Original Paper

Triggering of Eryptosis, the Suicidal Erythrocyte Death by Mammalian Target of Rapamycin (mTOR) inhibitor Temsirolimus

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Phosphatidylserine • Eryptosis • Staurosporine • Oxidative stress • Calcium • Ceramide

Abstract

Background/Aims: The mammalian target of rapamycin (mTOR) inhibitor temsirolimus is utilized for the treatment of malignancy. Temsirolimus is at least in part effective by triggering suicidal tumor cell death. The most common side effect of temsirolimus treatment is anemia. At least in theory, the anemia following temsirolimus treatment could result from stimulation of eryptosis, the suicidal erythrocyte death. Hallmarks of eryptosis include cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Signaling involved in the orchestration of eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), oxidative stress, ceramide, as well as activation of staurosporine and chelerythrine sensitive protein kinase C, SB203580 sensitive p38 kinase, D4476 sensitive casein kinase 1, and zVAD sensitive caspases. The purpose of the present study was to test whether temsirolimus influences eryptosis and, if so, to shed light on the signaling involved. **Methods:** Flow cytometry was employed to estimate cell volume from forward scatter, phosphatidylserine exposure at the cell surface from annexin-V-binding, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, reactive oxygen species (ROS) abundance from DCFDA dependent fluorescence, and ceramide abundance utilizing specific antibodies. Hemolysis was determined from hemoglobin concentration in the supernatant. **Results:** A 48 hours exposure of human erythrocytes to temsirolimus (5 – 20 $\mu\text{g/ml}$) significantly decreased forward scatter and significantly increased the percentage of annexin-V-binding cells. Temsirolimus significantly increased Fluo3-fluorescence, DCFDA fluorescence and ceramide abundance at the erythrocyte surface. The effect of temsirolimus on annexin-V-binding was significantly blunted but not abolished by removal of extracellular Ca^{2+} and by addition of staurosporine (1 μM) or chelerythrine (10 μM) but not significantly modified by addition of SB203580 (2 μM), D4476 (10 μM), or zVAD (10 μM). Chelerythrine (10 μM) further significantly blunted the effect of temsirolimus on DCFDA fluorescence but not ceramide formation. Removal of extracellular Ca^{2+} had no effect on temsirolimus induced ROS formation or ceramide abundance. **Conclusions:** Temsirolimus triggers eryptosis with

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cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect at least in part due to Ca^{2+} entry, oxidative stress, ceramide and activation of staurosporine/Chelerythrine sensitive kinase(s).

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Introduction

Temsirolimus, an ester derivative of sirolimus (rapamycin) and selective inhibitor of the kinase mammalian target of rapamycin (mTOR) [1-9], is a cytostatic drug widely used in the treatment of diverse malignancies including refractory mantle cell lymphoma [1, 10-15], non-Hodgkin lymphomas [15, 16], multiple myeloma [16], metastatic renal cell carcinoma [2, 17-44], and a variety of further solid tumors [16, 45, 46].

Temsirolimus is effective by inducing apoptosis of tumor cells [9, 47-57]. Signaling involved includes activation of caspases [47]. On the other hand temsirolimus may counteract apoptosis of hippocampal neurons [58]. The most common side effects of temsirolimus include the development of anemia [59, 60].

The anemia could result from suicidal death of erythrocytes or eryptosis [61], which is characterized by cell membrane scrambling with phosphatidylserine translocation to the cell surface [61]. Following most stimulators of eryptosis, the cell membrane scrambling is paralleled by cell shrinkage [62]. During eryptosis, the cell membrane typically remains tight thus preventing cellular release of hemoglobin with subsequent glomerular filtration and tubular precipitation of hemoglobin, occlusion of nephrons and renal injury [63]. Necroptosis-like suicidal erythrocyte death disrupts the cell membrane integrity by plasma membrane pore formation [64].

Signaling participating in the orchestration of eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [61], ceramide [65], caspases [61, 66, 67], G-protein Galphai2 [68], casein kinase 1 α [69], Janus-activated kinase JAK3 [70], protein kinase C [71], and p38 kinase [72]. Eryptosis is suppressed by AMP activated kinase AMPK [73], cGMP-dependent protein kinase [74], mitogen and stress activated kinase MSK1/2 [75], PAK2 kinase [76] and sorafenib/sunitinib sensitive kinases [77, 78]. Eryptosis is triggered by a wide variety of conditions including hyperosmotic shock [61], oxidative stress [61], energy depletion [61], or exposure to diverse xenobiotics [61, 79-137]. Several clinical disorders are associated with enhanced eryptosis, such as iron deficiency [61], dehydration [138], hyperphosphatemia [139], chronic kidney disease (CKD) [140-143], hemolytic-uremic syndrome [144], diabetes [145], hepatic failure [79], malignancy [146, 147], arteriitis [148], sepsis [149], sickle-cell disease [61], beta-thalassemia [61], Hb-C and G6PD-deficiency [61], Wilsons disease [149], as well as advanced age [150]. Eryptosis further increases following storage of blood for transfusion [151].

