Original Paper

Sonidegib, a Novel Inhibitor of Suicidal Erythrocyte Death

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Key Words

Phosphatidylserine • Eryptosis • Energy depletion • Oxidative stress • Hyperosmotic shock • Ionomycin • Calcium

Abstract

Background/Aims: The Hedgehog pathway disrupting drug sonidegib is used in the treatment of basal cell carcinoma. Side effects of sonidegib include anemia, which could result either from impaired erythropoiesis or from loss of erythrocytes e.g. due to suicidal erythrocyte death or eryptosis, which is characterized by cell membrane scrambling with phosphatidylserine translocation to the cell surface and by cell shrinkage. Eryptosis is stimulated by cell stress, including energy depletion, hyperosmotic shock, oxidative stress and excessive increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). The present study explored, whether sonidegib exerts an effect on eryptosis. **Methods:** Human erythrocytes have been treated with 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 sonidegib (2–6 $\mu\text{g}/\text{ml}$). After treatment flow cytometry was employed to quantify phosphatidylserine exposure at the cell surface from annexin-V-binding, and cell volume from forward scatter. Hemolysis was estimated from the hemoglobin concentration in the supernatant. **Results:** In the absence of cell stress exposure to sonidegib did not significantly modify annexin-V-binding or forward scatter, but triggered hemolysis. 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. Sonidegib significantly blunted the effect of energy depletion, hyperosmotic shock, and oxidative stress, but not of ionomycin on annexin-V-binding. Sonidegib further significantly blunted the effect of energy depletion, but not of hyperosmotic shock, oxidative stress, and ionomycin on forward scatter. **Conclusions:** Sonidegib is a novel inhibitor of erythrocyte cell membrane scrambling following energy depletion, hyperosmotic shock and oxidative stress.

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Introduction

The smoothened (SMO) inhibitor sonidegib interferes with the Hedgehog pathway [1] and is used in the treatment of basal cell carcinoma [1-11]. Side effects of the drug include anemia [1], which, at least in theory, could result from stimulation of eryptosis, the suicidal erythrocyte death [12-16]. A hallmark of eryptosis is cell membrane scrambling with phosphatidylserine translocation to the cell surface, which is characterized by cell membrane scrambling with phosphatidylserine translocation to the cell surface [12]. A second hallmark of eryptosis is cell shrinkage due to activation of ion channels in the cell membrane [17]. Stimulators of eryptosis include energy depletion [12], hyperosmotic shock [12], and oxidative stress [12]. Signaling participating in the orchestration of eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [12], ceramide [18], caspases [12, 19, 20], G-protein $\text{G}\alpha_{i2}$ [21], CDK4 [22] casein kinase 1 α [12], Janus-activated kinase JAK3 [12], protein kinase C [12], and p38 kinase [12]. Signaling mechanisms inhibiting eryptosis include the kinases AMPK [12], cGMP-dependent protein kinase [12], MSK1/2 [23], PAK2 [12] and sorafenib/sunitinib sensitive kinases [12]. Eryptosis is stimulated by a wide variety of xenobiotics [12, 23-77] and enhanced eryptosis is observed in several clinical conditions including iron deficiency [12], dehydration [78], hyperphosphatemia [68], vitamin D excess [32], chronic kidney disease (CKD) [79-83] hemolytic-uremic syndrome [84], diabetes [85], hepatic failure [86, 87], malignancy [88, 89], autoimmune hemolytic anemia [90], lack of sialic acid [91], arteriitis [92], sepsis [93], sickle-cell disease [12], beta-thalassemia [12], Hb-C and G6PD-deficiency [12], Wilsons disease [94], as well as advanced age [95]. Eryptosis further increases following storage for transfusion [96-98].

The present study explored, whether sonidegib influences eryptosis. Thus, human erythrocytes have been exposed to sonidegib without or with additional exposure to energy depletion, hyperosmotic shock, oxidative stress, as well as Ca^{2+} loading. In order to identify eryptotic erythrocytes, phosphatidylserine surface abundance and cell volume were determined by flow cytometry.

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 sonidegib (MedChem Express, Princeton, USA).

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 CaCl_2 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 sonidegib 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 Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM $CaCl_2$ and 5 μ M Fluo-3/AM. The cells were incubated at 37°C for 30 min. Ca^{2+} -dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Hemolysis

Following incubation, the erythrocyte suspension was centrifuged for 3 min at 1600 rpm, 4°C, and the supernatant harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration in the supernatant was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

Statistics

Data are expressed as arithmetic means $\pm$ 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 addressed a putative influence of sonidegib on eryptosis, the suicidal erythrocyte death. Thus, the key features of eryptosis, i.e. cell membrane scrambling and cell shrinkage were quantified by flow cytometry. Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding to phosphatidylserine, cell volume was estimated utilizing forward scatter.

