Original Paper

Inhibition of Suicidal Erythrocyte Death by Volasertib

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Key Words

Phosphatidylserine • Eryptosis; energy depletion • Oxidative stress • Hyperosmotic shock • Ionomycin • Calcium • Volasertib

Abstract

Background/Aims: The Polo-like kinase 1 (Plk1) inhibitor volasertib is used in the treatment of malignancy. Volasertib is partially effective by triggering suicidal death or apoptosis of tumor cells. Similar to apoptosis of nucleated cells, erythrocytes may enter suicidal cell death or eryptosis, which is characterized by cell membrane scrambling with phosphatidylserine translocation to the cell surface and by cell shrinkage. Stimulators of eryptosis include energy depletion, hyperosmotic shock, oxidative stress and excessive increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). The present study explored, whether volasertib impacts on eryptosis.

Methods: Human erythrocytes have been exposed to energy depletion (glucose withdrawal for 48 hours), hyperosmotic shock (addition of 550 mM sucrose for 6 hours), oxidative stress (addition of 0.3 mM tert-butylhydroperoxide [tBOOH] for 50 min) or Ca^{2+} ionophore ionomycin (1 μM for 60 min) in absence and presence of volasertib (0.5-1.5 $\mu\text{g/ml}$) and flow cytometry was employed to quantify phosphatidylserine exposure at the cell surface from annexin-V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3 fluorescence, reactive oxygen species from 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence and ceramide abundance utilizing antibodies. For comparison, annexin-V-binding and forward scatter were determined following a 48 hours exposure of human leukemic K562 cells in RPMI-1640 medium to volasertib. **Results:** Treatment with volasertib alone did not significantly modify annexin-V-binding or forward scatter in mature erythrocytes. Energy depletion, hyperosmotic shock, oxidative stress and ionomycin, all markedly and significantly increased the percentage of annexin-V-binding erythrocytes, and decreased the forward scatter. Volasertib significantly blunted the effect of energy depletion and hyperosmotic shock, but not of oxidative stress and ionomycin on annexin-V-binding. Volasertib did not significantly influence the effect of any maneuver on forward scatter. In K562 cells, volasertib enhanced annexin-V-binding and

decreased the forward scatter. **Conclusions:** Volasertib is a novel inhibitor of erythrocyte cell membrane scrambling following energy depletion and hyperosmotic shock, effects contrasting the stimulation of K562 cell apoptosis.

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Introduction

The Polo-like kinase 1 (Plk1) inhibitor volasertib (BI6727) [1-10] has been successfully used in preclinical and clinical studies for the treatment of several malignancies [1, 2, 7-14] including acute myeloid leukaemia (AML) [2-9, 11, 15-20]. Volasertib is in part effective by triggering suicidal death or apoptosis of tumor cells [1, 7]. Reported side effects of treatment with volasertib or combinations of volasertib with other cytostatic drugs include anemia [12, 13, 21].

At least in theory, the anemia following volasertib treatment could result from stimulation of suicidal erythrocyte death or eryptosis [22, 23], which is characterized by cell membrane scrambling with phosphatidylserine translocation to the cell surface [22]. Another hallmark of eryptosis is cell shrinkage [24]. Eryptosis could be stimulated by cell stress, e.g. by energy depletion [22], hyperosmotic shock [22], and oxidative stress [22]. Signaling mechanisms triggering eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [22], ceramide [25], caspases [22, 26, 27], G-protein $\text{G}\alpha_2$ [28], casein kinase 1α [22], Janus-activated kinase JAK3 [22], protein kinase C [22], and p38 kinase [22]. Signaling mechanisms inhibiting eryptosis include the kinases AMPK [22], cGMP-dependent protein kinase [22], MSK1/2 [29], PAK2 [22] and sorafenib/sunitinib sensitive kinases [22]. Eryptosis is stimulated by a myriad of xenobiotics [22, 29-83] and enhanced eryptosis is observed in a variety of clinical conditions including iron deficiency [22], dehydration [84], hyperphosphatemia [74], vitamin D excess [38], chronic kidney disease (CKD) [85-89] hemolytic-uremic syndrome [90], diabetes [91], hepatic failure [92, 93], malignancy [94, 95], arteritis [96], sepsis [97], sickle-cell disease [22], beta-thalassemia [22], Hb-C and G6PD-deficiency [22], Wilsons disease [98], as well as advanced age [99]. Eryptosis further increases following erythrocyte storage for transfusion [100].