The present study explored, whether temsirolimus modifies eryptosis. To this end, human erythrocytes from healthy volunteers were exposed to temsirolimus and phosphatidylserine surface abundance, cell volume, $[\text{Ca}^{2+}]_i$, 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 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 temsirolimus (Sigma Aldrich, Hamburg, Germany). In order to estimate the impact of Ca^{2+} entry on temsirolimus induced

eryptosis, erythrocytes were exposed to temsirolimus in the presence and absence of extracellular Ca^{2+} . To test for an involvement of kinases, erythrocytes were exposed for 48 hours to a combination of temsirolimus and p38 kinase inhibitor SB203580 (Tocris bioscience, Bristol, UK), protein kinase C inhibitor staurosporine (Enzo Life Sciences, Lörrach, Germany), specific protein kinase C inhibitor chelerythrine (Enzo Life Sciences, Lörrach, Germany) or casein kinase inhibitor D4476 (Tocris Bioscience, Bristol, UK). The impact of caspases was elucidated utilizing the 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 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 temsirolimus 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 μM 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. Afterwards, the geomean of the Ca^{2+} 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 DCFDA at a final concentration of 10 μM . 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 $\mu\text{g}/\text{ml}$ 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 cytometry with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Finally, the geomean of the ceramide-dependent fluorescence was determined.

Hemolysis

For the determination of hemolysis, the samples were centrifuged (3 min. at 1600 rpm in room temperature) after incubation, 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.

Statistics

Data are expressed as arithmetic means $\pm$ SD. 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 the impact of tamsirolimus on eryptosis, the suicidal erythrocyte death. Hallmarks of eryptosis include erythrocyte shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

Cell volume was estimated from forward scatter determined by flow cytometry. The measurements were made after an incubation of the erythrocytes for 48 hours in Ringer solution without or with tamsirolimus (5 – 20 µg/ml). As illustrated in Fig. 1, a 48 hours exposure to tamsirolimus significantly decreased the forward scatter at each of the concentrations applied. It further increased the percentage of shrunken erythrocytes, an effect reaching statistical significance at 20 µg/ml tamsirolimus. Moreover, the exposure to tamsirolimus decreased the percentage of swollen erythrocytes at each of the concentrations applied.

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding to phosphatidylserine, as determined by flow cytometry. Annexin-V-binding was analysed following an incubation of the erythrocytes for 48 hours in Ringer solution without or

Fig. 1. Effect of Tamsirolimus 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 20 µg/ml Tamsirolimus. B. Arithmetic means $\pm$ SD ($n = 11$) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Tamsirolimus (5 - 20 µg/ml) or solvent DMSO alone (grey bar). C,D. Arithmetic means $\pm$ SD of the 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) Tamsirolimus (5 - 20 µg/ml) or solvent DMSO alone (grey bar). * ($P < 0.05$), *** ($p < 0.001$) indicates significant difference from the absence of tamsirolimus (ANOVA).

Fig. 2. Effect of temsirolimus 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 20 $\mu\text{g/ml}$ temsirolimus. B. Arithmetic means $\pm$ SD ($n = 16$) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) temsirolimus (5 - 20 $\mu\text{g/ml}$). For comparison, the effect of the solvent DMSO is shown (grey bar). ***($p < 0.001$) indicates significant difference from the absence of temsirolimus (ANOVA).

Fig. 3. Effect of temsirolimus on cytosolic Ca^{2+} activity. A. Original histogram of Fluo3 fluorescence of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 20 $\mu\text{g/ml}$ temsirolimus. B. Arithmetic means $\pm$ SD ($n = 16$) of erythrocyte Fluo3 fluorescence following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) temsirolimus (5 - 20 $\mu\text{g/ml}$). For comparison, the effect of the solvent DMSO is shown (grey bar). ***($p < 0.001$) indicates significant difference from the absence of temsirolimus (ANOVA).

with temsirolimus (5 - 20 $\mu\text{g/ml}$). As shown in Fig. 2, a 48 hours exposure to temsirolimus increased the percentage of phosphatidylserine exposing erythrocytes at each of the concentrations applied.