The percentage of human erythrocytes binding annexin-V after 48 hours incubation in standard, glucose containing, Ringer was low ($1.58 \pm 0.08\%$, n = 14) and not significantly

Fig. 1. Sonidegib 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 area), Ringer solution without glucose (black solid line) and Ringer solution without glucose and presence of sonidegib (6 μ g/ml) (black dashed line). B. Arithmetic means $\pm$ SEM (n = 14) of the percentage annexin-V-binding erythrocytes after a 48 hours treatment with Ringer solution with glucose (white bar) or without glucose in the absence (black bar) and presence (grey bars) of sonidegib (2-6 μ g/ml). Striped bar is in absence of glucose and presence of DMSO. ***($p < 0.001$) indicates significant difference from the presence of glucose, ###($p < 0.001$) indicates significant difference from the absence of sonidegib (ANOVA).

Fig. 2. Sonidegib 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 area), Ringer solution without glucose (black solid line) and Ringer solution without glucose and presence of sonidegib (6 µg/ml) (black dashed line). B. Arithmetic means $\pm$ SEM ($n = 14$) of the erythrocyte forward scatter after a 48 hours treatment with Ringer solution with glucose (white bar) or without glucose in the absence (black bar) and presence (grey bars) of sonidegib (2-6 µg/ml). Striped bar is in absence of glucose and presence of DMSO. ***($p < 0.001$) indicates significant difference from the presence of glucose, ###($p < 0.001$) indicates significant difference from the absence of sonidegib (ANOVA).

altered by addition of 6 µg/ml sonidegib ($3.45 \pm 0.50\%$, $n = 14$). The percentage of hemolytic erythrocytes after 48 hours incubation in standard, glucose containing, Ringer was similarly low ($3.11 \pm 0.36\%$, $n = 5$), but significantly increased by addition of 6 µg/ml sonidegib ($28.47 \pm 3.37\%$, $n = 5$). Accordingly, in standard glucose containing Ringer solution, sonidegib did not appreciably influence erythrocyte cell membrane scrambling, but triggered hemolysis. As shown in Fig. 1, glucose removal was followed by a marked increase of the percentage of annexin-V-binding erythrocytes. At all sonidegib concentrations tested (2-6 µg/ml), the addition of the drug blunted the increase of the percentage annexin-V-binding erythrocytes following glucose deprivation. However, even in the presence of sonidegib, energy depletion significantly increased the percentage of phosphatidylserine exposing erythrocytes (Fig. 1). Accordingly, energy depletion triggered cell membrane scrambling, an effect partially, but not completely blunted in the presence of sonidegib.

Erythrocyte forward scatter reflecting erythrocyte volume was, following a 48 hours exposure to Ringer solution in the presence of glucose, again similar in the absence (517.30 ± 4.76 a.u., $n = 14$) and presence (498.50 ± 7.14 a.u., $n = 14$) of sonidegib (6 µg/ml). Accordingly, in standard glucose containing Ringer solution, sonidegib did not appreciably modify erythrocyte volume. As illustrated in Fig. 2, glucose removal was followed by a marked decrease of erythrocyte forward scatter, an observation pointing to marked cell shrinkage. At each sonidegib concentration tested (2-6 µg/ml), the addition of the drug slightly, but significantly, blunted the decrease of forward scatter following glucose deprivation. However, even in the presence of sonidegib, energy depletion was followed by a significant decrease of forwards scatter (Fig. 2). Accordingly, energy depletion triggered cell shrinkage, an effect partially, but not completely blunted in the presence of sonidegib.

Fluo3 fluorescence was employed to test, whether sonidegib modifies the influence of energy depletion on cytosolic Ca^{2+} activity. As a result, a 48 hours glucose deprivation increased Fluo3 fluorescence from 20.91 ± 0.57 ($n = 14$) to similarly high values in the absence (61.19 ± 2.81 , $n = 14$) and presence of 2 (54.7 ± 2.76 , $n = 14$), 4 (50.08 ± 2.33 , $n = 14$), or 6 (51.89 ± 2.54 , $n = 14$) µg/ml sonidegib.

