Polo-like Kinase-1, Aurora Kinase A and Wee1 Kinase Are Novel Therapeutic Targets in Chronic Myeloid Leukemia

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Research

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Abstract

Background

In chronic myeloid leukemia (CML), Aurora kinase A and Polo like kinase 1 (Plk1), two serine-threonine kinases involved in the maintenance of a functional G2/M checkpoint, may cooperate with the constitutive tyrosine-kinase (TK) activity of the BCR-ABL1 fusion protein increasing DNA damage, promoting the occurrence of additional genomic alterations ultimately driving resistance to TK inhibitors (TKIs) and progression from chronic phase to blast crisis (BC).

Methods

In this study, we propose a new therapeutic strategy based on the combination of Aurora kinase A or Plk1 inhibition with danusertib or volasertib, respectively, and WEE1 inhibition with AZD1775. Apoptotic cell death was quantified by annexin V/propidium iodide staining and flow cytometry. Cell cycle distribution was assessed by PI staining and flow cytometry. Protein expression and activation was assessed by Western Blotting (WB) and Immunofluorescence. Clonogenic assays were performed to evaluate cells sensitivity to the different drugs.

Results

Danusertib and volasertib used as single drugs induced apoptosis and G2/M-phase arrest, associated with accumulation of phospho-WEE1. Subsequent addition of the WEE1 inhibitor AZD1775 in combination significantly enhanced the induction of apoptotic cell death in TKI-sensitive and -resistant cell lines as compared to both danusertib and volasertib alone and to the simultaneous combination. This schedule indeed induced a significant increase of the DNA double-strand break marker γH2AX, forcing the cells through successive replication cycles ultimately resulting in apoptosis. Finally, combination of danusertib or volasertib+AZD1775 significantly reduced the clonogenic potential of CD34+ CML progenitors.

Conclusions

Our results may have implications for the development of innovative therapeutic approaches based on Aurora kinase A or Plk1 inhibition associated with WEE1 blocking, aimed to improve the outcomes of patients with multi-TKI-resistant or BC CML.

Background

Chronic myeloid leukemia (CML) is characterized by the Philadelphia (Ph) chromosome resulting from the t(9;22)(q34;q11) balanced reciprocal translocation that generates the BCR-ABL1 oncogene. BCR-ABL1 encodes a chimeric protein that shows constitutive tyrosine kinase (TK) activity and drives clonal expansion of leukemic hematopoiesis [1]. Targeted therapy with TK inhibitors (TKIs) (imatinib [IM], nilotinib, dasatinib, bosutinib or ponatinib) has shown excellent results. However, the development of

resistance remains a critical problem in the management of CML [2]. Primary and secondary resistance to TKIs may be triggered by BCR-ABL1-independent signals sustaining the maintenance of a pool of transformed cells and potentially able to induce the auto-maintenance of the leukemic stem cells (LSCs) clone [3–11]. In these cells, mitogen-activated protein kinase (MAPK), PI3K, Hedgehog (Hh), WNT, polycomb gene BMI1 and Notch remain active despite BCR-ABL1 kinase inhibition [11–14].

Therefore, there is currently a great interest in the characterization of signals promoting BCR-ABL1-independent resistance, in an attempt to develop novel eradicating strategies for CML patients.

We have previously shown that the hyper-activation of Polo like kinase 1 (Plk1) serine/threonine kinase and FOXM1 transcription factor has a crucial role in maintenance and survival of CD34 + progenitors and in BCR-ABL1 + LSC persistence under TK inhibitor therapy [15].

In this study, we extended our investigations to Aurora kinase A and Plk1 inhibition, already used for clinical purposes in acute myeloid leukemia and myelodysplasias [16] starting from the evidences that the integrity of signaling pathways involved in cell cycle arrest, chromatin remodeling and DNA repair are critical for maintaining the fidelity of replicated DNA.

Normal cells repair damaged DNA during G1 arrest; in contrast, leukemic cells often have a deficient G1/S checkpoint and depend on a functional G2/M checkpoint for DNA repair. Aurora kinase A is a key component of centrosome cycle and polar spindle assembly required for regulated progression from G2 to M and throughout the M phase. It is frequently overexpressed in cancers, where it correlates with a poor prognosis [17–19]. Plk1 is essential for precisely regulating the cell division and maintaining genome stability in mitosis, spindle assembly, and DNA damage response [20–25]. Active Plk1 can phosphorylate WEE1, a tyrosine kinase that negatively regulates cyclin B-CDK1 complexes. WEE1 is an oncogenic nuclear kinase able to regulate cell cycle at the crucial G2/M checkpoint. WEE1 kinase is a key player in the DNA damage repair process and acts to inhibit mitotic entry in cells with damaged DNA. Overexpression of WEE1 has been identified in several malignant tumors including leukemias [26].

Aurora kinase A and Plk1 are involved in regulation of mitotic entry interfering with cyclin B1- CDK1 activity. Plk1 promotes the recruitment of Aurora kinase A to the centrosomes in G2 inducing Aurora kinase A-mediated recruitment of cyclin B1. Plk1 activates cyclin B1-CDK1 complex and the consequent degradation of the CDK-inhibitory kinase WEE1 [27].Aurora kinase A and Plk1 overexpression may thus be associated with genomic instability, one major trait of CML LSCs. Our hypothesis is that Aurora kinase A, together with Plk1, cooperates with the constitutive TK activity of the BCR-ABL1 fusion protein by increasing DNA damage, promoting the occurrence of additional genomic alterations and driving TKIs resistance and disease progression to blast crisis (BC). In this study, a new therapeutic strategy based on Aurora kinase A or Plk1 inhibition with PHA-739358 (danusertib) or BI6727 (volasertib), respectively, associated with WEE1 inhibition with AZD1775, was evaluated in cell lines displaying BCR-ABL1-independent TKI resistance and in primary cells from CML patients in BC.

Materials And Methods

Materials. Imatinib, danusertib, volasertib and AZD1775 were purchased from Selleck Chemicals. For Western blotting, 10% acrylamide gels, running buffer (MOPS), transfer buffer and polyvinylidene difluoride (PVDF) transfer membrane were bought from Thermo Scientific. For immunofluorescence, the FITC-conjugated anti-mouse IgG, the anti-rabbit conjugated with Alexa Fluor 568 antibodies and DAPI (6-diamidino-2-phenylindole) were purchased from Sigma-Aldrich.