Both, cell shrinkage and cell membrane scrambling could result from an increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Fluo3 fluorescence was employed in order to quantify $[\text{Ca}^{2+}]_i$. The erythrocytes were analysed after a 48 hours incubation in Ringer solution without or with temsirolimus (5 - 20 $\mu\text{g/ml}$). As illustrated in Fig. 3, a 48 hours exposure to temsirolimus increased the Fluo3 fluorescence, an effect reaching statistical significance at 20 $\mu\text{g/ml}$ temsirolimus.

In order to test whether entry of extracellular Ca^{2+} is required for the stimulation of cell membrane scrambling by temsirolimus, erythrocytes were incubated for 48 hours in the absence or presence of 20 $\mu\text{g/ml}$ temsirolimus in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 4, removal of extracellular Ca^{2+} significantly blunted the effect of temsirolimus on annexin-V-binding. However, even in the absence of extracellular Ca^{2+} , temsirolimus significantly increased the percentage of annexin-V-binding erythrocytes. Thus, temsirolimus triggered cell membrane scrambling in part but not exclusively by entry 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 illustrated in Fig. 5, a 48 hours exposure to temsirolimus (20 $\mu\text{g/ml}$) significantly increased the DCFDA fluorescence of erythrocytes. Thus, temsirolimus does trigger oxidative stress.

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was quantified utilizing specific antibodies. As shown in Fig. 6, a 48 hours exposure to temsirolimus (20 $\mu\text{g/ml}$) significantly increased the ceramide abundance at the erythrocyte surface.

Fig. 4. Ca^{2+} sensitivity of temsirolimus-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) temsirolimus (20 µg/ml) in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means $\pm$ SD ($n = 8$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) temsirolimus (20 µg/ml) 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 temsirolimus, ##($p < 0.01$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

Fig. 5. Effect of temsirolimus on reactive oxygen species. A. Original histogram of DCFDA fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 20 µg/ml temsirolimus. B. Arithmetic means $\pm$ SD ($n = 8$) of DCFDA fluorescence in erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bar) temsirolimus (20 µg/ml). *($p < 0.05$) indicates significant difference from the absence of temsirolimus (unpaired t test).

Fig. 6. Effect of Temsirolimus on ceramide abundance. A. Original histograms of ceramide abundance at the erythrocyte surface following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 20 µg/ml Temsirolimus. B. Arithmetic means $\pm$ SD ($n = 5$) of ceramide abundance at the erythrocyte surface following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Temsirolimus (20 µg/ml). ***($p < 0.001$) indicates significant difference from the absence of Temsirolimus (ANOVA).

To explore, whether the effects of temsirolimus involved kinase activity, the influence of temsirolimus on annexin-V-binding was tested in the presence of protein kinase C inhibitors staurosporine and chelerythrine, of p38 kinase inhibitor SB203580, or of casein kinase 1 inhibitor D4476. As illustrated in Fig. 7, staurosporine (1 µM) significantly blunted the

Fig. 7. Staurosporine sensitivity of temsirolimus-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) temsirolimus (20 µg/ml) in the absence (A) and presence (B) of protein kinase C inhibitor staurosporine (1 µM). C. Arithmetic means $\pm$ SD (n = 5) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) temsirolimus (20 µg/ml) in the absence (left bars, - staurosporine) and presence (right bars, + staurosporine) of protein kinase C inhibitor staurosporine (1 µM). ***($p < 0.001$) indicates significant difference from the absence of temsirolimus, ##($p < 0.01$) indicates significant difference from the absence of staurosporine (1 µM) (ANOVA).

Fig. 8. Sensitivity of Chelerythrine on temsirolimus-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) temsirolimus (20 µg/ml) in the absence (A) and presence (B) of protein kinase C inhibitor chelerythrine (10 µM). C. Arithmetic means $\pm$ SD (n = 29) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) temsirolimus (20 µg/ml) in the absence (left bars, - chelerythrine) and presence (right bars, + chelerythrine) of protein kinase C inhibitor chelerythrine (10 µM). ***($p < 0.001$) indicates significant difference from the absence of temsirolimus, #($p < 0.05$) indicates significant difference from the absence of chelerythrine (10 µM) (ANOVA).

effect of temsirolimus (20 µg/ml) on annexin-V-binding. Similarly, chelerythrine (10 µM) significantly blunted the effect of temsirolimus (20 µg/ml) on annexin-V-binding (Fig. 8). However, even in the presence of staurosporine or chelerythrine, temsirolimus significantly increased the percentage of annexin-V-binding erythrocytes. Accordingly, temsirolimus-induced cell membrane scrambling was in part but not fully dependent on staurosporine and chelerythrine sensitive kinases.

