Original Paper

Stimulation of Suicidal Erythrocyte Death by the CDC25 Inhibitor NSC-95397

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Key Words

Phosphatidylserine • Cell volume • Eryptosis • Calcium • NSC-95397 • Staurosporine • Necrostatin

Abstract

Background/Aims: The CDC25B inhibitor NSC-95397 triggers apoptosis of tumor cells and is thus considered for the treatment of malignancy. The substance is effective in part by modification of gene expression. Similar to apoptosis of nucleated cells erythrocytes may undergo eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Eryptosis may be triggered by increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), oxidative stress, ceramide, as well as activation of protein kinases. The present study explored, whether NSC-95397 induces eryptosis and, if so, to shed some light on the mechanisms involved. **Methods:** Phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, ROS formation from DCFDA dependent fluorescence, and ceramide abundance utilizing specific antibodies. **Results:** A 48 hours exposure of human erythrocytes to NSC-95397 significantly increased the percentage of annexin-V-binding cells ($\geq 1 \mu\text{M}$), significantly decreased forward scatter ($\geq 2.5 \mu\text{M}$), and significantly increased Fluo3-fluorescence ($\geq 1 \mu\text{M}$), DCFDA fluorescence ($5 \mu\text{M}$) and ceramide abundance ($\geq 5 \mu\text{M}$). The effect of NSC-95397 ($5 \mu\text{M}$) on annexin-V-binding was slightly, but significantly blunted by removal of extracellular Ca^{2+} and by addition of the protein kinase C inhibitor staurosporine ($1 \mu\text{M}$). **Conclusions:** NSC-95397 triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect in part requiring entry of Ca^{2+} and activation of staurosporine sensitive kinase(s).

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Introduction

NSC95397 (2,3-bis-[(2-hydroxyethyl)thio]-1,4-naphthoquinone), a dual-specificity phosphatase cell division cycle 25 B (CDC25B) inhibitor [1-10], counteracts inflammation

[10] and cancer [10]. NSC95397 is in part effective by inhibition of the kinases AKT, IKK α / β , MKK7, and TBK1 [10], inhibition of the Ca²⁺ binding protein S100A4 [11, 12], suppression of the transcription factors NF- κ B (p65) and AP-1 (c-Fos/c-Jun) [10] and downregulation of tumor necrosis factor (TNF)- α , inducible NO synthase (iNOS), cyclooxygenase (COX)-2, and interferon (IFN)- β expression [10]. NSC95397 influences transcription by disrupting the interaction between Carboxyl-terminal binding protein (CtBP) and its partner proteins [13]. NSC95397 interferes with transcription of viral proteins [14] and decreases the expression of the epithelial Na⁺ channel ENaC [15]. NSC95397 triggers apoptosis of diverse tumor cells and is thus considered for the treatment of malignancy [6, 16, 17].

Even though lacking nuclei and mitochondria, key regulators of apoptosis, erythrocytes may enter suicidal death or eryptosis [18], which is characterized by cell shrinkage [19] and cell membrane scrambling with breakdown of phospholipid asymmetry of the cell membrane apparent from phosphatidylserine exposure at the cell surface [18]. Eryptosis may be triggered by increase of cytosolic Ca²⁺ activity ([Ca²⁺]_i) [18], ceramide [20], oxidative stress [18], energy depletion [18], activated caspases [18, 21, 22], as well as stimulation of casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, and/or p38 kinase [18]. Eryptosis is inhibited by activation of AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase, and sorafenib/sunitinib sensitive kinases [18]. Eryptosis could be elicited by a wide variety of xenobiotics [18, 23-58].