The anti-caspase 3, anti-caspase 9, anti-Bax, anti-CHK1, anti-phospho-CHK1 (S317), anti-CHK2, anti-phospho-CHK2 (T68), anti-cyclin B1, anti-phospho-cyclin B1 (S133), anti CDC25C, anti-phospho-CDC25C (S198), anti-WEE1, anti-phospho-WEE1 (S642), anti-CDK1, anti-phospho-CDK1 (Y15), anti-phospho-H2AX (S139), anti-Aurora kinase A, anti-phospho-Aurora kinase A (T288), anti-Plk1, anti-phospho-Plk1 (T210) and anti-PARP antibodies were purchased from Cell Signaling Technology. The anti-β-actin antibody used as loading control was purchased from Santa Cruz Biotechnology.

Cell lines, patients and drug treatments. The parental K562 BCR-ABL1 + cell line (K562-S) was maintained in RPMI 1640 medium (Lonza) supplemented with 10% fetal calf serum (FCS, Gibco), 1% L-Glutamine and antibiotics in 5% CO2 and fully humidified atmosphere at 37°C. K562-R cells were generated by progressive increase of imatinib concentration (from 0.05 μM to 10 μM) in the culture medium. Drug resistance was confirmed by clonogenic assays and BCR-ABL1 dephosphorylation (indicating the survival in the presence of imatinib was sustained by a BCR-ABL1-independent mechanism of TKI resistance) was confirmed by western blotting [15].

Four CML patients in BC showing resistance to multiple lines of TKI therapy were included in our study; samples were collected with informed consent according to institutional guidelines. This study was conducted according to the principles of the Declaration of Helsinki and was approved by the Independent Ethics Committee of the “S. Orsola-Malpighi” University Hospital of Bologna (protocol 112/2014/U/Tess). The mononuclear cell fraction (MCF) from bone marrow or peripheral blood samples of patients and peripheral blood apheresis products of 8 healthy donors (HD) were obtained by Ficoll-Hypaque gradient centrifugation. CD34 + cells were isolated by immunomagnetic separation (mini-MACS, Miltenyi Biotec). Briefly, the MCF was incubated at 4° C for 30’ with magnetic microbeads coated with anti-CD34 antibody (Miltenyi Biotec). CD34 + cells were flown through a separation column in a magnetic field. Cell purity was confirmed using flow cytometric analysis of anti-CD34-FITC antibody (BD Biosciences); it was > 84% in all cases (data not shown).

Imatinib (1 μM), danusertib (1 μM) and volasertib (1 μM) were used to inhibit BCR-ABL1, Aurora kinase A and Plk1 respectively. The WEE1 inhibitor AZD1775 (1 μM) was used alone or in combination with danusertib (500 nM) and volasertib (500 nM).

Analysis of cell cycle distribution and apoptosis. Cell cycle distribution was performed on 5×10^5 cells fixed overnight in 70% ethanol and treated with 1 μg/μL propidium iodide (PI) and RNase (both from Sigma) at 37°C for 30 min. Apoptotic cells were recognized by cytofluorimetric analysis of fluorescinated

Annexin V and PI uptake (Roche). Cell fluorescence and PI uptake were measured by mean of a FACScan flow cytometer (set at 488 nm excitation and 530 nm bandpass filter wave length for fluorescin detection or > 580 nm for PI detection) and two different dedicated softwares were used to analyze results obtained (Modfit and Diva software from Beckton Dickinson) [33].

Protein analyses. Whole cell lysates were used for protein analyses (Western blot) and evaluation of protein post-translational modifications. Briefly, $10x10^6$ cells before and after treatments, were resuspended in 200µl of lysis solution (20 mM Tris-HCl, pH 7.5) and maintained in constant agitation for 30 min at 4°C. Lysates were then centrifuged in a microcentrifuge at 4°C for 30 min at 12.000 rpm. The supernatant was aspirated and placed in a fresh tube kept on ice, and the pellet discarded. 40µg of the whole cell lysates obtained were resolved by SDS PAGE. Gels were removed from the electrophoresis apparatus and transferred on a PVDF transfer membrane. Signal intensities in single blots obtained in three separate experiments were revealed by means of ChemiDoc XRS + Gel Imaging System (BioRad) equipped with a dedicated software (Image Lab, BioRad).

Clonogenic assays. Drug cytotoxicity was evaluated in cell lines (K562-S and K562-R) and CD34 + progenitor cells from BC patients or HD by clonogenic assays. The reduction of colony number (generated in 0.9% methylcellulose supplemented with 30% fetal calf serum) in the presence of increasing doses of imatinib (0.025-0.1 µM), danusertib (0.025-0.1 µM) and volasertib (0.025-0.1 µM) was assessed after 14 days of incubation at 37°C in a fully humidified atmosphere and 5% CO2. Nonlinear regression analyses (GraphPad Prism; GraphPad Software Inc.) were used to calculate the lethal dose (LD50) of the different drugs and combinations.

Immunofluorescence (IF) analysis. Cells set on poly-L-lysine-coated glass slides were fixed with 3% paraformaldehyde for 10’ at 37°C, washed with 0.1M glycine in phosphate-buffered saline (PBS), permeabilized in 70% ice-cold ethanol for 2’ at -20° C and incubated overnight at 4°C with primary anti-H2AX and anti-RAD51 antibodies. Slides were then stained with a secondary anti-rabbit antibody conjugated with Alexa Fluor 568 for 2 h at room temperature and a subsequent anti-mouse antibody fluoresceinated. 6-diamidino-2-phenylindole (DAPI) was used to stain the nuclear compartment. IF analyses were performed using an Axiovert 40 CFL microscope (Zeiss). Images were acquired with a 100X objective and analyzed with the AxioVision software.

Statistics. Data are presented as mean values ± SD and were analyzed for statistical significance by Student t-test (GraphPad Prism Software). A P value of < 0.05 was considered as statistically significant.