The present study explored, whether volasertib impacts on eryptosis. To this end, human erythrocytes from healthy volunteers were exposed to volasertib in the absence and presence of energy depletion, hyperosmotic shock, oxidative stress, as well as Ca^{2+} loading. Phosphatidylserine surface abundance and cell volume were determined by flow cytometry. For comparison, the effect of volasertib was tested on annexin-V-binding and forward scatter of human leukemic K562 cells.

Materials and Methods

Erythrocytes, solutions and chemicals

Fresh Li-Heparin-anticoagulated blood samples were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003 V). The blood was centrifuged at 120 g for 20 min at 21 °C and the platelets and leukocytes-containing supernatant was disposed. Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, 1 CaCl_2 , at 37°C for 48 hours. Where indicated, erythrocytes were exposed for 48 hours to glucose depleted Ringer solution, for 6 hours to hypertonic Ringer (addition of 550 mM sucrose, Sigma Aldrich, Hamburg, Germany), for 50 minutes to the oxidant tert-butyl-hydroperoxide (0.3 mM, Sigma Aldrich, Hamburg, Germany), or for 60 minutes to Ca^{2+} ionophore ionomycin (1 μM , Merck Millipore, Darmstadt, Germany), each in the absence and presence of volasertib (MedChem Express, Princeton, USA).

K562 cell culture and treatments

The K562 human leukaemia cells (Sigma Aldrich, Hamburg, Germany) were cultured using RPMI-1640 medium (Biochrom GmbH, Berlin, Germany) supplemented with 2.0 g/l NaHCO_3 , without L-glutamine. The

cells were cultured at 37°C in a moistened incubator with 5% CO₂. Where indicated, volasertib (0.5 – 1.5 µg/ml) was added to the cell culture medium.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 150 µl cell suspension was washed in Ringer solution containing 5 mM CaCl₂ and then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37°C for 15 min under protection from light. The annexin-V-abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). Annexin-V-binding was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A marker (M1) was placed to set an arbitrary threshold between annexin-V-binding cells and control cells. The same threshold was used for untreated and volasertib treated erythrocytes. A dot plot of forward scatter (FSC) vs. side scatter (SSC) was set to linear scale for both parameters. The threshold of forward scatter was set at the default value of “52”.

Intracellular Ca²⁺

After incubation, erythrocytes were washed in Ringer solution and loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM CaCl₂ and 5 µM Fluo-3/AM. The cells were incubated at 37°C for 30 min. Ca²⁺-dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur. Afterwards, the geomean of the Ca²⁺ dependent fluorescence was determined.

Reactive oxygen species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 150 µl suspension of erythrocytes was washed in Ringer solution and stained with DCFDA (Sigma, Aldrich, Germany) in Ringer solution containing 10 µM DCFDA. Erythrocytes were incubated at 37°C for 30 min in the dark and washed two times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 µl Ringer solution and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD). Subsequently, the geomean of the DCFDA dependent fluorescence was determined.

Ceramide abundance

For the determination of ceramide, a monoclonal antibody-based assay was used. To this end, cells were stained for 1 hour at 37°C with 1 µg/ml anti ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. The samples were washed twice with PBS-BSA. The cells were stained for 30 minutes with polyclonal fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. The samples were analysed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Finally, the geomean of the ceramide-dependent fluorescence was determined.

Statistics

Data are expressed as arithmetic means ± SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test. n denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study explored, whether volasertib stimulates or interferes with eryptosis, the suicidal erythrocyte death. The two hallmarks of eryptosis, i.e. cell membrane scrambling and cell shrinkage were determined by flow cytometry. Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding to phosphatidylserine, cell shrinkage was quantified utilizing forward scatter.

