Original Paper

Micafungin-Induced Suicidal Erythrocyte Death

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Key Words

Phosphatidylserine • Cell volume • Eryptosis • SB203580 • zVAD • Calcium • Hemolysis

Abstract

Background/Aims: The antifungal drug Micafungin is used for the treatment of diverse fungal infections including candidiasis and aspergillosis. Side effects of Micafungin treatment include microangiopathic hemolytic anemia and thrombocytopenia with microvascular thrombosis. The development of thrombosis may be fostered by stimulation of eryptosis, the suicidal death of erythrocytes characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Triggers of eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), oxidative stress, ceramide, activated protein kinase C (PKC), casein kinase 1 α or p38 kinase and activated caspases. The present study explored, whether Micafungin induces eryptosis. **Methods:** Flow cytometry was employed to estimate phosphatidylserine abundance at the erythrocyte surface from annexin-V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, abundance of reactive oxygen species (ROS) from DCFDA dependent fluorescence, and ceramide abundance at the erythrocyte surface utilizing specific antibodies. Hemolysis was quantified by measuring haemoglobin concentration in the supernatant. **Results:** A 48 hours exposure of human erythrocytes to Micafungin (10 - 25 $\mu\text{g/ml}$) significantly increased hemolysis and the percentage of annexin-V-binding cells, and significantly decreased forward scatter. Micafungin (25 $\mu\text{g/ml}$) did not significantly modify Fluo3-fluorescence, DCFDA fluorescence, or ceramide abundance. The effect of Micafungin on annexin-V-binding was not significantly modified by removal of extracellular Ca^{2+} , by PKC inhibitor staurosporine (1 μM), p38 kinase inhibitor SB203580 (2 μM), casein kinase 1 α inhibitor D4476 (10 μM) or pancaspase inhibitor zVAD (10 μM). **Conclusions:** Micafungin triggers hemolysis and eryptosis with cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane.

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Introduction

Micafungin has been employed for the treatment and prophylaxis of diverse fungal infections [1-10] including candidiasis [5-37] and aspergillosis [5-8, 12, 15, 24, 27-30, 32, 33, 36, 38, 39]. Side effects of Micafungin treatment include thrombotic thrombocytopenic purpura (TTP), a condition characterized by microangiopathic hemolytic anemia and thrombocytopenia with microvascular thrombosis [40].

Stimulators of thrombosis include triggering of eryptosis, the suicidal death of erythrocytes characterized by cell shrinkage [41] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [42]. Phosphatidylserine exposing erythrocytes further adhere to the vascular wall [43], stimulate blood clotting and thus foster thrombosis [44-46]. Accordingly, stimulation of eryptosis may lead to impairment of microcirculation [44, 47-51].

Signaling mechanisms stimulating eryptosis include Ca^{2+} entry with increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [42], ceramide [47], caspases [42, 52, 53], as well as several kinases including casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, and p38 kinase [42]. Eryptosis is inhibited by AMP activated kinase AMPK, cGMP-dependent protein kinase, and PAK2 kinase and sorafenib/sunitinib sensitive kinases [42].

Eryptosis is triggered by oxidative stress [42], energy depletion [42] and a wide variety of xenobiotics [42, 54-84].

The present study explored whether Micafungin could stimulate eryptosis. To this end, human erythrocytes were exposed to Micafungin and phosphatidylserine surface abundance, cell volume, $[\text{Ca}^{2+}]_i$, abundance of reactive oxygen species (ROS) and ceramide 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 120xg for 20 min at 20 °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 Micafungin (Selleckchem, Munich, Germany). To test for an involvement of oxidative stress, erythrocytes were exposed for 48 hours to a combination of Micafungin and antioxidant N-acetylcysteine (Sigma Aldrich, Hamburg, Germany). Involvement of protein kinase C or p38 kinase was analyzed by exposure of erythrocytes for 48 hours to a combination of Micafungin and either PKC inhibitor staurosporine (Tocris bioscience, Bristol, UK) or p38 kinase inhibitor SB 203580 (Tocris bioscience). A putative role of casein kinase 1 α was elucidated by exposure of erythrocytes for 48 hours to a combination of Micafungin and casein kinase 1 α inhibitor D4476 (Sigma Aldrich, Hamburg, Germany). Involvement of caspases was tested by exposure of erythrocytes for 48 hours to a combination of Micafungin and pancaspase inhibitor zVAD (Enzo Life Sciences, Lörrach, Germany).