Results

Inhibition of Aurora kinase A or Plk1 activity arrests cell proliferation and favors apoptosis in both TKI-sensitive and -resistant CML cells

We have already demonstrated that the serine/threonine kinase Plk1 is hyper-activated in the progenitor compartment of CML, consisting of CD34+/BCR-ABL1 + cells, and that together with up-modulation, it

contributes to TKI resistance [15]. To better understand the role of Plk1 and Aurora kinase A in sustaining proliferation of CML cells, we performed clonogenic assays in K562-Sensitive (K562S) and K562-Resistant (K562-R) cells in the presence of danusertib or volasertib (that inhibit Aurora kinase A and Plk1, respectively). While imatinib spared K562-R cells even at the dose of 8 μM, both Aurora kinase A and Plk1 inhibition induced a dramatic dose-dependent reduction in colony formation with LD50 ranging from 0.0282 to 0.0362 μM in both K562-S and K562-R cells (Fig. 1A). In agreement with our previous results, inhibition of cell proliferation was associated with increased apoptosis and caspase activation (Fig. 1B and C). Interestingly, the greatest increase of the apoptotic activator Bax was observed in K562-R cells following danusertib and volasertib treatments, indicating that both Aurora kinase A and Plk1 play a prominent role in sustaining resistant CML cells, since their inhibition is a stressful condition that triggers apoptosis.

Aurora kinase A or Plk1 inhibition induces cell cycle arrest and affects G2/M checkpoint proteins

To further investigate the role of Aurora kinase A and Plk1 in sustaining CML cell proliferation, we next examined the impact of their inhibition on cell cycle. Unlike imatinib, both danusertib and volasertib treatment dramatically increased G2/M phase cells regardless of imatinib sensitivity (Fig. 2A). Due to the role of Aurora kinase A and Plk1 in driving cell-cycle progression, in normal cells their expression and activity is tightly controlled, peaking during G2 and mitosis and dropping at mitotic exit [28]. We have previously reported that both Aurora kinase A and Plk1 are up-modulated and hyper-activated in imatinib-resistant BCR-ABL1 positive CML cells [15]. To confirm the efficacy of danusertib and volasertib on Aurora kinase A and Plk1 inhibition and to assess if molecular effects of drugs administration are on-target, we performed Western blotting assays testing Aurora kinase A and Plk1 expression and phosphorylation (Fig. 2B). Our results showed that both danusertib and volasertib induce Aurora kinase A and Plk1 down-modulation and de-phosphorylation. To determine the functional consequence of Aurora kinase A and Plk1 inhibition, we used Western blotting to assess the phosphorylation of several proteins involved in cell cycle control including Chk-1, Chk-2, cyclin B1, WEE1 and CDK1 (Fig. 2B). We observed increased levels of active phospho (p)-Chk1/2 proteins, that are required for checkpoint-mediated cell cycle arrest to ensure the integrity of the genome before DNA replication, and of the nuclear p-cyclin B1, that regulates G2/M transition. As expected, p-cyclin B1 on S133 and p-CDC25C on S198 were reduced after Plk1 inhibition. These observations confirm the on-target effects of volasertib 1 μM for 24 h. Moreover, active p-WEE1, that regulates CDK1 activity, was found to be increased, thus preventing the progression into mitosis. Collectively, these data support the hypothesis that Aurora kinase A and Plk1 over-expression and hyper-activation induce DNA damage causing G2/M cell cycle arrest. A time kinetic profiling of cell cycle after Aurora kinases inhibition (24, 36, 48 hours) by danusertib significantly induced polyploidy in all survival cells, thus causing definitively apoptotic cell death (data not shown).

WEE1 inhibition following Aurora kinase A or Plk1 inhibition dramatically increases DNA damage

Given that Aurora kinase A and Plk1 inhibition induced p-WEE1 up-modulation, we hypothesized that blocking WEE1 could promote mitosis and trigger apoptosis as a result of DNA damage propagation. First, we investigated the DNA damage induced by Aurora kinase A, Plk1 and WEE1 inhibition following

treatment with danusertib, volasertib or AZD1775, respectively, as single agents. As detected by immunofluorescence, in both K562-S and K562-R cell lines danusertib and volasertib treatments perturbed the mitotic process and induced the nuclear foci formation of γ-H2AX and Rad51 (Fig. 3A), indicating the presence of DNA double strand breaks and repair activity, respectively. Next, we evaluated a schedule whereby 24h treatment with danusertib or volasertib was followed by 24h combination with AZD1775 to force K562-S and K562-R cell entry in mitosis. This approach dramatically increased the DNA double strand break marker γ-H2AX (Fig. 3B). These data indicate that WEE1 blockage following Aurora kinase A or Plk1 inhibition propagates genomic instability in both imatinib-sensitive and -resistant cells through successive replication cycles.

Combined WEE1 and Aurora kinase A or Plk1 inhibition exerts additive effects and dramatically affects LSC viability

To further investigate the impact of damaged DNA accumulation, we used flow cytometry to verify apoptosis induction. As to the combinations, we examined and compared the effects of two different schedules: a) 48h-co-treatment with either danusertib or volasertib + AZD1775, and b) 24 h-treatment with danusertib or volasertib followed by 24 h-treatment with danusertib or volasertib + AZD1775. As illustrated in Fig. 4A and B, while the first schedule induced only a modest increase in apoptotic cells in both K562-S and K562-R cells, the second one induced a drastic induction of caspase-dependent apoptosis (Fig. 4A-D), indicating that in K562-S and K562-R cells the apoptotic response is triggered to avoid the propagation of mutations.

To ascertain whether the combination of danusertib or volasertib and AZD1775 could be an useful strategy for BC CML patients, we assessed the effects of Aurora kinase A, Plk1 and WEE1 inhibition on the clonogenic potential of CD34+/BCR-ABL1 + cells isolated from four multi-TKI-resistant BC CML patients. Indeed, this cellular fraction is enriched in leukemic stem cells (LSC) that are intrinsically resistant to TKIs and are supposed to be a source of relapse [9]. We found that the combinations significantly reduced the proliferation of all CD34+/BCR-ABL1 + primary blasts. Importantly, neither the single drugs nor the combined treatments significantly impacted on the viability of cells derived from an healthy donor, tested as control. Collectively, our findings suggest that combined administration of Aurora kinase A or Plk1 and WEE1 inhibitors could be a promising strategy to enhance apoptosis in TKI-resistant CML cells.