Fig. 1. Volasertib sensitivity of phosphatidylserine exposure following energy depletion. A. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to glucose containing Ringer solution (grey areas), Ringer solution without glucose (green areas) and Ringer solution without glucose and presence of volasertib (1.5 µg/ml) (blue areas). B. Arithmetic means $\pm$ SEM ($n = 14$) of the percentage of annexin-V-binding erythrocytes after a 48 hours treatment with Ringer solution with glucose (grey bar) or without glucose in the absence (green bar) and presence (blue bars) of volasertib (0.5-1.5 µg/ml). Striped bar is in absence of glucose and presence of DMSO. ***($p < 0.001$) indicates significant difference from the presence of glucose, ##($p < 0.01$), ###($p < 0.001$) indicates significant difference from the absence of volasertib (ANOVA).

Fig. 2. Volasertib sensitivity of erythrocyte shrinkage following energy depletion. A. Original histograms of forward scatter of erythrocytes following exposure for 48 hours to glucose containing Ringer solution (grey areas), Ringer solution without glucose (green areas) and Ringer solution without glucose and presence of volasertib (1.5 µg/ml) (blue areas). B. Arithmetic means $\pm$ SEM ($n = 14$) of the erythrocyte forward scatter after a 48 hours treatment with Ringer solution with glucose (grey bar) or without glucose in the absence (green bar) and presence (blue bars) of volasertib (0.5-1.5 µg/ml). Striped bar is in absence of glucose and presence of DMSO. ***($p < 0.001$) indicates significant difference from the presence of glucose (ANOVA).

The percentage of human erythrocytes binding annexin-V after 48 hours incubation in standard, glucose containing, Ringer was low ($2.4 \pm 0.3\%$, $n = 14$) and not significantly modified by the presence of 1.5 µg/ml volasertib ($2.5 \pm 0.4\%$, $n = 14$). Thus, in standard glucose containing Ringer solution, volasertib did not appreciably modify erythrocyte cell membrane scrambling. As illustrated in Fig. 1, glucose removal was followed by a marked increase of the percentage of annexin-V-binding erythrocytes. The addition of volasertib (0.5-1.5 µg/ml) blunted the increase of the percentage of annexin-V-binding erythrocytes following glucose deprivation, an effect reaching statistical significance at 1 and 1.5 µg/ml volasertib. Nevertheless, even in the presence of volasertib, energy depletion significantly increased the percentage of phosphatidylserine exposing erythrocytes (Fig. 1). Volasertib thus partially blunted cell membrane scrambling following energy depletion.

Fig. 3. Effect of Volasertib on phosphatidylserine exposure of K562 cells. A. Original histograms of annexin-V-binding of K562 cells following exposure for 48 hours to RPMI 1640 medium without (grey area) and with presence of volasertib (1.5 µg/ml) (blue areas). B. Arithmetic means $\pm$ SEM (n = 10) of the percentage of annexin-V-binding K562 cells following incubation for 48 hours to RPMI 1640 medium without (grey bar) or with (blue bars) Volasertib (0.5 – 1.5 µg/ml). For comparison, the effect of the solvent DMSO is shown (Striped bar). ***($p < 0.001$) indicates significant difference from the absence of volasertib (ANOVA).

Fig. 4. Effect of Volasertib on K562 cell forward scatter (FSC). A. Original histograms of forward scatter reflecting cell volume of K562 cells following exposure for 48 hours to RPMI 1640 medium without (grey area) and with presence of volasertib (1.5 µg/ml) (blue areas). B. Arithmetic means $\pm$ SEM (n = 10) of K562 cell forward scatter (FSC) following incubation for 48 hours to RPMI 1640 medium without (grey bar) or with (blue bars) volasertib (0.5 – 1.5 µg/ml). For comparison, the effect of the solvent DMSO is shown (Striped bar). *($p < 0.05$) indicates significant difference from the absence of volasertib (ANOVA).