Additional experiments were performed to test whether the effects of extracellular Ca²⁺ removal and protein kinase C inhibition were additive. To this end, erythrocytes were

Fig. 9. Effects of Ca²⁺ removal without and with addition of chelerythrine on temsirolimus-induced phosphatidylserine exposure. A,B,C. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) temsirolimus (20 µg/ml) in the presence (A) and absence (B) of extracellular Ca²⁺ as well as (C) simultaneous absence of extracellular Ca²⁺ and presence of chelerythrine (10 µM). D. Arithmetic means ± SD (n = 5) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) temsirolimus (20 µg/ml) in the presence (left bars) and absence (middle bars) of Ca²⁺ as well as the absence (right bars) of Ca²⁺ and presence of chelerythrine. *** (p<0.001) indicates significant difference from the absence of temsirolimus, # (p<0.05), ### (p<0.001) indicates significant difference from the presence of Ca²⁺ (ANOVA).

Fig. 10. Chelerythrine sensitivity of temsirolimus-induced reactive oxygen species formation. A,B. Original histogram of DCFDA fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) temsirolimus (20 µg/ml) in the absence (A) and presence (B) of protein kinase C inhibitor chelerythrine (10 µM). C. Arithmetic means ± SD (n = 16) of DCFDA fluorescence in erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) temsirolimus (20 µg/ml) in the absence (left bars, - Chelerythrine) and presence (right bars, + Chelerythrine) of protein kinase C inhibitor Chelerythrine (10 µM). *** (p<0.001) indicates significant difference from the absence of temsirolimus, ## (p<0.01) indicates significant difference from the absence of Chelerythrine (10 µM) (ANOVA).

incubated for 48 hours in the absence or presence of temsirolimus (20 µg/ml) in the presence or nominal absence of extracellular Ca²⁺ with or without presence of chelerythrine. As illustrated in Fig. 9, the annexin-V-binding was significantly blunted by Ca²⁺ removal and further blunted in the additional presence of chelerythrine (10 µM). However, even in the absence of extracellular Ca²⁺ and presence of chelerythrine temsirolimus still significantly increased cell membrane scrambling, an observation pointing to involvement of additional mechanisms.

Further experiments tested whether temsirolimus induced reactive oxygen species (ROS) formation is sensitive to extracellular Ca²⁺ removal and/or chelerythrine. To this end, erythrocytes were incubated for 48 hours in the absence or presence of 20 µg/ml temsirolimus in the absence or presence of chelerythrine (10 µM) and in the presence or nominal absence

Fig. 11. Effect of temsirolimus on hemolysis. Arithmetic means $\pm$ SD ($n = 5$) of the percentage of hemolysed erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) temsirolimus (5 – 20 $\mu\text{g/ml}$). ***($p < 0.001$) indicates significant difference from the absence of temsirolimus (ANOVA).

of extracellular Ca^{2+} . As illustrated in Fig. 10, the formation of ROS was significantly blunted in the presence of chelerythrine. Thus temsirolimus may induce oxidative stress in part by activation of protein kinase C. In contrast, temsirolimus induced DCFDA fluorescence was similar in the presence (from 19.1 ± 0.5 a.u. to 23.5 ± 1.1 a.u., $n = 24$) and in the absence (from 22.1 ± 0.6 a.u. to 24.6 ± 1.5 a.u., $n = 24$) of extracellular Ca^{2+} .

Additional experiments explored whether the effect of temsirolimus on ceramide abundance was sensitive to chelerythrine and/or removal extracellular Ca^{2+} . To this end, erythrocyte ceramide abundance was determined following incubation for 48 hours in the absence or presence of 20 $\mu\text{g/ml}$ temsirolimus in the absence or presence of chelerythrine (10 μM) and in the presence or nominal absence of Ca^{2+} . As a result, the increase of ceramide dependent fluorescence in erythrocytes following temsirolimus was similar in the absence (from 10.6 ± 0.3 a.u. to 14.8 ± 2.7 a.u., $n = 6$) and in the presence (from 10.7 ± 0.4 a.u. to 15.1 ± 2.6 a.u., $n = 6$) of chelerythrine (10 μM), as well as in the presence (from 10.9 ± 0.1 a.u. to 15.3 ± 3.2 a.u., $n = 5$) and absence (from 12.1 ± 0.4 a.u. to 16.2 ± 3.2 a.u., $n = 5$) of extracellular Ca^{2+} .