The present study explored, whether and how NSC-95397 stimulates eryptosis. To this end, erythrocytes from healthy volunteers were treated with NSC-95397 and phosphatidylserine surface abundance, cell volume, [Ca²⁺]_i, ROS formation, and ceramide abundance 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 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, 1 CaCl₂, at 37°C for 48 hours. Where indicated, erythrocytes were exposed for 48 hours to NSC-95397 (MedChem Express, Princeton, USA), Ca²⁺-free solution without or with addition of Ca²⁺ chelator 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) BAPTA-AM (Thermo Fisher Scientific, Darmstadt, Germany), necroptosis inhibitor necrostatin-1 (Sigma, Schnellendorf, Germany), p38 kinase inhibitor SB 203580 (Tocris bioscience, Bristol, UK), protein kinase C inhibitor staurosporine (Sigma Aldrich, Hamburg, Germany), casein kinase inhibitor D4476 (Sigma Aldrich, Hamburg, Germany), and pan-caspase inhibitor zVAD (Tocris bioscience, Bristol, UK).

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 150 μ l 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 NSC-95397 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".

Hemolysis

For the determination of hemolysis, the samples were centrifuged (10 min at 2000 rpm, room temperature) after incubation under the respective experimental conditions and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatant was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

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.

Reactive oxygen species (ROS)

ROS abundance was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 150 μ l suspension of erythrocytes was washed in Ringer solution and stained with DCFDA (Sigma, Schnellendorf, Germany) in Ringer solution containing DCFDA at a final concentration of 10 μ M. 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 μ l 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).

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 μ g/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 analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. As a control, secondary antibody alone was used.

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 and *t* test as appropriate. *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 NSC-95397 stimulates eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

Erythrocyte volume was estimated from forward scatter which was determined utilizing flow cytometry. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with NSC-95397 (0.5 – 5 μ M). As illustrated in Fig. 1A,B, NSC-95397 decreased erythrocyte forward scatter, an effect reaching statistical significance at 2.5 μ M NSC-95397 concentration. NSC-95397 further increased the percentage of shrunken erythrocytes, an effect reaching statistical significance at 0.5 μ M NSC-95397 concentration (Fig. 1C). As shown in Fig. 1D, 0.5 and 1 μ M NSC-95397 increased in addition significantly the percentage of swollen erythrocytes.

Fig. 1. Effect of NSC-95397 on erythrocyte forward scatter. A. Original histograms of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 5 μ M NSC-95397. B. Arithmetic means $\pm$ SEM (n = 9) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) NSC-95397 (0.5 - 5 μ M). C,D. Percentage of erythrocytes with (C) FSC < 200 or (D) FSC > 800 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) NSC-95397 (0.5 - 5 μ M). *(<0.05), ***(<0.001) indicates significant difference from the absence of NSC-95397 (ANOVA).

Fig. 2. Effect of NSC-95397 on phosphatidylserine exposure. A. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 5 μ M NSC-95397. B. Arithmetic means $\pm$ SEM (n = 9) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) NSC-95397 (0.5 - 5 μ M). **(<0.01), ***(<0.001) indicates significant difference from the absence of NSC-95397 (ANOVA). ###(<0.001) indicates significant difference between hemolysis and eryptotic death (ANOVA).

Fig. 3. Effect of NSC-95397 on cytosolic Ca^{2+} activity. A. Original histograms of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 5 μ M NSC-95397. B. Arithmetic means $\pm$ SEM (n = 9) of Fluo3 fluorescence in erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) NSC-95397 (0.5 - 5 μ M). **(<0.01), ***(<0.001) indicates significant difference from the absence of NSC-95397 (ANOVA).

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with NSC-95397 (0.5 - 5 μ M). As shown in Fig. 2, a 48 hours exposure to NSC-95397 increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 1.0 μ M NSC-95397.

Fig. 4. Ca^{2+} sensitivity of NSC-95397-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) NSC-95397 (5 μM) in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means $\pm$ SEM ($n = 9$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) NSC-95397 (5 μM) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . ***($p < 0.001$) indicates significant difference from the absence of NSC-95397, #($p < 0.05$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

Fig. 5. Ca^{2+} and BAPTA-AM sensitivity of NSC-95397-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) NSC-95397 (5 μM) in the absence (A) and presence (B) of Ca^{2+} chelator BAPTA-AM (50 μM). C. Arithmetic means $\pm$ SEM ($n = 10$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) NSC-95397 (5 μM) in the absence (left bars, $-\text{BAPTA-AM}$) and presence (right bars, $+\text{BAPTA-AM}$) of Ca^{2+} chelator BAPTA-AM. ***($p < 0.001$) indicates significant difference from the absence of NSC-95397, #($p < 0.05$) indicates significant difference from the absence of BAPTA-AM (ANOVA).