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 150 μl cell suspension of erythrocytes was centrifuged at 630xg for 3 min and, after trashing the supernatant, the erythrocytes were stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in Ringer solution containing 5 mM CaCl_2 at 37°C for 20 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 Micafungin 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".

Confocal microscopy

In order to visualize the effect of Micafungin on F-actin in human erythrocytes, Phalloidin eFluor® 660 (eBioscience, San Diego, USA) was used. Briefly, treated erythrocytes were stained with Phalloidin eFluor® 660 at a dilution of 1:100 for 45 mins in the dark at 37°C. In order to visualize phosphatidylserine exposure, erythrocytes were additionally stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) for 30 mins in the dark at 37°C. The erythrocytes were washed twice and were finally resuspended in 200 µl Ringer solution containing 5 mM CaCl₂. For confocal microscopy, 20 µl of each sample were spread onto a glass slide and dried for 15 mins at RT. The slides were covered with PROlong Gold antifade reagent (Invitrogen, Darmstadt, Germany). Subsequently, confocal images were taken on a Zeiss LSM 5 Exciter confocal laser-scanning microscope.

Intracellular Ca²⁺

After incubation, erythrocytes were washed in Ringer solution and then 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 and washed once in Ringer solution containing 5 mM CaCl₂. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer solution. Then, Ca²⁺-dependent fluorescence intensity was measured in FL-1 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 then stained with DCFDA (Sigma Aldrich) in Ringer solution containing DCFDA at a final concentration of 10 µM. Erythrocytes were incubated at 37°C for 30 min in the dark and then washed two times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 µl Ringer solution, and ROS-dependent fluorescence intensity was measured in FL-1 at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD).

Ceramide abundance

To determine the ceramide abundance at the erythrocyte surface, a monoclonal antibody was used. After incubation, cells were stained for 1 h at 37°C with 1 µg/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in phosphate-buffered saline (PBS) containing 0.1 % bovine serum albumin (BSA) at a dilution of 1:10. After two washing steps with PBS-BSA, cells were stained for 30 min with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG/IgM (concentration 0.5 mg/ml) specific antibody (BD Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. Samples were then analyzed by flow cytometric analysis in FL-1 at 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 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 Micafungin is able to trigger eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and by phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface. To this end, erythrocytes drawn from healthy volunteers were incubated for 48 hours in Ringer solution without or with Micafungin (10 – 25 µg/ml).

Fig. 1. Effect of Micafungin on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 25 µg/ml Micafungin. B. Arithmetic means $\pm$ SEM ($n = 14$) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Micafungin (10–25 µg/ml). C. Arithmetic means $\pm$ SEM ($n = 14$) of the percentage of erythrocytes with forward scatter (FSC) < 200 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Micafungin (10–25 µg/ml). D. Arithmetic means $\pm$ SEM ($n = 14$) of the percentage of erythrocytes with forward scatter (FSC) > 800 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Micafungin (10–25 µg/ml). ***($p < 0.001$) indicates significant difference from the absence of Micafungin (ANOVA).

Erythrocyte volume was estimated from forward scatter, which was determined utilizing flow cytometry. As illustrated in Fig. 1, Micafungin (10–25 µg/ml) significantly decreased erythrocyte forward scatter. Moreover, Micafungin significantly increased the percentage of shrunken erythrocytes (Fig. 1C), an effect reaching statistical significance at 10 µg/ml Micafungin concentration. In contrast, Micafungin treatment decreased the percentage of swollen erythrocytes (Fig. 1D), an effect reaching statistical significance at 10 µg/ml Micafungin concentration.

The percentage of hemolytic erythrocytes was estimated from hemoglobin concentration in the supernatant. As illustrated in Fig. 2, a 48 hours exposure to Micafungin (10–25 µg/ml) increased the percentage of hemolytic erythrocytes.

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. As illustrated in Fig. 3, a 48 hours exposure to Micafungin (10–25 µg/ml) significantly increased the percentage of phosphatidylserine exposing erythrocytes.

Confocal microscopy was employed to visualize the annexin binding and the actin filaments of erythrocytes. As illustrated in Fig. 4, confocal microscopy confirmed the increase of annexin-V-binding. The actin filaments were apparently resistant to the Micafungin treatment (Fig. 4).