Discussion

CML is a hematologic neoplasm sustained by the constitutive activation of the BCR-ABL1 fusion kinase that can be successfully targeted with TKIs, so that most CML patients nowadays obtain durable remissions. However, tumor escape mechanisms may be activated that compromise TKI efficacy. The most extensively investigated mechanism is the selection of point mutations in the BCR-ABL1 kinase domain, that turn on BCR-ABL1 TKI activity again by impairing inhibitor binding [29, 30]. To counteract this mechanism, two generations of more potent or selective TKIs have been developed beyond imatinib,

and switching from one TKI to another may rescue response in patients harboring mutations [31]. However, resistance may also be driven by BCR-ABL1-independent mechanisms that sustain the proliferation and survival of CML cells – including the BCR-ABL1-positive quiescent LSC pool – despite BCR-ABL1 inhibition, finally resulting in disease relapse and eventually progression [1, 2]. Much less is known about these mechanisms. Therefore, in order to identify novel therapeutic approaches to treat TKI-resistant patients, it becomes extremely important to dissect the cellular signals implicated in BCR-ABL1-independent drug resistance as well as in LSC proliferation and maintenance. This is especially true for the setting of patients who progress from CP to BC, in whom targeting BCR-ABL1 alone is not sufficient anymore and prognosis has remained poor all over the years [32]. In our study, we prove the efficacy of a novel drug combination based on Aurora kinase A or Plk1 inhibition in association with WEE1 inhibition, to treat CML. Most importantly, this therapeutic approach seems to be particularly effective against progenitor cells from CML patients in BC.

Aurora kinase A or Plk1 are key components of cell cycle regulation. We have previously demonstrated that both Aurora kinase A and Plk1 are hyper-activated in CML resistant cell lines [15]. In a previous work, we proved that drug resistance displayed by the K562-R cell line was not dependent upon BCR-ABL1 tyrosine kinase activity by assessing the phosphorylation status of the protein and of its downstream substate Crk-L. Indeed, we found that although BCR-ABL1 is expressed in K562-R cells, it is not phosphorylated and neither is Crk-L. Very interestingly, we finally proved that Aurora kinase A and Plk1 are over-expressed and hyper-activated in the CD34 + compartment, where LSCs reside [15]. Here, we describe the anti-proliferative effects of danusertib and volasertib, two selective inhibitors of Aurora kinase A and Plk1, respectively. In both TKI-sensitive and -resistant CML cell lines, these two inhibitors were able to impair proliferation and induce a significant degree of caspase-dependent apoptosis. Interestingly, as compared to imatinib, these two drugs act by exploiting alternative pathways. Indeed, both danusertib and volasertib induced a dramatic increase of cells in G2/M phase. The next step of our study aimed to test different drugs combination between danusertib or volasertib and a WEE1 inhibitor (AZD1775) with the purpose to increase the percentage of apoptotic cell death and to block cell escape mechanisms and eventually drugs resistance. It is well known from the literature, and it has been confirmed by our data, that WEE1 is an over-expressed and hyper-activated protein kinase in the G2 phase. We therefore decided to use a WEE1 inhibitor at the exact timepoint in the cell cycle when WEE1 is hyper-activated: after 24 hours of inhibition of Aurora kinase A or Plk1. The combination of drugs thus designed was found to be very efficient if compared with the administration of single drugs.

Accordingly, we detected a significant modulation in post-translational modifications of several proteins involved in G2/M transition regulated by Aurora kinase A or Plk1 such as cyclin B1 (phosphorylated on ser 133) and cdc25C. Notably, this was also found in our cell line model, selected for BCR-ABL1-independent resistance. Indeed, we had previously demonstrated that in our K562-R cell line, surviving imatinib at 10 μM, BCR-ABL1 and its downstream target Crk-L are both dephosphorylated (data not shown). All the effects we have observed in the present study are therefore attributable solely to Aurora kinase A or Plk1 inhibition, suggesting that employing danusertib or volasertib would be an alternative strategy to treat CML relapsed/resistant patients. Importantly, in addition to CML cell lines, the

combination of danusertib or volasertib + AZD1775 was also effective in CD34+/BCR-ABL1 + cells isolated from for BC CML patients and representative of the LSC compartment.

Our mechanistic studies explain why Aurora kinase A and Plk1 inhibition alone or in combination with WEE1 inhibition induce apoptosis in TKI-sensitive and -resistant BCR-ABL + cell lines and in multi-TKI-resistant BC CML patient cells. We propose a model that describes the roles of Aurora kinase A and Plk1 over-expression and hyper-activation in signaling pathways that operate in CML cells and the effects of their inhibition. In this model, our findings suggest that both Aurora kinase A and Plk1 have important roles in activating proliferation and miscontrolled cell division and in inducing genomic instability. Based on our findings, we propose that combined targeting of Aurora kinase A or Plk1 + WEE1 may be an excellent strategy for inducing apoptosis in CML cells whose resistance is driven by BCR-ABL1-independent mechanisms, or where all TKIs have failed, including advanced stages of CML like BC. Thus, danusertib or volasertib associated with AZD1775 (or other, equivalent inhibitors that could rapidly be repurposed) may have potential to effectively address some remaining key unmet needs in CML treatment.

Conclusions

Overall, our results demonstrate at a preclinical level that the combination of danusertib or volasertib and AZD1775 is a promising option that would deserve further investigation at the clinical level in an attempt to improve the poor outcome of multi-TKI-resistant and BC CML patients.

Abbreviations

CML: chronic myeloid leukemia; Ph: Philadelphia; TK: tyrosine kinase; TKIs:TK inhibitors; IM: imatinib; LSC: leukemic stem cells; MAPK: mitogen-activated protein kinase, PI3K: Phosphatidylinositol-3-Kinase; Hh: hedgehog; Plk1: Polo-like kinase 1; CDK1: Cyclin dependent kinase 1; BC: blast crisis; PVDF: polyvinylidene difluoride; FITC: fluorescein isothiocyanate; DAPI: 6-diamidino-2-phenylindole; MCF: mononuclear cell fraction; HD: healthy donor; PI: propidium iodide; SDS PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; IF: immunofluorescence; PBS: phosphate-buffered saline; K562-S: K562 IM sensitive; K562-R: K562 IM resistant.