Temsirolimus (20 $\mu\text{g/ml}$) further increased the percentage of phosphatidylserine exposing erythrocytes to similar values following incubation in the absence (from 1.28 ± 0.10 % to 8.55 ± 0.85 %, $n = 19$) and in the presence (from 1.22 ± 0.07 % to 7.85 ± 1.22 %, $n = 19$) of SB203580 (2 μM). Temsirolimus (20 $\mu\text{g/ml}$) further increased the percentage of phosphatidylserine exposing erythrocytes to similar values following incubation in the absence (from 1.36 ± 0.14 % to 11.79 ± 0.94 %, $n = 15$) and in the presence (from 0.90 ± 0.03 % to 10.07 ± 1.28 %, $n = 15$) of D4476 (10 μM).

To test whether the effects of temsirolimus involved caspases, the influence of temsirolimus on annexin-V-binding was tested in the presence of pancaspase inhibitor zVAD. As a result, temsirolimus (20 $\mu\text{g/ml}$) increased the percentage of phosphatidylserine exposing erythrocytes to similar values following incubation in the absence (from 1.19 ± 0.12 % to 10.51 ± 0.98 %, $n = 5$) and in the presence (from 2.40 ± 0.81 % to 13.54 ± 1.15 %, $n = 5$) of zVAD (10 μM).

In order to explore the effect of temsirolimus on hemolysis, the percentage of hemolytic cells was quantified from the hemoglobin concentration in the supernatant. As illustrated in Fig. 11, a 48 hours exposure to temsirolimus increased the percentage of hemolytic erythrocytes, an effect reaching statistical significance at 20 $\mu\text{g/ml}$ temsirolimus concentration.

Discussion

The present study uncovers a novel effect of temsirolimus, i.e. triggering of erythrocyte shrinkage and of erythrocyte cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Erythrocyte shrinkage and cell membrane scrambling are the hallmarks of eryptosis, the suicidal erythrocyte death.

The effect of temsirolimus on cell shrinkage and cell membrane scrambling was paralleled by an increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) and the cell membrane scrambling was blunted by removal of extracellular Ca^{2+} . The cell shrinkage was at least in part due to increase

of $[Ca^{2+}]_i$ with subsequent activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [61]. An increase of $[Ca^{2+}]_i$ is further known to stimulate a scramblase [61]. Comparison of the Figs. 1 and 2 with Fig. 3 would suggest, however that higher concentrations of temsirolimus are needed to increase $[Ca^{2+}]_i$ than those required to trigger cell shrinkage and cell membrane scrambling. More importantly, temsirolimus triggered cell membrane scrambling even in the nominal absence of extracellular Ca^{2+} , an observation pointing to the involvement of additional mechanisms contributing to temsirolimus induced cell membrane scrambling. Temsirolimus treatment increased the ceramide abundance, which is known to sensitize erythrocytes for the scrambling effect of Ca^{2+} [61]. Moreover, temsirolimus significantly enhanced the abundance of reactive oxygen species, a well known trigger of eryptosis [61]. Temsirolimus is therefore partially effective by inducing oxidative stress. The effect of temsirolimus was significantly blunted by staurosporine and chelerythrine, an observation pointing to the involvement of protein kinase C. Protein kinase C participates for instance in the orchestration of eryptosis following hyperosmotic shock [61]. Apparently, chelerythrine further blunts the temsirolimus induced oxidative stress. The effect of temsirolimus on annexin V binding apparently does not require SB203580 sensitive p38 kinase, D4476 sensitive casein kinase 1, or zVAD sensitive caspases.

Phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood and stimulation of eryptosis may lead to anemia as soon as the loss of erythrocytes surpasses the formation of new erythrocytes by erythropoiesis [61]. The temsirolimus concentrations required for the stimulation of eryptosis are well in the range of concentrations (102 μ g/ml) encountered in the plasma of patients [60]. The stimulation of eryptosis may thus well explain the anemia following temsirolimus treatment [59, 60]. Phosphatidylserine exposing erythrocytes further adhere to the vascular wall [152], stimulate blood clotting and trigger thrombosis [153-155], and may thus interfere with microcirculation [65, 153, 156-159]. Along those lines thrombosis has been observed in some patients receiving temsirolimus treatment [160, 161].

In conclusion, temsirolimus triggers eryptosis with erythrocyte shrinkage and erythrocyte cell membrane scrambling, an effect in part due to Ca^{2+} entry, oxidative stress, ceramide and staurosporine sensitive kinase(s).

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Disclosure Statement

The authors of this manuscript declare that they have no conflicts of interests.

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