data 2). Principal component analysis distinguished the treatments (DMSO, 25 nM volasertib and 500 nM volasertib), while the replicates grouped together (Figure S3A). Differential expression analysis of the qMS data was performed using DEqMS. A number of proteins were differentially expressed after 25 nM volasertib treatment (1.5 log fold change, adjusted \( P \leq .01 \)) (Figure 5B, Supplementary Data 3) and a significant increase in PLK1 expression and in the expression of several other proteins involved in cell cycle regulation was observed in both concentrations of volasertib. For example, KIF2A, a

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**TABLE 1** Significantly enriched categories GO: Biological processes and Reactome pathways in TPP after treatment with volasertib

| Term ID | Description                                      | Gene count | FDR   | Matching proteins in the network |
|---------|--------------------------------------------------|------------|-------|---------------------------------|
| HSA-556833 | Metabolism of lipids                             | 27/721     | 0.015 | ACBD7, ASAH1, CBR4, DDHD2, DECR1, GLA, HEXA, HEXB, LGMN, LSS, MED1, MED15, MED17, MED24, MED28, MED30, MED4, MED6, PI4KA, PIK3C2A, PIK3C2B, PIKFYVE, PIP4K2A, PIP4K2C, PPT1, PTGR2, STARD4 |
| HSA-1989781 | PPARA activates gene expression                   | 8/114      | 0.025 | MED1, MED15, MED17, MED24, MED28, MED30, MED4, MED6 |
| HSA-400206 | Regulation of lipid metabolism by PPAR-alpha     | 8/115      | 0.026 | MED1, MED15, MED17, MED24, MED28, MED30, MED4, MED6 |
| HSA-8847453 | Synthesis of PIPs in the nucleus                 | 2/4        | 0.041 | PIP4K2A, PIP4K2C |

**FIGURE 3** ZADH2 is stabilized by volasertib. A, MHH-CALL2 cells were treated with 10 µM volasertib or DMSO for 90 minutes and subjected to heating at indicated temperatures, followed by western blotting with anti-ZADH2 and anti-β-actin antibodies. Absolute band quantification by ImageJ is presented below the blot. B, IDTR of MHH-CALL2 live cells, treated with volasertib at the indicated concentrations for 90 minutes followed by heating to 60°C and western blotting with the indicated antibodies. Absolute band quantification is presented below the blot.
phosphorylation substrate of PLK1 and Aurora kinase A (AURKA) that directly phosphorylates PLK1 at Th210, was also increased by volasertib (Figure 5C, upper panel). The expression of CDKN1B, E2F1, and CCNE2 was reduced compared to DMSO-treated cells (Figure 5C, lower panel).

Using Gene Set Enrichment Analysis (GSEA), the Kyoto Encyclopedia of Genes and Genomes (KEGG), and hallmark pathways analysis, we identified the top 25 pathways enriched after volasertib treatment (Figure 5D). As expected, these included pathways relevant for cell cycle regulation: E2F targets, G2/M checkpoint, and mitotic spindle regulation. Additionally, citrate/tricarboxylic acid cycle, peroxisome formation, oxidative phosphorylation, phosphatidylinositol, and fatty acid metabolism pathways were affected (Figures 5D, S3B-5E). Interestingly, we detected an increase in the level of proteins from phosphatidylinositol and fatty acid metabolism pathways that were found to be likely volasertib off-targets in the TPP and CETSA as described above. Namely, the expression of PIP4K2A (Figure 6A) and ZADH2 (Figure 6B) was elevated after 24 hours of exposure to volasertib (Table 1). Thus, qMS analysis of the proteome affected by volasertib predictably identified changes in the pathways regulating cell division, but also showed that phosphatidylinositol and fatty acid metabolism pathways altered by volasertib. This finding is in line with the results of our TPP analysis that indicate that volasertib directly binds to PIP4K2A and ZADH2 resulting in off-target activity.