Declarations

Author Contributions

Conceptualization, Manuela Mancini and Simona Soverini; Data curation, Manuela Mancini and Sara De Santis; Formal analysis, Manuela Mancini and Annalisa Lonetti; Funding acquisition, Manuela Mancini, Michele Cavo, Gabriele Gugliotta and Simona Soverini; Investigation, Manuela Mancini, Sara De Santis and Cecilia Monaldi; Methodology, Sara De Santis, Cecilia Monaldi and Samantha Bruno; Project administration, Manuela Mancini, Michele Cavo, Gabriele Gugliotta and Simona Soverini; Resources,

Manuela Mancini, Elisa Dan, Barbara Sinigallia, Gabriele Gugliotta and Simona Soverini; Software, Manuela Mancini and Annalisa Lonetti; Supervision, Fausto Castagnetti, Michele Cavo and Simona Soverini; Validation, Sara De Santis, Cecilia Monaldi and Samantha Bruno; Visualization, Cecilia Monaldi, Fausto Castagnetti, Samantha Bruno, Elisa Dan, Barbara Sinigallia and Gianantonio Rosti; Writing – original draft, Manuela Mancini and Annalisa Lonetti; Writing – review & editing, Fausto Castagnetti, Michele Cavo, Gabriele Gugliotta and Simona Soverini. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article and relate materials are available upon request.

Ethics approval and consent to participate

Sample and data collection were approved by the Institutional Review Boards of the S. Orsola-Malpighi Hospital (protocol 112/2014/U/Tess).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

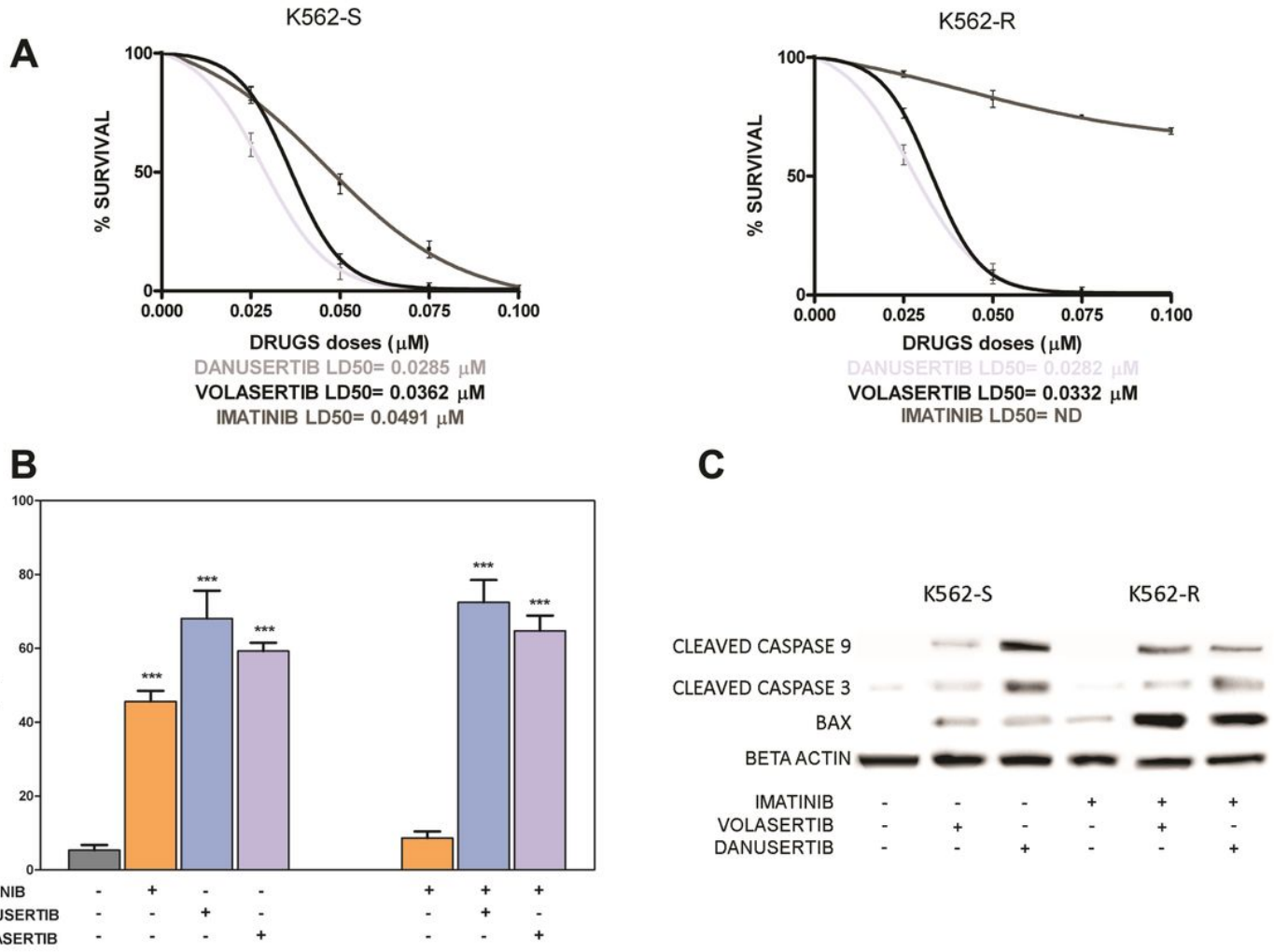

Figure 1

Effects of Aurora kinase A and Plk1 inhibition on CML cell proliferation. (A) Dose-dependent inhibition of K562-S (left panel) and K562-R (right panel) cells in 14-days methylcellulose colony-forming assay. Cells were treated with increasing concentrations of imatinib, danusertib (Aurora kinase A inhibitor) and volasertib (Plk1 inhibitor). (B) Flow cytometry analysis of Annexin-V/PI positive cells. K562-S and K562-R cells were treated with imatinib (1 μM), danusertib (1 μM) and volasertib (1 μM) for 24 hours. (C) Western blot analysis of apoptosis-related proteins in K562-S and K562-R cells treated with 1 μM of imatinib, danusertib and volasertib for 24 hours. An antibody against beta-actin was used as loading control. Clonogenic assays on K562-S and K562-R cells were repeated three times in an independent way: results were represented as a mean of three biological replicates. Western blotting assays were performed starting from lysates obtained by three independent drug treatments for each sample.