Erythrocyte forward scatter was, following a 48 hours exposure to Ringer solution in the presence of glucose, again similar in the absence ($517 \pm 6\%$, n = 14) and presence ($522 \pm 5.4\%$, n = 14) of volasertib (1.5 µg/ml). Accordingly, in standard glucose containing Ringer solution, volasertib did not appreciably modify erythrocyte volume. As illustrated in Fig. 2, a 48 hours exposure to glucose-depleted Ringer solution was followed by a marked decrease of forward scatter, reflecting erythrocyte shrinkage. The decline of erythrocyte forward scatter was, following a 48 hours exposure to Ringer solution in the absence of glucose, virtually identical in the absence and presence of volasertib (0.5–1.5 µg/ml). Accordingly, neither in energy repleted nor in energy depleted erythrocytes did volasertib significantly modify erythrocyte volume.

As volasertib did not, in contrast to previous observations in nucleated cells, significantly stimulate eryptosis, further experiments were performed exploring whether

Fig. 5. Volasertib sensitivity of cytosolic Ca^{2+} activity following energy depletion. A. Original histograms of Fluo3 fluorescence reflecting cytosolic Ca^{2+} activity of erythrocytes following exposure for 48 hours to glucose containing Ringer solution (grey areas), Ringer solution without glucose (green areas) and Ringer solution without glucose and presence of volasertib (1.5 $\mu\text{g/ml}$) (blue areas). B. Arithmetic means $\pm$ SEM ($n = 14$) of erythrocyte Fluo3 fluorescence after a 48 hours treatment with Ringer solution with glucose (grey bar) or without glucose in the absence (green bar) and presence (blue bars) of volasertib (0.5–1.5 $\mu\text{g/ml}$). Striped bar is in absence of glucose and presence of DMSO. ***($p < 0.001$) indicates significant difference from the presence of glucose.

volasertib stimulated apoptosis of K562 cells. As shown in Fig. 3, a 48 hours exposure to volasertib increased the percentage of phosphatidylserine exposing K562 cells at each of the concentrations applied (0.5–1.5 $\mu\text{g/ml}$) and as illustrated in Fig. 4, volasertib triggered cell shrinkage, an effect reaching statistical significance at 0.5 $\mu\text{g/ml}$ and 1.5 $\mu\text{g/ml}$ volasertib.

Further experiments addressed the influence of volasertib on cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), which was estimated from Fluo3 fluorescence. As illustrated in Fig. 5, a 48 hours exposure to glucose depleted Ringer solution was followed by a marked significant increase of $[\text{Ca}^{2+}]_i$ both, in the absence and presence of volasertib. The addition of volasertib (0.5–1.5 $\mu\text{g/ml}$) did not significantly modify $[\text{Ca}^{2+}]_i$ in erythrocytes without or with energy depletion.

Reactive oxygen species (ROS) was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After 48 hours incubation in standard glucose containing Ringer, the ROS was similar in the absence (19.07 ± 0.64 , $n = 5$) and presence (18.63 ± 0.46 , $n = 5$) of 1.5 $\mu\text{g/ml}$ volasertib. As illustrated in Fig. 6, removal of glucose was followed by a marked significant increase of DCFDA fluorescence in the absence and presence of volasertib (1.5 $\mu\text{g/ml}$). The addition of volasertib did not significantly modify DCFDA fluorescence following glucose depletion.

The abundance of ceramide at the erythrocyte surface was quantified utilizing specific antibodies. The abundance of ceramide was similar following a 48 hours incubation in standard glucose containing Ringer (10.11 ± 0.24 , $n = 5$) and in glucose depleted Ringer without (10.1 ± 0.2 , $n = 5$) and with (9.8 ± 0.2 , $n = 5$) addition of volasertib.

A next series of experiments addressed the effect of hyperosmotic shock (addition of 550 mM sucrose) for 6 hours. As illustrated in Fig. 7, hyperosmotic shock was followed by a marked increase of the percentage of annexin-V-binding erythrocytes. The addition of volasertib (0.5–1.5 $\mu\text{g/ml}$) blunted the increase of the percentage of annexin-V-binding erythrocytes following hyperosmotic shock, an effect reaching statistical significance at 1 and 1.5 $\mu\text{g/ml}$ volasertib. Again, even in the presence of volasertib, hyperosmotic shock significantly increased the percentage of phosphatidylserine exposing erythrocytes (Fig. 7). Volasertib thus partially blunted cell membrane scrambling following hyperosmotic shock.