4 | DISCUSSION

In the present work, we evaluated the proteome-wide effect of small molecule PLK1 inhibitor volasertib. As volasertib is actively analyzed in clinical trials of leukemia patients, we aimed to explore its potential off-targets. First, we investigated the thermal response of the proteins upon treatment using TPP assay. In our data, the $T_{agg}$ of PLK1 in the DMSO control was found to be 45°C. Volasertib destabilized PLK1, as the $T_{agg}$ of drug-bound PLK1 decreased compared to unbound PLK1. Treatment with another small molecule ATP-competitive PLK1 inhibitor, onvansertib, also decreased the $T_{agg}$ of PLK1, indicating that when these two small molecules bind to PLK1, it tends to get destabilized and aggregate when the temperature increases. More commonly, small molecule ligand binding leads to the thermal
**FIGURE 5** qMS analysis of proteins regulated by volasertib. Jurkat cells were treated with DMSO, 25 nM or 500 nM of volasertib for 24 hours. After that, qMS was performed. A, Heatmap of qMS analysis, DMSO (yellow), 25 nM volasertib (green), and 500 nM Volasertib (magenta). B, Volcano plot of the proteins detected after 25 nM volasertib treatment. In color—proteins that were downregulated 1.5-fold (red) or upregulated 1.5-fold (green) with $P < .01$. C, Violin plots representing log2 fold change of protein quantities detected in the qMS analysis (blue—DMSO, red—volasertib 25 nM, green—volasertib 500 nM). D, Summary of the top 25 pathways analyzed by GSEA ranked by false discovery rate (FDR) (red—highest significance; green—lowest significance).
stabilization of a protein, and hence, to an increase in $T_{\text{agg}}$. However, thermal destabilization of target proteins has been described previously.\textsuperscript{32,33} The thermal destabilization of proteins could be due to numerous reasons such as the loss of protein-protein interactions, changes in post-translational modification status or protein structure-specific mechanisms. Binding of ligands to non-native protein states at different stoichiometry has been shown to cause the thermal destabilization of the Polo-box domain of PLK1,\textsuperscript{34} however, more focused studies are needed to uncover the true mechanism for thermal destabilization of PLK1 upon binding to volasertib and onvansertib.

Other PLK family members, namely PLK2 and PLK3, are targeted by volasertib, although at higher concentrations than PLK1.\textsuperscript{8} Several studies have demonstrated that PLK2 and PLK3 have tumor-suppressive roles, therefore, their inhibition needs to be evaluated for some tumors and especially for normal cells. In hematological cancers, however, these PLKs are frequently silenced as we observed in T-ALL, B-ALL, and AML.\textsuperscript{12,24,35-37} In particular, the Jurkat cell lines do not express either of them,\textsuperscript{34} confirmed by the TPP analysis (data not shown). Therefore, we could not assess how their $T_{\text{agg}}$ changes upon volasertib and onvansertib treatment.

We found two groups of proteins that were stabilized by volasertib upon heating: PIP4K2s and PTGRs. The PIP4Ks are involved in oxidative stress signaling, fat metabolism, and insulin sensitivity in normal cells.\textsuperscript{38,39} PIP4K2A has a higher expression in healthy peripheral mononuclear cells derived from blood compared to cells from established leukemic cell lines.\textsuperscript{40} Initial studies of different subtypes of PIP4Ks indicated a potential role of PIP4K2A as an anticancer target in leukemia as it was shown that PIP4K2A knockdown in p53-mutated tumors reduced the tumor size and increased the tumor-free survival.\textsuperscript{42,43} However, recent studies indicate a potential tumor-suppressive role of PIP4K2A due to its silencing in leukemia and glioblastoma cells.\textsuperscript{50,44,45} Therefore, the question regarding the possibility of using PIP4K2A-targeted drugs remains open. In contrast to volasertib, onvansertib, another highly specific PLK1 inhibitor, did not affect PIP4K2A in our hands. The antitumor activity of onvansertib was investigated in several preclinical studies in both solid and hematological malignancies.\textsuperscript{23,46,47} The results of a Phase I open-label dose-escalation study in adult patients with advanced/metastatic solid tumors revealed that reversible thrombocytopenia, anemia, leukopenia, and neutropenia were the major dose-limiting toxicities\textsuperscript{37} similar to side effects seen in patients treated with volasertib in combination with cytarabine.\textsuperscript{48} Despite that 11 out of 16 patients faced disease progression, onvansertib was recommended for further evaluation in patients with solid tumors and hematological cancers. Since the role of PIP4K2A in leukemia is not fully understood it is unclear whether this effect of volasertib is beneficial or if it is responsible for any of the observed side effects. Therefore, more investigations are required to elucidate the effect of this binding.

Another member of the PIP4K family, PIP4K2C, was previously analyzed in mice.\textsuperscript{49} It was found that the absence of PIP4K2C after knockdown increased the pro-inflammatory cytokines IL-12 (p40, p70), IL-2, and interferon γ in plasma. At the same time, the concentration of IL-10 in plasma increased compared to wild-type mice. Therefore, it was concluded that PIP4K2C regulated the immune response in mammals. In humans, PIP4K2C gene transcripts were shown to correlate with the survival rate of adult patients with AML.\textsuperscript{43} PIP4K2C has previously been identified as an off-target to BI2536, a precursor of volasertib.\textsuperscript{50,51} BI2536 and volasertib both belong to the chemical class of dihydropteridinones. However, they interact with different residues of PLK1, sharing only Cys133 as a common binding site in the kinase domain.\textsuperscript{52} Herein, we show by TPP that volasertib also binds to PIP4K2C in addition to PIP4K2A, demonstrating the importance of complementary methods for target elucidations. In a different study, PIP4K2C (but not PIP4K2A) was found to be inhibited by fostamatinib that possibly contributed to the effects of fostamatinib.\textsuperscript{53}