Fig. 3. Sonidegib 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 (solid black line) or presence (black dashed line) of sonidegib (6 µg/ml). B. Arithmetic means $\pm$ SEM ($n = 15$) of the percentage annexin-V-binding erythrocytes after a 6 hours treatment with isotonic Ringer solution (white bar), or hypertonic Ringer solution (550 mM sucrose added) in the absence (black bar) and presence (grey bars) of sonidegib (2-6 µg/ml). Striped bar is in presence of hypertonic solution and DMSO. ***($p < 0.001$) indicates significant difference from isotonic Ringer, #($p < 0.05$), ##($p < 0.01$) indicates significant difference from the absence of sonidegib (ANOVA).

Fig. 4. Sonidegib 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 (solid black line) or presence (black dashed line) of sonidegib (6 µg/ml). B. Arithmetic means $\pm$ SEM ($n = 9$) of the percentage annexin-V-binding erythrocytes after a 50 min treatment with Ringer solution without tert-butylhydroperoxide (white bar) or with tert-butylhydroperoxide (0.3 mM) in the absence (black bar) and presence (grey bars) of sonidegib (2-6 µg/ml). Striped bar is in presence of tert-butylhydroperoxide and DMSO. ***($p < 0.001$) indicates significant difference from the absence of tert-butylhydroperoxide, #($p < 0.05$) indicates significant difference from the absence of sonidegib (ANOVA).

In order to explore the influence of sonidegib on the eryptosis following hyperosmotic shock the erythrocytes were exposed 6 hours to hypertonic Ringer (addition of 550 mM sucrose). As shown in Fig. 3, hyperosmotic shock was followed by a marked increase of the percentage of annexin-V-binding erythrocytes. The addition of sonidegib blunted the increase of the percentage annexin-V-binding erythrocytes following hyperosmotic shock at all sonidegib concentrations tested (2-6 µg/ml). However, even in the presence of sonidegib, hyperosmotic shock significantly increased the percentage of phosphatidylserine exposing

Fig. 5. Sonidegib 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 (solid black line) or presence (black dashed line) of sonidegib (6 $\mu\text{g}/\text{ml}$). B. Arithmetic means $\pm$ SEM ($n = 14$) of the percentage annexin-V-binding erythrocytes after a 60 min treatment with Ringer solution without ionomycin (white bar) or with ionomycin (1 μM) in the absence (black bar) and presence (grey bars) of sonidegib (2–6 $\mu\text{g}/\text{ml}$). Striped bar is in presence of ionomycin and DMSO. ***($p < 0.001$) indicates significant difference from the absence of ionomycin (ANOVA).

erythrocytes (Fig. 3). Accordingly, hyperosmotic shock triggered cell membrane scrambling, an effect partially, but not completely blunted in the presence of sonidegib.

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 (378.5 ± 10.58 , $n = 15$) and presence of 2 (394.2 ± 10.3 , $n = 15$), 4 (389.9 ± 9.69 , $n = 15$), or 6 (384 ± 9 , $n = 15$) $\mu\text{g}/\text{ml}$ sonidegib. Accordingly, sonidegib did not significantly interfere with the effect of hyperosmotic shock on erythrocyte volume.

Further experiments addressed the influence of sonidegib on the eryptosis following oxidative stress. To this end, oxidative stress was induced by addition of 0.3 mM tert-butylhydroperoxide (tBOOH) for 50 minutes. As illustrated in Fig. 4, oxidative stress was followed by a marked increase of the percentage of annexin-V-binding erythrocytes. The addition of sonidegib blunted the increase of the percentage annexin-V-binding erythrocytes following hyperosmotic shock at all sonidegib concentrations tested (2–6 $\mu\text{g}/\text{ml}$). However, even in the presence of sonidegib, oxidative stress significantly increased the percentage of phosphatidylserine exposing erythrocytes (Fig. 5). Accordingly, hyperosmotic shock triggered cell membrane scrambling, an effect partially, but not completely blunted in the presence of sonidegib.

Oxidative stress was further followed by a marked decrease of forward scatter, reflecting erythrocyte shrinkage. The erythrocyte shrinkage following oxidative stress was virtually identical in the absence (353.6 ± 5.09 , $n = 9$) and presence of 2 (355.3 ± 3.62 , $n = 9$), 4 (357.5 ± 6.40 , $n = 9$), or 6 (356.1 ± 4.48 , $n = 9$) $\mu\text{g}/\text{ml}$ sonidegib. Accordingly, sonidegib did not significantly interfere with the effect of oxidative stress on erythrocyte volume.

A next series of experiments explored the influence of sonidegib on the eryptosis following Ca^{2+} entry, which was achieved by a 60 min exposure of erythrocytes to Ca^{2+} ionophore ionomycin (1 μM). As illustrated in Fig. 5, 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 sonidegib, an effect, however, not reaching statistical significance. Instead, even in the presence of sonidegib, ionomycin significantly increased the percentage of phosphatidylserine exposing erythrocytes (Fig. 5).