Fluo3 fluorescence was taken as a measure of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). As a result, the Fluo3 fluorescence was similar following incubation of the erythrocytes in the absence of Micafungin (21.16 ± 0.56 a.u., $n = 14$) and in the presence of 10 µg/ml (20.77 ± 0.85 a.u., $n = 14$), 15 µg/ml (19.78 ± 0.72 a.u., $n = 14$) and 25 µg/ml (20.04 ± 0.57 a.u., $n = 14$) Micafungin. Thus, Micafungin did not appreciably modify $[\text{Ca}^{2+}]_i$.

Fig. 2. Effect of Micafungin on hemolysis. Arithmetic means $\pm$ SEM ($n = 8$) of the percentage of hemolytic erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Micafungin (10 - 25 $\mu\text{g}/\text{ml}$). * ($p < 0.05$), *** ($p < 0.001$) indicate significant difference from the absence of Micafungin (ANOVA).

Fig. 3. Effect of Micafungin on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 25 $\mu\text{g}/\text{ml}$ Micafungin. B. Arithmetic means $\pm$ SEM ($n = 14$) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Micafungin (10 - 25 $\mu\text{g}/\text{ml}$). *** ($p < 0.001$) indicates significant difference from the absence of Micafungin (ANOVA).

Fig. 4. Confocal microscopy on effects of Micafungin on phosphatidylserine externalization and F-actin. Confocal microscopy of annexin V fluorescence (left panels), F-actin abundance (middle panels) and overlay of both stainings (right panels) in erythrocytes following exposure to Ringer solution (upper panels) or to 10 $\mu\text{g}/\text{ml}$ micafungin for 48 hours (lower panels).

A next series of experiments explored whether the Micafungin-induced translocation of phosphatidylserine was sensitive to extracellular Ca^{2+} . To this end, erythrocytes were incubated for 48 hours in the absence or presence of 25 $\mu\text{g}/\text{ml}$ Micafungin in the presence

Fig. 5. Ca^{2+} insensitivity of Micafungin-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) Micafungin (25 $\mu\text{g}/\text{ml}$) in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means $\pm$ SEM ($n = 16$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) Micafungin (25 $\mu\text{g}/\text{ml}$) in the presence (left bars, + Calcium) and absence (right bars, - Calcium) of Ca^{2+} . ***($p < 0.001$) indicates significant difference from the absence of Micafungin (ANOVA).

or nominal absence of extracellular Ca^{2+} . As shown in Fig. 5, removal of extracellular Ca^{2+} did not significantly blunt the effect of Micafungin on the percentage of annexin-V-binding erythrocytes and even in the absence of extracellular Ca^{2+} , Micafungin significantly increased the percentage of annexin-V-binding erythrocytes. Thus, Micafungin-induced cell membrane scrambling was not appreciably modified by removal of extracellular Ca^{2+} .

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As shown in Fig. 6 A&B, the DCFDA fluorescence was significantly ($p < 0.001$) lower in the presence of Micafungin than in the absence of Micafungin. Thus, Micafungin rather decreased oxidative stress.

To explore, whether the effects of Micafungin could be modified by the redox state, the influence of Micafungin on annexin-V-binding was tested in the absence or presence of antioxidant N-acetylcysteine (1 mM). As a result, Micafungin (25 $\mu\text{g}/\text{ml}$) increased the percentage of phosphatidylserine exposing erythrocytes to similar values in the absence (from $2.19 \pm 0.29\%$ to $19.49 \pm 0.83\%$, $n = 16$) and in the presence (from $1.58 \pm 0.16\%$ to $17.16 \pm 0.88\%$, $n = 16$) of N-acetylcysteine (1 mM).

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As shown in Fig. 6 C&D, the ceramide abundance was significantly lower following exposure to Micafungin, than in the absence of Micafungin. Thus, Micafungin rather decreased ceramide abundance.

In order to elucidate a possible role of p38 kinase, the influence of Micafungin on annexin-V-binding was quantified in the absence or presence of p38 kinase inhibitor SB 203580 (2

Fig. 6. Effect of Micafungin on ROS and ceramide abundance. A. Original histogram of DCFDA fluorescence of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 25 µg/ml Micafungin. B. Arithmetic means $\pm$ SEM ($n = 5$) of erythrocyte DCFDA fluorescence following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Micafungin (10 - 25 µg/ml). C. Original histogram of ceramide abundance of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 25 µg/ml Micafungin. D. Arithmetic means $\pm$ SEM ($n = 4$) of erythrocyte ceramide abundance following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Micafungin (10 - 25 µg/ml). **($p < 0.01$), ***($p < 0.001$) indicate significant difference from the absence of Micafungin (ANOVA).

