Stimulation of Suicidal Erythrocyte Death by Tafenoquine

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Abstract

Background/Aims: The 8-aminoquinoline tafenoquine has been shown to be effective against Plasmodia, Leishmania and Trypanosoma. The substance is at least in part effective by triggering apoptosis of the parasites. Similar to apoptosis, erythrocytes may enter suicidal death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Signaling involved in the regulation of eryptosis include increase of cytosolic Ca^{2+} activity ([Ca^{2+}]_i), oxidative stress, ceramide, zVAD sensitive caspases, SB203580 sensitive p38 kinase, staurosporine sensitive protein kinase C as well as D4476 sensitive casein kinase. The present study explored, whether tafenoquine induces eryptosis and aimed to possibly identify cellular mechanisms involved. Methods: Flow cytometry was employed to estimate phosphatidylserine exposure at the cell surface from annexin-V-binding, cell volume from forward scatter, [Ca^{2+}]_i from Fluo3-fluorescence, ROS formation from 2’,7’-dichlorodihydrofluorescein diacetate (DCFDA) dependent fluorescence, and ceramide abundance utilizing specific antibodies. Results: A 48 hours exposure of human erythrocytes to tafenoquine (500 ng/ml) significantly increased the percentage of annexin-V-binding cells, significantly decreased forward scatter, significantly increased Fluo3-fluorescence, and significantly increased DCFDA fluorescence. Tafenoquine did not significantly modify ceramide abundance. The effect of tafenoquine on annexin-V-binding was significantly blunted but not abolished by removal of extracellular Ca^{2+}. The effect of tafenoquine on annexin-V-binding was not significantly blunted by zVAD (10 µM), SB203580 (2 µM) or staurosporine (1 µM). The effect of tafenoquine on annexin-V-binding was significantly blunted but not abolished by D4476 (10 µM). Conclusions: Tafenoquine triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect at least in part due to stimulation of Ca^{2+} entry, oxidative stress and possibly activation of casein kinase.
Introduction

The 8-aminoquinoline tafenoquine, an antimalarial drug [1-8] effective against all stages of the Plasmodium vivax life cycle including the liver stage, is considered mainly for the prevention of relapsing Plasmodium vivax infection [1, 8-27]. The substance is further effective against trypanosoma brucei [28, 29] and leishmania [29-33]. In leishmania, tafenoquine has been shown to trigger apoptosis [30].

In analogy to apoptosis of nucleated cells, erythrocytes may enter suicidal death or eryptosis, which is characterized by cell shrinkage [34] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [35]. Signaling mediating stimulation of eryptosis include increase of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_i\)) [35], energy depletion [35], caspases [35, 37, 38], casein kinase 1α, Janus-activated kinase JAK3, protein kinase C, and p38 kinase [35]. Inhibiting signaling molecules include nitric oxide [35], AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase and sorafenib/sunitinib sensitive kinases [35]. Eryptosis is triggered by a multitude of xenobiotics [35, 39-80].

The present study explored, whether tafenoquine influences eryptosis. To this end, human erythrocytes from healthy volunteers were exposed to tafenoquine and phosphatidylserine surface abundance, cell volume, [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 x 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 tafenoquine (MedChem Express, Princeton, USA). In order to explore the involvement of caspases, erythrocytes were exposed for 48 hours to a combination of tafenoquine and pancaspase inhibitor zVAD (Enzo Life Sciences, Lörrach, Germany). To test for an involvement of kinases, erythrocytes were exposed for 48 hours to a combination of tafenoquine and p38 kinase inhibitor SB203580 (Tocris bioscience, Bristol, UK), protein kinase C inhibitor staurosporine (Enzo Life Sciences, Lörrach, Germany), or casein kinase inhibitor D4476 (Tocris bioscience, Bristol, UK).

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 tafenoquine 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, Schnelldorf, 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 geometric mean of the DCFDA dependent fluorescence was determined.

Ceramide abundance

For the determination of ceramide, a monoclonal antibody-based assay was used. To this end, a 100 µl suspension of erythrocytes was stained for 1 hour at 37°C with 1 µg/ml anti ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. The samples were washed twice with PBS-BSA. The cell suspension was subsequently 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. Finally, the geometric mean 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 tafenoquine influences the suicidal erythrocyte death or eryptosis, which is characterized by cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

Forward scatter in flow cytometry was taken as a measure of erythrocyte volume. Prior to the measurements, erythrocytes were incubated for 48 hours in Ringer solution without or with tafenoquine (250 – 500 ng/ml). As illustrated in Fig. 1, 500 ng/ml tafenoquine significantly decreased erythrocyte forward scatter.

Fig. 1. Effect of tafenoquine 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 500 ng/ml tafenoquine. (B) Arithmetic means ± SEM (n = 14) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) tafenoquine (250 - 500 ng/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). *** (p<0.001) indicates significant difference from the absence of tafenoquine (ANOVA).
Annexin-V-binding determined by flow cytometry was utilized to identify phosphatidylserine exposing erythrocytes. Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with tafenoquine (250–500 ng/ml). As shown in Fig. 2, a 48 hours exposure to 500 ng/ml tafenoquine significantly increased the percentage of phosphatidylserine exposing erythrocytes.