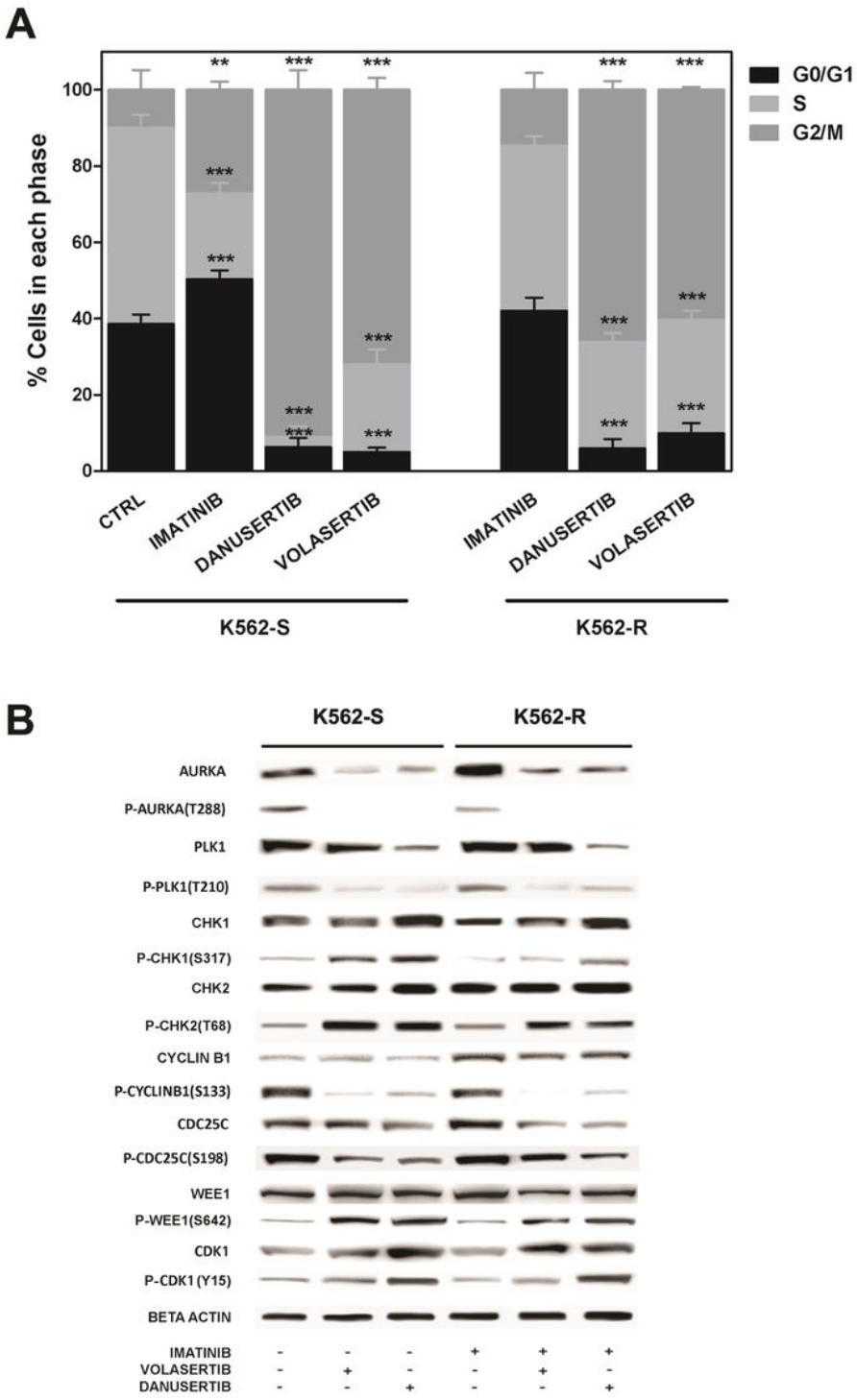

Figure 2

Effects of Aurora kinase A and Plk1 inhibition on cell cycle. (A) Flow cytometry analysis of cell cycle. K562-S and K562-R cells were treated with 1 μM of imatinib, danusertib or volasertib for 24 hours. Asterisks indicate a significant difference between control (for K562-S) and imatinib-treated (for K562-R) cells and between treatments with danusertib and volasertib. (B) Western blot analysis of cell cycle-related proteins in K562-S and K562-R cells treated with 1 μM of imatinib, danusertib and volasertib for

24 hours. Beta-Actin was used as loading control. Cell cycle distribution assays were represented as a mean of three biological replicates. Similarly, all Western blotting assays were performed starting from lysates obtained by three independent drug treatments for each sample.