The second group identified by the TPP analysis, namely the PTGRs (including PTGR2 and ZADH2), is involved in the metabolism of prostaglandins, peroxisome, and adipose cell metabolism.\textsuperscript{54,55} Both kinases are NADP-dependent oxidoreductases that catalyze reversible oxidation of alcohols to aldehydes or ketones. ZADH2, also called PTGR3, is an important regulator of adipogenesis and is associated with
cardiac diseases and nephropathy.\textsuperscript{55-57} ZAHD2 was also identified as a target for cancer treatment as it was overexpressed in chronic lymphocytic leukemia and mantle cell lymphoma.\textsuperscript{58} Moreover, ZADH2 was suggested to play a role in cancer metabolism due to its binding properties to Peroxisome proliferator-activated receptor γ (PPARγ). It was shown that the interferon production by natural killer cells (NK-cells) in the B-cell lymphoma environment is decreased due to excess fatty acids as an energy source for tumor cells.\textsuperscript{59} Therefore, if volasertib suppresses the function of ZADH2, it may lead to increased adipogenesis and consequently to an impaired function of NK cells.

The other member of the PTGR family, PTGR2, is ubiquitously expressed in human tissues, except adipose tissue, and to a lower extent in muscles and skin. PTGR2 is responsible for NADH-dependent metabolization of 15-keto-prostaglandin E2 (PGE2) to 13,14-dihydro-15-keto-PGE2.\textsuperscript{60} One study showed that PTGR2 expression is reduced in chronic lymphocytic leukemia, contributing to accumulation and increased activity of PGE2 that results in immunosuppression through the inactivation of macrophages, T and B cells, and upregulation of IL-10.\textsuperscript{61} At the same time, Ptgr2 gene knockdown in mice results in the inactivation of pro-inflammatory NFκB pathway and improved survival in the murine septic models.\textsuperscript{62} Therefore, the observation that volasertib affects these groups of proteins that are downstream regulators of the immune response, could be important to consider in patients with disrupted immune activity.

We\textsuperscript{63} and others\textsuperscript{64} have shown that RNA interference-mediated knockdown of PLK1 results in less toxic effects in non-transformed BJ cells and in normal, dividing cells in mice, respectively, compared to malignant cells. Volasertib, moreover, induced some cell cycle arrest and apoptosis in BJ fibroblasts.\textsuperscript{65} Thus, toxicity caused by on-target mechanisms from the PLK1 inhibitors cannot be excluded, although it is likely that the clinically observed side effects are also due to the off-target activity of volasertib. Some of the most notable side effects in AML patients treated with volasertib in combination with cytarabine were grade 3-4 adverse effects: febrile neutropenia, leukopenia, anemia, thrombocytopenia, and severe infections as a consequence. Our study identified a number of potential targets of volasertib that could be responsible for these effects, as PIP4Ks and PTGRs are involved in immune regulation. However, to exactly pinpoint the role of these proteins in the survival and functioning of leukemic and normal hematopoietic cells, proper functional studies should follow. Other proteins from our TPP hit least (eg, BRD4, PARP14) could also contribute to the reported side effects, therefore, we cannot currently attribute the clinical phenomena directly to one protein or group of proteins. In addition, drug interaction studies of volasertib and cytarabine should be performed to ascertain that the adverse reactions are not due to the combination treatment.

Previously, qMS was applied to study the proteomic profile of volasertib in melanoma cells.\textsuperscript{66} The study showed that the drug downregulates the expression of multiple proteins involved in cell metabolism and proteasomal activity. Our data supported the previous publication as we also found volasertib to affect metabolic processes, including the metabolism of nucleic acids/RNA and lysosomal function.

In summary, we identified proteins that do not belong to the PLK family and have no previously known interactions with PLK1 that are affected by PLK1-small molecule inhibitor volasertib. Due to their important role in the immune response regulation and still unclear role in leukemia, more studies are needed to uncover the mechanism of the complex interaction between the drug and its potential off-targets in future volasertib patients.

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**CONFLICT OF INTEREST**

The authors declare no competing financial or non-financial interests.

**AUTHOR CONTRIBUTIONS**

CPA and RJ conceived, designed, and supervised the study; OG, IK, EK, LV, MP, and AA performed the experiments; CPA and RJ conceived, designed, and supervised the study; OG, IK, EK, LV, MP, and AA performed the experiments; CPA and RJ conceived, designed, and supervised the study; OG, IK, and CPA wrote the manuscript; RJ edited the manuscript; all authors proofread and approved the manuscript.

**DATA AVAILABILITY STATEMENT**

The MS data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository, identifier PXD023757 for TPP data and PXD024040 for qMS data.

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