Fluo3 fluorescence was taken as a measure of cytosolic Ca²⁺ activity ([Ca²⁺]ᵢ). Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with tafenoquine (250–500 ng/ml). As displayed in Fig. 3, a 48 hours exposure to 500 ng/ml tafenoquine significantly increased the Fluo3 fluorescence.

In order to test whether triggering of tafenoquine-induced annexin-V-binding required entry of extracellular Ca²⁺, erythrocytes were incubated for 48 hours in the absence or presence of 500 ng/ml tafenoquine in the presence or nominal absence of extracellular Ca²⁺. As illustrated in Fig. 4, removal of extracellular Ca²⁺ significantly blunted the effect of tafenoquine (500 ng/ml) on annexin-V-binding. However, even in the absence of extracellular Ca²⁺, tafenoquine significantly increased the percentage of annexin-V-binding erythrocytes. Thus, the tafenoquine-induced cell membrane scrambling was in part but not exclusively triggered by entry of extracellular Ca²⁺.
Fig. 4. Ca\(^{2+}\) sensitivity of tafenoquine-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) tafenoquine (500 ng/ml) in the presence (A) and absence (B) of extracellular Ca\(^{2+}\). (C) Arithmetic means ± SEM (n = 9) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) tafenoquine (500 ng/ml) in the presence (left bars, +Ca\(^{2+}\)) and absence (right bars, -Ca\(^{2+}\)) of Ca\(^{2+}\). ***(p<0.001) indicates significant difference from the absence of tafenoquine, ###(p<0.01) indicates significant difference from the presence of Ca\(^{2+}\) (ANOVA).

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with tafenoquine (500 ng/ml). As shown in Fig. 5, a 48 hours exposure to tafenoquine significantly increased the DCFDA fluorescence reflecting induction of oxidative stress.

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As a result, the ceramide abundance was similar following exposure to 500 ng/ml tafenoquine (18.68 ± 0.28 a.u., n = 5) and in the absence of tafenoquine (19.06 ± 0.32 a.u., n = 5). Thus, tafenoquine did not appreciably affect ceramide abundance.

In order to test whether the effect of tafenoquine on cell membrane scrambling required caspase activity, the influence of tafenoquine on annexin-V-binding was tested in the presence of pancaspase inhibitor zVAD. As a result, tafenoquine (500 ng/ml) increased...
Fig. 6. D4476 sensitivity of tafenoquine-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) tafenoquine (500 ng/ml) in the absence (A) and presence (B) of casein kinase inhibitor D4476 (10 µM). (C) Arithmetic means ± SEM (n = 9) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (left bars) or with (right bars) tafenoquine (500 ng/ml) in the absence (-D4476) and presence (+D4476) of casein kinase inhibitor D4476 (10 µM). ***(p<0.001) indicates significant difference from the absence of tafenoquine, ###(p<0.001) indicates significant difference from the absence of D4476 (10 µM) (ANOVA).

Fig. 7. D4476 sensitivity of tafenoquine-induced Ca²⁺ entry. (A,B) Original histograms of Fluo3 fluorescence of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) tafenoquine (500 ng/ml) in the absence (A) and presence (B) of casein kinase inhibitor D4476 (10 µM). (C) Arithmetic means ± SEM (n = 5) of Fluo3 fluorescence of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) tafenoquine (500 ng/ml) in the absence (left bars, -D4476) and presence (right bars, +D4476) of casein kinase inhibitor D4476 (10 µM). * (p<0.05) and ***(p<0.001) indicate significant difference from the absence of tafenoquine, ###(p<0.001) indicates significant difference from the absence of D4476 (10 µM) (ANOVA).

the percentage of phosphatidylserine exposing erythrocytes to similar values in the absence (from 1.12 ± 0.09 % to 37.11 ± 3.00 %, n = 26) and in the presence (from 2.60 ± 0.46 % to 32.34 ± 2.76 %, n = 26) of zVAD (10 µM).

To explore whether the effects of tafenoquine involved kinase activity, the influence of tafenoquine on annexin-V-binding was tested in the presence of p38 kinase inhibitor...
Tafenoquine (500 ng/ml) increased the percentage of phosphatidylserine exposing erythrocytes to similar values in the absence (from 1.00 ± 0.09 % to 33.83 ± 2.84 %, n = 28) and in the presence (from 0.98 ± 0.05 % to 30.57 ± 2.71 %, n = 28) of SB203580 (2 µM). Moreover, tafenoquine (500 ng/ml) increased phosphatidylserine exposure to similar values in the absence (from 1.01 ± 0.11 % to 44.31 ± 4.24 %, n = 9) and in the presence (from 4.14 ± 0.44 % to 47.37 ± 3.02 %, n = 9) of staurosporine (1 µM). As illustrated in Fig. 6, addition of D4476 (10 µM) significantly blunted the effect of tafenoquine (500 ng/ml) on annexin-V-binding. However, even in the presence of D4476, tafenoquine significantly increased the percentage of annexin-V-binding erythrocytes. Thus, tafenoquine-induced cell membrane scrambling was in part but not fully dependent on casein kinase activity. As shown in Fig. 7 & 8, addition of D4476 (10 µM) further significantly blunted the effect of tafenoquine (500 ng/ml) on Fluo3 fluorescence and DCFDA fluorescence. Thus, the tafenoquine-induced Ca^{2+} entry and oxidative stress were partially dependent on casein kinase activity.