Hyperosmotic shock was further followed by a marked decrease of forward scatter, reflecting erythrocyte shrinkage. The erythrocyte shrinkage following hyperosmotic shock was virtually identical in the absence and presence of volasertib (0.5–1.5 $\mu\text{g/ml}$) (Fig. 8). Accordingly, volasertib did not significantly interfere with the effect of hyperosmotic shock on erythrocyte volume.

Fig. 6. Volasertib sensitivity of reactive oxygen species following energy depletion. A. Original histograms of DCFDA fluorescence reflecting reactive oxygen species in erythrocytes following exposure for 48 hours to glucose containing Ringer solution (grey areas), Ringer solution without glucose (green areas) and Ringer solution without glucose and presence of volasertib (1.5 µg/ml) (blue areas). B. Arithmetic means $\pm$ SEM ($n = 5$) of DCFDA fluorescence in erythrocytes following a 48 hours treatment with Ringer solution with glucose (grey bar) or without glucose in the absence (green bar) and presence (blue bars) of volasertib (1.5 µg/ml). **($p < 0.01$) indicates significant difference from the presence of glucose.

Fig. 7. Volasertib sensitivity of phosphatidylserine exposure following hyperosmotic shock. A. Original histograms of annexin-V-binding of erythrocytes following exposure for 6 hours to isotonic Ringer solution (grey area), or hypertonic Ringer solution (550 mM sucrose added) in absence (green areas) or presence (blue areas) of volasertib (1.5 µg/ml). B. Arithmetic means $\pm$ SEM ($n = 10$) of the percentage of annexin-V-binding erythrocytes after a 6 hours treatment with isotonic Ringer solution (grey bar), or hypertonic Ringer solution (550 mM sucrose added) in the absence (green bar) and presence (blue bars) of volasertib (0.5-1.5 µg/ml). Striped bar is in presence of tert-butylhydroperoxide and DMSO. ***($p < 0.001$) indicates significant difference from isotonic Ringer, ##($p < 0.01$) indicates significant difference from the absence of volasertib (ANOVA).

A next series of experiments addressed the effect of oxidative stress due to addition of 0.3 mM tert-butylhydroperoxide (tBOOH) for 50 minutes. As illustrated in Fig. 9, oxidative stress was followed by a marked increase of the percentage of annexin-V-binding erythrocytes. The addition of volasertib (0.5-1.5 µg/ml) did not significantly interfere with the increase of the percentage of annexin-V-binding erythrocytes following oxidative stress. Instead, oxidative stress significantly increased the percentage of phosphatidylserine exposing erythrocytes both, in the absence and presence of volasertib (Fig. 9).

Oxidative stress was further followed by a marked decrease of forward scatter, reflecting erythrocyte shrinkage. The erythrocyte shrinkage following oxidative stress was virtually

Fig. 8. Volasertib sensitivity of erythrocyte shrinkage following hyperosmotic shock. A. Original histograms of erythrocyte forward scatter following exposure for 6 hours to isotonic Ringer solution (grey area), or hypertonic Ringer solution (550 mM sucrose added) in absence (green areas) or presence (blue areas) of volasertib (1.5 µg/ml). B. Arithmetic means $\pm$ SEM ($n = 10$) of erythrocyte forward scatter after a 6 hours treatment with isotonic Ringer solution (grey bar), or hypertonic Ringer solution (550 mM sucrose added) in the absence (green bar) and presence (blue bars) of volasertib (0.5-1.5 µg/ml). Striped bar is in presence of tert-butylhydroperoxide and DMSO. ***($p < 0.001$) indicates significant difference from isotonic Ringer (ANOVA).

