Original Paper

Effects of Antimalarial Tafenoquine on
Blood Platelet Activity and SurvivalHang Cao^a Rosi Bissinger^a Anja T. Umbach^a Abdulla Al Mamun Bhuyan^a
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Key Words

Cytosolic Ca²⁺ concentration • Phosphatidylserine translocation • P-selectin • Integrin • Caspase • Cell volume

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. Moreover, tafenoquine has been shown to trigger eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The effect of tafenoquine on eryptosis is in part due to stimulation of Ca²⁺ entry and oxidative stress. Ca²⁺ entry is a critical event in the activation of blood platelets by thrombin and collagen related peptide (CRP). The present study explored, whether tafenoquine influences Ca²⁺ entry, activation and apoptosis of blood platelets.**Methods:** Platelets isolated from wild-type mice were exposed for 30 minutes to tafenoquine (2.5 µg/ml) without or with an additional treatment with thrombin (0.01 U/ml) or CRP (2 µg/ml or 5 µg/ml). Flow cytometry was employed to estimate cytosolic Ca²⁺-activity ([Ca²⁺]_i) from Fluo-3 fluorescence, platelet degranulation from P-selectin abundance, integrin activation from α_{IIb}β₃ integrin abundance, phosphatidylserine abundance from annexin-V-binding, relative platelet volume from forward scatter, reactive oxygen species (ROS) from DCF fluorescence, caspase 3 activity with an active caspase-3 Staining kit, and aggregation utilizing staining with CD9-APC and CD9-PE. **Results:** Both, thrombin (0.01 U/ml) and CRP (2 µg/ml or 5 µg/ml), significantly increased [Ca²⁺]_i, P-selectin abundance, active α_{IIb}β₃ integrin, and annexin-V-binding, and both significantly decreased platelet volume, activated caspase 3 and stimulated aggregation. Administration of tafenoquine (2.5 µg/ml, 30 min) significantly decreased [Ca²⁺]_i both, in the absence and presence of thrombin and CRP. Tafenoquine significantly blunted the effect of thrombin and CRP on [Ca²⁺]_i, P-selectin abundance, and active α_{IIb}β₃ integrin, but significantly increased ROS and annexin-V-binding, significantly augmented the effect of thrombin on caspase 3 activity and platelet volume and significantly enhanced platelet aggregation. **Conclusions:** Tafenoquine counteracts thrombin and CRP induced increase of cytosolic Ca²⁺ activity and platelet activation, but enhances platelet apoptosis and platelet aggregation.© 2017 The Author(s)
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Introduction

The antimalarial drug tafenoquine [1-8] is effective against all stages of the *Plasmodium vivax* life cycle including the liver stage and is thus mainly considered for prevention of relapsing *Plasmodium vivax* infection [1, 8-27]. Tafenoquine could be further used for the treatment of *trypanosoma brucei* [28, 29] and *leishmania* [29-33] infection. Most recently, tafenoquine has been shown to trigger eryptosis, the suicidal death of erythrocytes characterized by cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane [34]. The effect was due to stimulation of Ca^{2+} entry and oxidative stress [34].

Ca^{2+} entry is a critical event in the activation of blood platelets by thrombin and collagen related peptide (CRP), which contribute to primary hemostasis following vascular injury and by the same token are involved in the pathophysiology of acute thrombotic occlusion [35]. Ca^{2+} entry into platelets is triggered by Ca^{2+} release from intracellular stores [36] and subsequent activation of Ca^{2+} release-activated channel Orai1 in the plasma membrane [37-40]. The increase of cytosolic Ca^{2+} activity triggers activation of $\alpha_{\text{IIb}}\beta_3$ integrin and degranulation, the latter being apparent from increase of P-selectin abundance [41, 42]. Ca^{2+} entry may further be followed by stimulation of platelet apoptosis, which is apparent from caspase activation, cell membrane scrambling and platelet shrinkage [43, 44].

The present study explored, whether tafenoquine influences Ca^{2+} entry, activation and apoptosis of blood platelets. To this end, murine platelets were exposed to tafenoquine prior to and following activation with thrombin or CRP and cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), P-selectin abundance, active $\alpha_{\text{IIb}}\beta_3$ integrin, reactive oxygen species, cell membrane scrambling, platelet volume, caspase activity, and aggregation determined by flow cytometry.

Materials and Methods

Mice

All animal experiments were conducted according to the German law for the welfare of animals and were approved by the authorities of the state of Baden-Württemberg. Experiments were performed with blood platelets isolated from wild type mice. The mice had free access to water and control chow (Ssniff, Soest, Germany).

Preparation of mouse platelets

Platelets were obtained from 10- to 12-week-old mice of either sex. The mice were anesthetized and 800 μl blood was drawn from the retro-orbital plexus into tubes with 200 μl acid-citrate-dextrose buffer before the mice were sacrificed [45]. Platelet rich plasma (PRP) was obtained by centrifugation at 260 g for 5 minutes. Afterwards, PRP was centrifuged at 640 g for 5 minutes to pellet the platelets. Where necessary, apyrase (0.02 U/ml; Sigma-Aldrich) and prostaglandin E_2 (0.5 μM ; Calbiochem) were added to the PRP to prevent activation of platelets during isolation. After two washing steps, the pellet of washed platelets was resuspended in modified Tyrode-HEPES buffer (pH 7.4, supplemented with 1 mM CaCl_2). Where indicated, thrombin (Roche, Basel, Switzerland) was added at the indicated concentrations [46].