**Discussion**

The present observations demonstrate that tafenoquine triggers suicidal erythrocyte death or eryptosis. Tafenoquine treatment is followed by appearance of the two hallmarks of eryptosis, i.e. cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The tafenoquine concentration required for the stimulation of eryptosis is in the range of concentrations encountered in the plasma of tafenoquine-treated patients [81-84]. The effect of tafenoquine treatment on eryptosis may be enhanced in clinical conditions with accelerated eryptosis, such as iron deficiency [35], dehydration [85], hyperphosphatemia [86], chronic kidney disease (CKD) [87-90], hemolytic-uremic syndrome [91], diabetes [92], hepatic failure [93], malignancy [35], sepsis [94], malaria [35], sickle-cell disease [35], beta-thalassemia [35], Hb-C-deficiency [35], glucose-6-phosphate dehydrogenase deficiency [35], and many others.

Fig. 8. D4476 sensitivity of tafenoquine-induced oxidative stress. (A,B) Original histograms of DCFDA fluorescence of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) tafenoquine (500 ng/ml) in the absence (A) and presence (B) of casein kinase inhibitor D4476 (10 µM). (C) Arithmetic means ± SEM (n = 9) of DCFDA fluorescence of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) tafenoquine (500 ng/ml) in the absence of D4476 and presence of casein kinase inhibitor D4476 (10 µM). ***(p<0.001) indicates significant difference from the absence of tafenoquine, ###(p<0.001) indicates significant difference from the absence of D4476 (10 µM) (ANOVA).
dehydrogenase (G6PD) deficiency [35], and Wilsons disease [95]. Notably, tafenoquine toxicity is enhanced in patients with G6PD-deficiency [96].

The present observations shed some light on the cellular mechanisms accounting for the stimulation of phosphatidylserine translocation. The effect of tafenoquine on cell membrane scrambling was paralleled by an increase of cytosolic Ca^{2+} activity ([Ca^{2+}]). Moreover, the stimulation of cell membrane scrambling was in part dependent on Ca^{2+} entry from the extracellular space, as removal of extracellular Ca^{2+} significantly blunted the tafenoquine induced phosphatidylserine translocation. However, even in the absence of extracellular Ca^{2+}, tafenoquine triggered significant phosphatidylserine translocation, an observation pointing to involvement of additional mechanisms. As a matter of fact, tafenoquine significantly increased DCFDA fluorescence, reflecting oxidative stress, a well known stimulator of eryptosis [35]. Tafenoquine did not significantly modify ceramide abundance, another stimulator of eryptosis [35]. The effect of tafenoquine on annexin-V-binding was apparently insensitive to inhibition of caspases by zVAD, to inhibition of p38 kinase by SB203580 and to inhibition of protein kinase C by staurosporine. However, the effect of tafenoquine on annexin-V-binding was slightly but significantly blunted following casein kinase inhibition with D4476.

The effect of tafenoquine on cell shrinkage could have resulted from Ca^{2+} entry with subsequent increase of [Ca^{2+}], activation of Ca^{2+} sensitive K⁺ channels, K⁺ exit, cell membrane hyperpolarization, Cl⁻ exit, and thus cellular loss of KCl with water [35].

Phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood. In view of the present data, it is tempting to speculate that the acceleration of eryptosis by tafenoquine may contribute to the beneficial effect of the drug in malaria. *Plasmodium* triggers Ca^{2+} entry into the host erythrocyte which in turn stimulates eryptosis with subsequent sequestration of the infected erythrocytes [35]. Thus, accelerated eryptosis counteracts the development of parasitemia by clearance of infected erythrocytes. Along those lines, several clinical conditions associated with accelerated eryptosis confer some protection against a severe course of the disease [35].

The elimination of eryptotic erythrocytes may, on the other hand, lead to anemia as soon as the loss of erythrocytes surpasses the formation of new erythrocytes by erythropoiesis [35]. Phosphatidylserine exposing erythrocytes may further impair microcirculation due to adherence to the vascular wall, stimulation of blood clotting and triggering of thrombosis [36, 97-104].

**Conclusion**

Tafenoquine triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect apparently involving Ca^{2+} entry, oxidative stress and activation of casein kinase.

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**Disclosure Statement**

None.

**References**


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