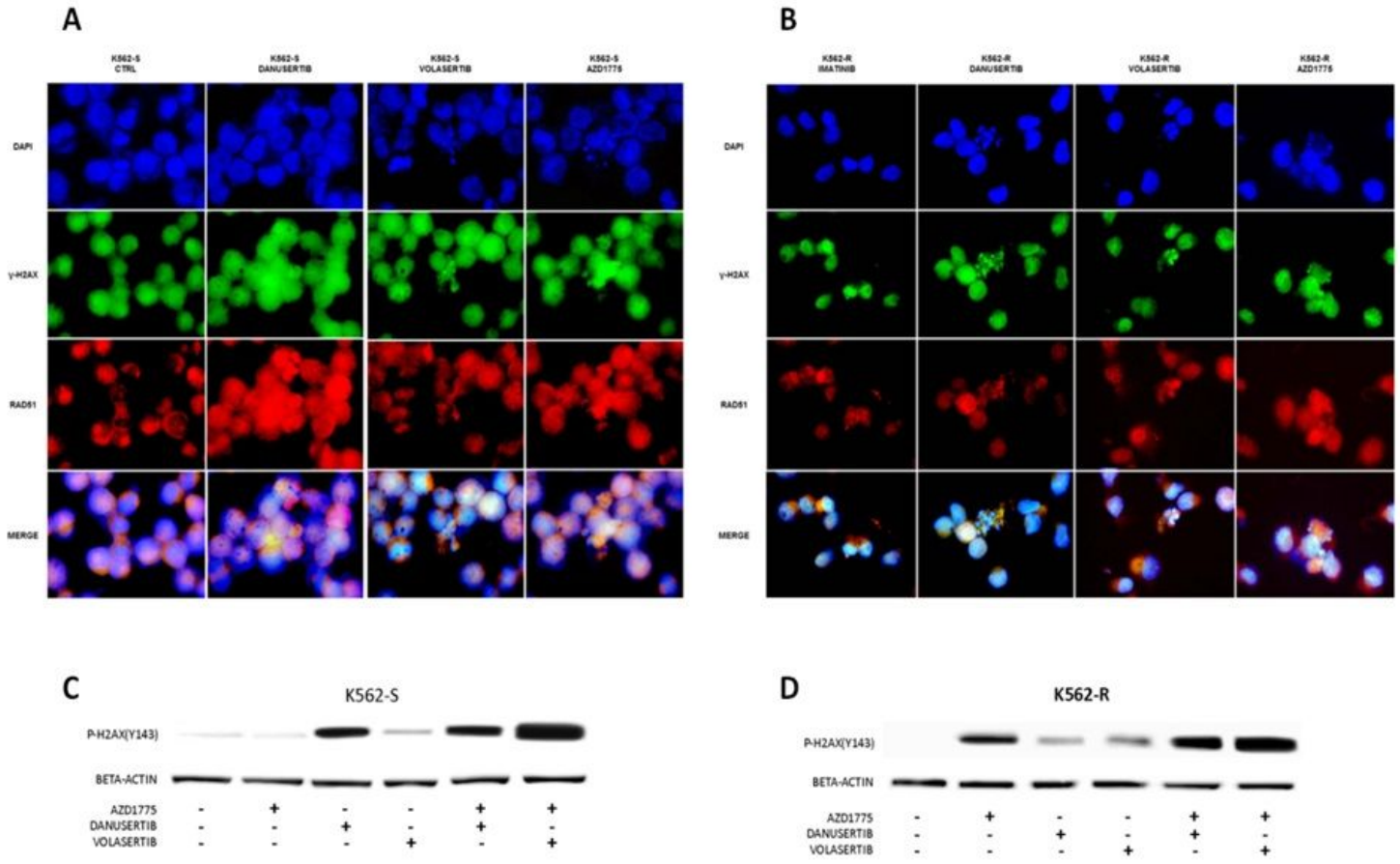

Figure 3

Effects of Aurora kinase A, Plk1 and WEE1 inhibition on DNA damage. Panels A and B show immunofluorescence analysis of phosphorylated histone 2A.X (γH2AX, green) and Rad51 (red) in K562-S (A) compared to K562-R (B) after 24 hours of treatment with danusertib, volasertib and AZD1775. Staining with DAPI (4',6-Diamidino-2-Phenylindole) indicates the nuclear localization of γH2AX and Rad51. Panels C and D show Western blot analysis of phosphorylated histone 2A.X revealing the effects on DNA damaged cells of danusertib or volasertib alone as compared to 24h priming with danusertib or volasertib alone + 24h combination of danusertib or volasertib+AZD1775. Beta-Actin was used as loading control. Western blotting assays were performed starting from lysates obtained by three independent drug treatments for each sample.