µM). As a result, Micafungin (25 µg/ml) increased the percentage of phosphatidylserine exposing erythrocytes to similar values in the absence (from 2.93 ± 0.70 % to 17.79 ± 0.85 %, $n = 8$) and in the presence (from 2.37 ± 0.58 % to 18.11 ± 1.27 %, $n = 8$) of SB 203580 (2 µM).

The involvement of protein kinase C was tested by quantifying the effect of Micafungin on annexin-V-binding in the absence or presence of protein kinase C inhibitor staurosporine (1 µM). As a result, Micafungin (25 µg/ml) increased the percentage of phosphatidylserine exposing erythrocytes to similar values in the absence (from 3.29 ± 0.58 % to 20.03 ± 0.96 %, $n = 16$) and in the presence (from 3.50 ± 0.53 % to 19.55 ± 1.29 %, $n = 16$) of staurosporine (1 µM).

A putative role of casein kinase 1 α was studied by determination of Micafungin-induced annexin-V-binding in the absence or presence of casein kinase 1 α inhibitor D4476 (10 µM). As a result, Micafungin (25 µg/ml) increased the percentage of phosphatidylserine exposing erythrocytes to similar values in the absence (from 1.59 ± 0.21 % to 22.68 ± 0.89 %, $n = 8$) and in the presence (from 1.54 ± 0.11 % to 21.83 ± 1.04 %, $n = 8$) of D4476 (10 µM).

A final series of experiments addressed the putative involvement of caspases in Micafungin induced eryptosis. To this end, the influence of Micafungin on annexin-V-binding was tested in the absence or presence of pancaspase inhibitor zVAD (10 µM). As a result, Micafungin (25 µg/ml) increased the percentage of phosphatidylserine exposing erythrocytes to similar values in the absence (from 2.15 ± 0.31 % to 17.42 ± 1.02 %, $n = 8$) and in the presence (from 2.12 ± 0.26 % to 19.61 ± 1.30 %, $n = 8$) of zVAD (10 µM).

Discussion

The present observations demonstrate that Micafungin stimulates eryptosis, the suicidal erythrocyte death. Treatment of erythrocytes from healthy individuals with Micafungin results in cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The concentrations required for this effect are in the range of peak plasma concentrations determined in Micafungin treated patients [85]. At least in theory the sensitivity to Micafungin induced eryptosis could be enhanced in clinical conditions with accelerated eryptosis, such as dehydration [86], hyperphosphatemia [87], chronic kidney disease (CKD) [88-91], hemolytic-uremic syndrome [92], diabetes [93], hepatic failure [94], malignancy [42], sepsis [95], sickle-cell disease [42], beta-thalassemia [42], Hb-C and G6PD-deficiency [42], as well as Wilsons disease [96].

Attempts to define the signaling of Micafungin on erythrocyte cell membrane scrambling were not successful. According to the present observations, the involvement of several well known triggers of eryptosis, such as Ca^{2+} entry, oxidative stress, ceramide, protein kinase C, p38 kinase and caspases [42] is unlikely. Micafungin-induced cell membrane scrambling was not paralleled by increase of Fluo3 fluorescence and was not significantly modified by removal of Ca^{2+} from extracellular space, indicating that the effect was not dependent on entry of extracellular Ca^{2+} . The effect of Micafungin on cell membrane scrambling was paralleled by a decrease rather than an increase of reactive oxygen species and was not significantly modified by the antioxidant N-acetylcysteine. Micafungin further decreased rather than increased the abundance of ceramide. The effect of Micafungin on cell membrane scrambling was not sensitive to staurosporine, SB203580 or D4476 and did thus apparently not require the activation of protein kinase C, p38 kinase or casein kinase 1 α . Moreover, Micafungin was effective even in the presence of caspase inhibitor zVAD. Thus, the cellular mechanism involved in the triggering of eryptosis remained elusive.

Besides its effect on eryptosis, micafungin triggered hemolysis. The effects of Micafungin are reminiscent to the hemolytic effects of surfactant [97]. It is actually the purpose of eryptosis to clear defective erythrocytes from circulating blood prior to hemolysis [42]. The removal of defective erythrocytes is important as hemolysis leads to release of hemoglobin, which passes the renal glomerular filter, precipitates in the acidic lumen of renal tubules, occludes nephrons and may thus lead to renal failure [98]. Eryptosis further accomplishes removal of infected erythrocytes from circulating blood [42].

Conclusion

In conclusion, Micafungin triggers eryptosis with cell shrinkage and cell membrane scrambling. In addition Micafungin and triggers hemolysis.

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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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