Fig. 9. Volasertib sensitivity of phosphatidylserine exposure following oxidative stress. A. Original histograms of annexin-V-binding of erythrocytes following exposure for 50 min to Ringer solution without tert-butylhydroperoxide (grey area), or Ringer solution with tert-butylhydroperoxide (0.3 mM) and absence (green areas) or presence (blue areas) of volasertib (1.5 µg/ml). B. Arithmetic means $\pm$ SEM ($n = 15$) of the percentage of annexin-V-binding erythrocytes after a 50 min treatment with Ringer solution without tert-butylhydroperoxide (grey bar) or with tert-butylhydroperoxide (0.3 mM) in the absence (green bar) and presence (blue bars) of volasertib (0.5-1.5 µg/ml). Striped bar is in presence of tert-butylhydroperoxide and DMSO. ***($p < 0.001$) indicates significant difference from the absence of tert-butylhydroperoxide (ANOVA).

identical in the absence and presence of volasertib (0.5-1.5 µg/ml). Accordingly, volasertib did not significantly interfere with the effect of oxidative stress on erythrocyte volume (Fig. 10).

A next series of experiments explored the effect of Ca^{2+} entry, which was achieved by a 60 min exposure of erythrocytes to Ca^{2+} ionophore ionomycin (1 µM). As illustrated in Fig. 11, exposure of the erythrocytes for 60 minutes to 1 µM ionomycin was followed by a sharp increase of the percentage of annexin-V-binding erythrocytes. The effect tended to be slightly blunted by volasertib, an effect, however, not reaching statistical significance. Instead, even in the presence of volasertib, ionomycin significantly increased the percentage of phosphatidylserine exposing erythrocytes (Fig. 11).

Exposure of the erythrocytes for 60 minutes to 1 µM ionomycin was followed by a sharp decrease of forward scatter (Fig. 12). The cell shrinkage was not significantly modified by volasertib (Fig. 12).

Fig. 10. Volasertib sensitivity of erythrocyte shrinkage following oxidative stress. A. Original histograms of erythrocyte forward scatter following exposure for 50 min to Ringer solution without tert-butylhydroperoxide (grey area), or Ringer solution with tert-butylhydroperoxide (0.3 mM) and absence (green areas) or presence (blue areas) of volasertib (1.5 µg/ml). B. Arithmetic means $\pm$ SEM (n = 15) of erythrocyte forward scatter after a 50 min treatment with Ringer solution without tert-butylhydroperoxide (grey bar) or with tert-butylhydroperoxide (0.3 mM) in the absence (green bar) and presence (blue bars) of volasertib (0.5-1.5 µg/ml). Striped bar is in presence of tert-butylhydroperoxide and DMSO. ***($p < 0.001$) indicates significant difference from absence of tert-butylhydroperoxide (ANOVA).

Fig. 11. Volasertib sensitivity of phosphatidylserine exposure following Ca^{2+} loading. A. Original histograms of annexin-V-binding of erythrocytes following exposure for 60 min to Ringer solution without Ca^{2+} ionophore ionomycin (grey area), or Ringer solution with ionomycin (1 µM) and absence (green areas) or presence (blue areas) of volasertib (1.5 µg/ml). B. Arithmetic means $\pm$ SEM (n = 10) of the percentage of annexin-V-binding erythrocytes after a 60 min treatment with Ringer solution without ionomycin (grey bar) or with ionomycin (1 µM) in the absence (green bar) and presence (blue bars) of volasertib (0.5-1.5 µg/ml). Striped bar is in presence of ionomycin and DMSO. ***($p < 0.001$) indicates significant difference from the absence of ionomycin (ANOVA).

Discussion

The present observations reveal a novel, unexpected effect of volasertib on erythrocyte cell membrane scrambling, which is a hallmark of eryptosis, the suicidal erythrocyte death. Treatment with volasertib alone did not significantly modify annexin-V-binding or forward scatter, but volasertib significantly blunted the stimulating effect of energy depletion and hyperosmotic shock on cell membrane scrambling. The volasertib concentrations required for this effect were in the range of concentrations (up to 1.45 µg/ml) encountered in the plasma of patients [21]. Volasertib did not significantly interfere with the stimulating effect of oxidative stress and ionomycin on cell membrane scrambling and did not significantly interfere with the cell shrinkage following energy depletion, hyperosmotic shock, oxidative stress and ionomycin.