Exposure of the erythrocytes for 60 minutes to 1 μM ionomycin was further followed by a sharp decrease of forward scatter to similarly low values in the absence (118.8 ± 8.89 , $n = 14$) and presence of 2 (98.48 ± 2.08 , $n = 14$), 4 (99.52 ± 1.78 , $n = 14$), or 6 (99.33 ± 1.55 ,

n = 14) $\mu\text{g/ml}$ sonidegib. The ionomycin induced cell shrinkage was thus not significantly modified by sonidegib.

Discussion

The present observations reveal a novel, completely unexpected effect of sonidegib on suicidal erythrocyte death. Treatment with sonidegib alone did not significantly modify the phosphatidylserine asymmetry of the cell membrane and cell volume. Instead, sonidegib significantly blunted the stimulating effect of energy depletion, hyperosmotic shock, and oxidative stress on cell membrane scrambling. The sonidegib concentrations required for this effect were in the range of concentrations (778 - 6024 ng/ml) encountered in the plasma of patients treated with sonidegib [99]. Sonidegib did not significantly interfere with the stimulating effect of ionomycin on cell membrane scrambling and did not significantly interfere with the cell shrinkage following hyperosmotic shock, oxidative stress and ionomycin. The insensitivity of cell volume to sonidegib may be due to the lack of effect on cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). A major mechanism accounting for cell shrinkage during eryptosis is increase of $[\text{Ca}^{2+}]_i$ with subsequent activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water. The mechanism accounting for the inhibitory effect of sonidegib on cell membrane scrambling remained, however elusive.

The present study did not elucidate the sonidegib dependent signaling accounting for or contributing to the inhibitory effect of sonidegib on cell membrane scrambling following energy depletion, hyperosmotic shock and oxidative stress. At least in theory, sonidegib could have been effective by inhibition of smoothened and thus by interference with the hedgehog pathway, which plays a decisive role in erythroid differentiation [100, 101]. How hedgehog signaling could interfere with triggering of cell membrane scrambling following energy depletion, hyperosmotic shock and oxidative stress, remains elusive. Clearly, the effect of sonidegib on cell membrane scrambling was not due to an effect on $[\text{Ca}^{2+}]_i$. Moreover, sonidegib did not interfere with the effect of ionomycin. Possibly, sonidegib is effective by modifying Ca^{2+} sensitivity of cell membrane scrambling, an effect overridden by ionomycin.

As sonidegib is inhibiting rather than stimulating eryptosis, the enhanced incidence of anemia following treatment with sonidegib cannot be explained by stimulation of eryptosis. As phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood [12], anemia may result from stimulation but not inhibition of eryptosis. Presumably, the anemia following sonidegib treatment is the result of hemolysis or of impaired erythroid differentiation and erythropoiesis [100, 101].

Sonidegib slightly, but significantly, interfered with cell shrinkage following energy depletion. The purpose of erythrocyte shrinkage during eryptosis is the avoidance of hemolysis with release of hemoglobin, which otherwise could pass the renal glomerular filter, precipitate in the acidic lumen of renal tubules, occlude nephrons and thus trigger renal failure [102]. Inhibition of eryptosis may, at least in theory, augment hemolysis and thus enhance renal injury. This may be particularly true for sonidegib, as it stimulates hemolysis.

The inhibitory effect of sonidegib on cell membrane scrambling could be favourable for the vascular system, as phosphatidylserine exposing erythrocytes adhere to the vascular wall [103], stimulate blood clotting/thrombosis [104-106], and interfere with microcirculation [18, 104, 107-110].

Moreover, inhibition of eryptosis may counteract anemia, a common clinical disorder of malignancy and its treatment [111-119]. Anemia in malignancy is at least in part due to eryptosis, which is triggered by both, the disease and by cytostatic treatment [13, 15, 44, 47, 50, 53-55, 62, 65, 74, 76, 88, 111, 120-128]. Anemia correlates with unfavourable outcome [112, 113, 129-132] and drugs are thus needed which counteract tumor growth and by the same token inhibit eryptosis. Despite its inhibitory effect on eryptosis, sonidegib causes,

however, anemia [100, 101]. According to the present observations, the anemia could at least in part be due to stimulation of hemolysis.

In conclusion, sonidegib inhibits erythrocyte cell membrane scrambling following energy depletion, hyperosmotic shock and oxidative stress as well as cell shrinkage following energy depletion and thus counteracts suicidal erythrocyte death.

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Disclosure Statement

The authors declare to have no conflict of interests.

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