Following a 48 hours incubation, the percentage of hemolytic erythrocytes was similarly low in the absence of NSC-95397 ($1.7 \pm 0.2\%$, $n = 9$) and in the presence of 0.5 μM ($2.1 \pm 0.1\%$, $n = 9$), 1 μM ($2.5 \pm 0.2\%$, $n = 9$), 2.5 μM ($3.7 \pm 0.3\%$, $n = 9$) and 5 μM ($3.8 \pm 0.5\%$, $n = 9$) NSC-95397.

Fluo3 fluorescence was taken as a measure of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). As illustrated in Fig. 3, following a 48 hours incubation the Fluo3 fluorescence was higher in the presence than in the absence of NSC-95397, a difference reaching statistical significance at 1.0 μM NSC-95397.

Fig. 6. Effect of NSC-95397 on ROS formation. A. Original histogram of DCF fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of NSC-95397 (5 μ M). B. Arithmetic means $\pm$ SEM (n = 9) of the DCF fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) NSC-95397 (5 μ M). *(p<0.05) indicates significant difference from the absence of NSC-95397 (ANOVA).

Fig. 7. Effect of NSC-95397 on ceramide abundance at the erythrocyte surface. A. Original histogram of ceramide abundance in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of NSC-95397 (5 μ M). B. Arithmetic means $\pm$ SEM (n = 9) of the ceramide abundance (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) NSC-95397 (5 μ M). *** (p<0.001) indicates significant difference from the absence of NSC-95397 (ANOVA).

A next series of experiments explored whether the NSC-95397-induced annexin-V-binding required entry of extracellular Ca^{2+} . To this end, erythrocytes were incubated for 48 hours in the absence or presence of 5 μ M NSC-95397 in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 4, removal of extracellular Ca^{2+} slightly but significantly blunted the effect of NSC-95397 on annexin-V-binding. However, even in the absence of extracellular Ca^{2+} , NSC-95397 significantly increased the percentage of annexin-V-binding erythrocytes. Thus, NSC-95397-induced annexin-V-binding was only in small part triggered by entry of extracellular Ca^{2+} . As illustrated in Fig. 5, similar observations were made, when in nominally Ca^{2+} free solutions Ca^{2+} was in addition bound to Ca^{2+} chelator BAPTA-AM.

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2',7'-dichlorodihydrofluorescein (DCF) diacetate. As illustrated in Fig. 6, following a 48 hours incubation the DCF fluorescence was significantly higher in the presence than in the absence of 5 μ M NSC-95397. Thus, NSC-95397 induced oxidative stress.

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As illustrated in Fig. 7, following a 48 hours incubation the ceramide abundance was significantly higher in the presence than in the absence of 5 μ M NSC-95397. Thus, NSC-95397 enhanced the ceramide abundance.

As illustrated in Fig. 8, the effect of NSC-95397 on annexin-V-binding was insensitive to necroptosis-inhibitor necrostatin (100 μ M), casein kinase inhibitor D4476 (10 μ M), pan-caspase inhibitor zVAD (10 μ M), and p38 kinase inhibitor SB203580 (2 μ M).

To explore, whether the effects of NSC-95397 involved protein kinase C activity, the influence of NSC-95397 on annexin-V-binding was tested in the presence of protein kinase C inhibitor staurosporine (1 μ M). Erythrocytes were incubated for 48 hours in the absence or presence of 5 μ M NSC-95397 in the absence and presence of staurosporine. As illustrated in Fig. 9, addition of staurosporine slightly but significantly blunted the effect of NSC-95397 on annexin-V-binding. However, even in the presence of staurosporine, NSC-95397 significantly

Fig. 8. Insensitivity of NSC-95397-induced phosphatidylserine exposure to Necrostatin, D4476, zVAD, and SB203580. Arithmetic means $\pm$ SEM (n = 10) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) NSC-95397 (5 μ M) in the absence (DMSO) and presence of 100 μ M necrostatin, 10 μ M D4476, 10 μ M zVAD, or 2 μ M SB203580. ***($p < 0.001$) indicates significant difference from the absence of NSC-95397 (ANOVA).