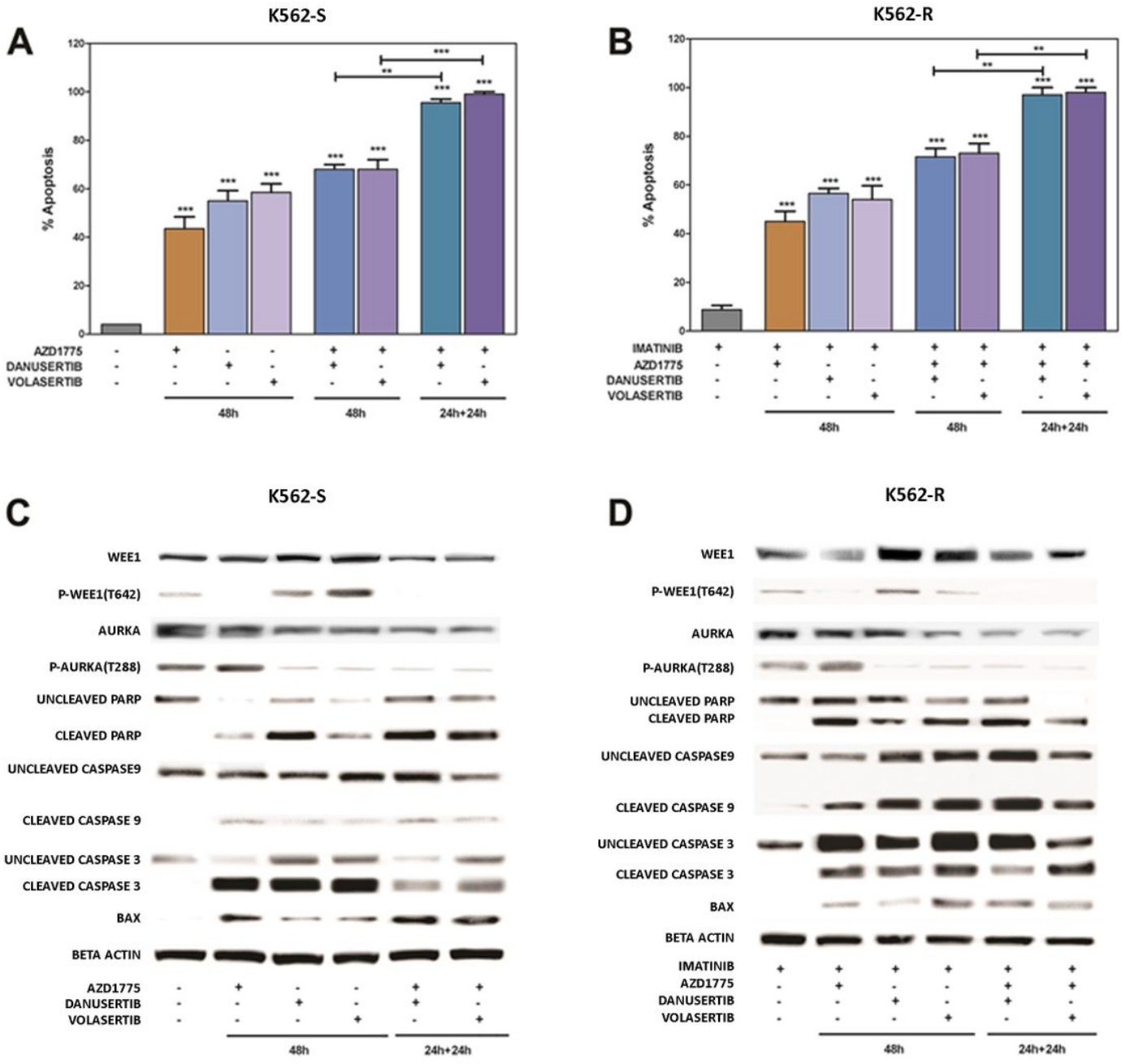

Figure 4

Effects of Aurora kinase A or Plk1 inhibition in combination with WEE1 inhibition. Flow cytometry analysis of apoptosis induction in K562-S (A) and K562-R (B) cells following 48h-co-treatment with danusertib or volasertib+AZD1775 (schedule a), or 24h-treatment with danusertib or volasertib followed by 24h-treatment with danusertib or volasertib+AZD1775 (schedule b). Western blot analysis of apoptosis-related proteins in K562-S (C) and K562-R (D) cells following treatment with Aurora kinase A, Plk1 or WEE1 inhibitors as single agents or in combination according to schedule a or b . Beta-actin was used as loading control. Apoptotic cell death evaluations were represented as a mean of three biological replicates. Similarly, all Western blotting assays were performed starting from lysates obtained by three independent drug treatments for each sample.

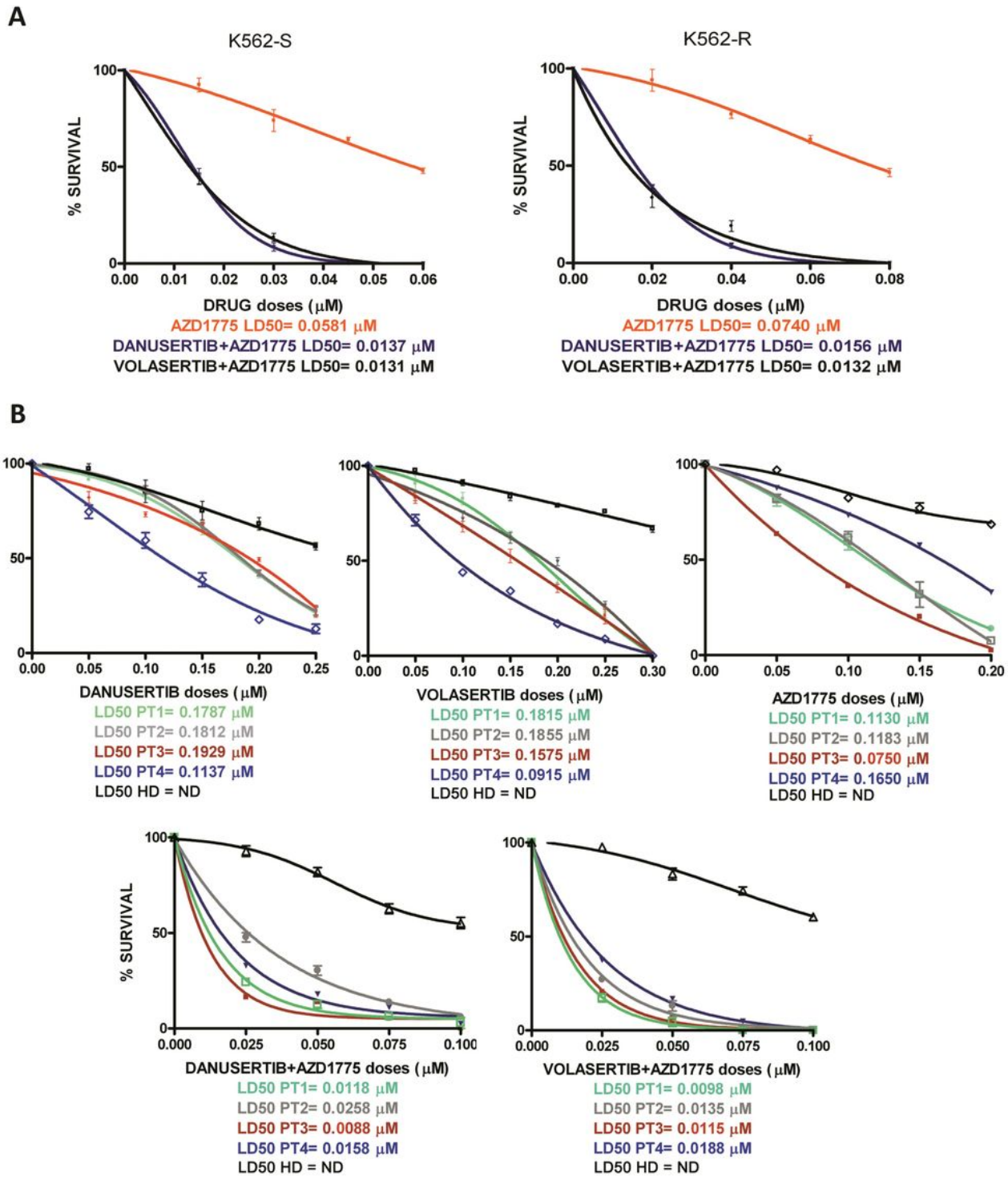

Figure 5

Effects of Aurora kinase A, Plk1 and WEE1 inhibition in K562-S and K562-R cell lines and in CD34+ cells derived from 4 BC CML patients and one healthy donor (HD). Dose-dependent inhibition of survival was assessed in 14-days methylcellulose colony-forming assays. (A) K562-S and -R cells and (B) CD34+ cells were treated with increasing concentrations of danusertib (Aurora kinase A inhibitor), volasertib (Plk1 inhibitor), AZD1775 (WEE1 inhibitor) danusertib+AZD1775, volasertib+AZD1775.