Fig. 12. Volasertib sensitivity of erythrocyte shrinkage following Ca^{2+} loading. A. Original histograms of erythrocyte forward scatter following exposure for 60 min to Ringer solution without ionomycin (grey area), or Ringer solution with ionomycin (1 μM) and absence (green areas) or presence (blue areas) of volasertib (1.5 $\mu\text{g}/\text{ml}$). B. Arithmetic means $\pm$ SEM ($n = 10$) of erythrocyte forward scatter after a 60 min treatment with Ringer solution without ionomycin (grey bar) or with ionomycin (1 μM) in the absence (green bar) and presence (blue bars) of volasertib (0.5-1.5 $\mu\text{g}/\text{ml}$). Striped bar is in presence of ionomycin and DMSO. ***($p < 0.001$) indicates significant difference from the absence of ionomycin (ANOVA).

The failure of volasertib to interfere with cell shrinkage could be explained by its inability to interfere with increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). An increase of $[\text{Ca}^{2+}]_i$ activates Ca^{2+} sensitive K^+ channels leading to K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [22].

The inhibitory effect of volasertib on cell membrane scrambling following energy depletion and hyperosmotic shock contrasts the stimulating effect of volasertib on apoptosis of nucleated cells, as shown here for K562 cells. While apoptosis and eryptosis share the eventual stimulation of cell membrane scrambling, the signaling triggering cell membrane scrambling differs considerably [22, 101]. Along those lines, apoptosis following inhibition of polo-like kinases may involve mitochondria [102], which are absent in mature erythrocytes. In view of the stimulation of tumor cell death and the simultaneous protection against eryptosis of energy or shrunken erythrocytes, volasertib may be particularly useful in the treatment of malignancies associated with anemia [103].

The present study did not uncover the signaling involved in the effect of volasertib on cell membrane scrambling following energy depletion and hyperosmotic shock. As shown for energy depletion, volasertib did not significantly interfere with the increase of cytosolic Ca^{2+} activity and did not modify ceramide formation. In theory, eryptosis could involve Polo-like kinase 1 (Plk1), which is specifically inhibited by volasertib [1-10]. However, the effects of volasertib are not restricted to the polo-like kinase 1 (Plk1) isoform but the substance inhibits, albeit to a lesser extent, the isoforms Plk2 and Plk3 [8]. Moreover, other effects unrelated to Polo-like kinases cannot be ruled out. Further experimental effort is required to identify the mechanisms accomplishing the inhibitory effect of volasertib on eryptosis following energy depletion and hyperosmotic shock. Moreover, additional analysis may be required to understand why volasertib is effective following energy depletion and hyperosmotic shock, but not following oxidative stress and ionomycin. The discrepancy points to volasertib sensitive signaling triggering cell membrane scrambling, which is shared by energy depletion and hyperosmotic shock but not by oxidative stress and Ca^{2+} overload.

Whatever mechanism involved, volasertib is inhibiting rather than stimulating erythrocyte cell membrane scrambling. The observed enhanced incidence of anemia in volasertib-combination treatments may be attributed to the effects of the combined drug. Alternatively, volasertib triggers apoptosis of progenitor cells thus compromising formation of new erythrocytes. If so, administration of the drug should decrease reticulocyte numbers in circulating blood. To the best of our knowledge, nothing is known about an

effect of volasertib on reticulocytes. In any case, inhibition of eryptosis counteracts anemia. Phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood and eryptosis leads to anemia as soon as the loss of eryptotic erythrocytes outcasts the formation of new erythrocytes by erythropoiesis [22].

As volasertib did not interfere with cell shrinkage, it presumably does not foster hemolysis. Hemoglobin released following hemolysis may otherwise pass the renal glomerular filter, precipitate in the acidic lumen of renal tubules, occlude nephrons and may thus lead to renal failure [104].

As phosphatidylserine exposing erythrocytes further adhere to the vascular wall [105], stimulate blood clotting/thrombosis [106-108], and interfere with microcirculation [25, 106, 109-112], the inhibition of eryptosis may decrease cardiovascular events in tumor patients.

In conclusion, volasertib inhibits erythrocyte cell membrane scrambling following energy depletion and hyperosmotic shock and thus rather counteracts suicidal erythrocyte death and development of anemia.

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Disclosure Statement

The authors of this manuscript state that they have no conflicts of interest to declare.

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