Fig. 9. Sensitivity of NSC-95397-induced phosphatidylserine exposure to protein kinase C inhibition. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) NSC-95397 (5 μ M) in the absence (A) and presence (B) of protein kinase C inhibitor (PKC i) staurosporine (1 μ M). C. Arithmetic means $\pm$ SEM (n = 9) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) NSC-95397 (5 μ M) in the absence (left bars, -PKC i) and presence (right bars, +PKC i) of protein kinase C inhibitor staurosporine (1 μ M). ***($p < 0.001$) indicates significant difference from the absence of NSC-95397, #($p < 0.05$) indicates significant difference from the absence of PKC i staurosporine (ANOVA).

increased the percentage of annexin-V-binding erythrocytes. Thus, NSC-95397-induced annexin-V-binding was only in small part dependent on protein kinase C activity.

NSC-95397 decreased the average forward scatter to a similar extent in the absence (from 492.8 ± 34.3 to 379 ± 42.4 , n = 9) and in the presence (from 463.3 ± 18.7 to 414 ± 38.4 , n = 9) of staurosporine (1 μ M).

Discussion

The present observations reveal that the CDC25B inhibitor NSC-95397 triggers suicidal erythrocyte death or eryptosis. Treatment of erythrocytes drawn from healthy volunteers with NSC-95397 is followed by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface.

The effect of NSC-95397 on cell membrane scrambling was paralleled by increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) and was slightly, but significantly blunted by removal of extracellular Ca^{2+} . However, even in the nominal absence of extracellular of Ca^{2+} NSC-95397

treatment was followed by a significant stimulation of eryptosis, an observation pointing to involvement of Ca^{2+} independent mechanisms. Those mechanisms apparently include oxidative stress, as NSC-95397 increased the DCF fluorescence. Oxidative stress is a powerful stimulator of eryptosis [18]. NSC-95397 further increased the erythrocyte surface abundance of ceramide, which could sensitize erythrocytes for the scrambling effect of Ca^{2+} [18]. The effect of NSC-95397 may further involve activation of staurosporine sensitive kinases such as protein kinase C. Protein kinase C is a strong stimulator of eryptosis [18].

NSC-95397 did not significantly modify hemolysis. Eryptosis allows the clearance of defective erythrocytes from circulating blood prior to hemolysis [18]. To the extent that eryptosis precedes hemolysis, it prevents the release of hemoglobin, which may otherwise be filtered in renal glomerula, precipitate in the acidic lumen of renal tubules, occlude nephrons and thus trigger renal failure [59].

The stimulation of eryptosis were expected to foster development of anemia, as phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood. Anemia develops as soon as the loss of erythrocytes outcasts the formation of new erythrocytes by erythropoiesis [18]. Accordingly, side effects of NSC-95397 treatment may include anemia. The risk of anemia may be enhanced in several clinical conditions with enhanced susceptibility of erythrocytes to eryptosis, including iron deficiency [18], dehydration [60], hyperphosphatemia [61], chronic kidney disease (CKD) [62-65], hemolytic-uremic syndrome [66], diabetes [67], hepatic failure [36], malignancy [18], sepsis [68], sickle-cell disease [18], beta-thalassemia [18], Hb-C and G6PD-deficiency [18], as well as Wilsons disease [69]. The adherence of phosphatidylserine exposing erythrocytes to the vascular wall [70] and the stimulation of blood clotting may trigger thrombosis [71-73] and impair microcirculation [20, 71, 74-77]. The anemia and impairment of microcirculation may, at least in theory, limit the use of the drug.

In conclusion, NSC-95397 triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect in part due to Ca^{2+} entry, oxidative stress, ceramide and presumably activation of protein kinase C.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Tanja Loch. The study was supported by the Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of Tuebingen University.

Disclosure Statement

None.

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