Cytosolic calcium

For the measurement of the cytosolic Ca^{2+} concentration, the platelet preparation was washed once in Tyrode buffer (pH 7.4), stained with 3 μM Fluo-3AM (Biotinim, USA) in the same buffer and incubated at 37°C for 30 minutes. Following the indicated experimental treatment, fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm utilizing a BD FACSCalibur (BD Biosciences, Heidelberg, Germany) [46].

P-selectin and activated integrin abundance

Fluorophore-labeled antibodies were utilized for the detection of P-selectin surface abundance (Wug. E9-FITC) and the active form of $\alpha_{\text{IIb}}\beta_3$ integrin (JON/A-PE). Washed mouse platelets (1×10^6) were suspended in modified Tyrode buffer (pH 7.4) containing 1 mM CaCl_2 and antibodies (1:10 dilution) and subsequently

exposed to the respective treatments and for the indicated time periods at room temperature (RT). The reaction was stopped by addition of PBS and the samples were immediately analyzed on a BD FACSCalibur [47].

Phosphatidylserine exposure and forward scatter

Phosphatidylserine exposure was determined in platelets with and without 10 minutes thrombin or CRP treatment. To this end, the platelet preparation was centrifuged at 660 g for 5 minutes followed by washing once with Tyrode buffer (pH 7.4) with 1 mM CaCl_2 , staining with 1:20 dilution of Annexin-V FITC (Mabtag, Germany) in Tyrode buffer (pH 7.4) with 2 mM CaCl_2 and incubation at 37°C for 30 minutes. Annexin-V binding reflecting surface exposure of phosphatidylserine was evaluated by flow cytometry utilizing a BD FACSCalibur. In parallel, the forward scatter (FSC) of the platelets was determined by flow cytometry as a measure of platelet size.

Quantification of reactive oxygen species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein (DCF) diacetate. Washed platelets were incubated for 10 minutes (37°C) with 0.01 U/ml thrombin and 2 µg/ml CRP, and washed two times with 350 µl Tyrode buffer after stimulation by agonists. Subsequently, the platelets were stained with DCF (10 µM; Sigma, Schnelldorf, Germany) in Tyrode buffer at 37°C for 30 min and washed once in 150 µl Tyrode buffer. The DCF-loaded platelets were resuspended in 200 µl Tyrode buffer and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a BD FACSCalibur.

Caspase-3 activity

Caspase 3 activity was determined utilizing a CaspGlow Fluorescein Active Caspase-3 Staining kit from BioVision (CA, USA) according to the manufacturer's instruction. Fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm in a BD FACSCalibur (BD Biosciences, USA).

Platelet aggregation

Aggregation was determined utilizing flow cytometry as previously described [39, 48]. To this end, platelets were labeled with CD9-APC and CD9-PE monoclonal antibodies (1:100 dilution, Abcam) for 15 minutes at room temperature. Following incubation, differently labeled samples were washed twice, mixed 1:1 and then pre-incubated at 37°C while shaking at 600 rpm for 10 min. Pre-incubated platelets were activated with thrombin or collagen related peptide at 37°C while shaking at 1000 rpm. At the indicated time points, samples were fixed by addition of 0.5% paraformaldehyde (Carl Roth, Germany) in phosphate-buffered saline. The fixed samples were measured utilizing a BD FACSCalibur (BD Biosciences, Heidelberg, Germany). For quantification, a quadrant was set in the dot plot of respective channels on non-stimulated platelets. The appearance of double-colored events in the upper right quadrant (Q2) was taken as evidence for aggregation and quantified as percentage of total amount of labeled events ($Q1+Q2+Q4$) at every time point analyzed.

Statistical analysis

Data are provided as means $\pm$ SEM; n represents the number of independent experiments. All data were tested for significance using ANOVA with Tukey's test as post-test or unpaired student's t-test as appropriate. Differences with $p < 0.05$ were considered statistically significant.

Results

The present study explored, whether tafenoquine triggers activation and apoptosis of blood platelets. To this end, murine platelets were isolated from wild type mice and exposed to thrombin (0.01 U/ml) or collagen related peptide (2 µg/ml or 5 µg/ml) in the absence and presence of tafenoquine (2.5 µg/ml).

In a first series of experiments, cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was determined utilizing Fluo-3 fluorescence. As illustrated in Fig. 1, both, thrombin and CRP triggered a sharp

Fig. 1. Tafenoquine sensitive cytosolic Ca^{2+} concentration. A-C. Original histogram overlays of Fluo-3 fluorescence reflecting cytosolic Ca^{2+} activity in murine platelets without (A) and with a treatment with (B) thrombin (100 s, 0.01 U/ml) or (C) CRP (150 s, 2 $\mu\text{g}/\text{ml}$) without (grey areas) and with (black lines) presence of tafenoquine (2.5 $\mu\text{g}/\text{ml}$, 30 min). D. Arithmetic means $\pm$ SEM ($n = 8$) of Fluo-3 fluorescence reflecting cytosolic Ca^{2+} activity in murine platelets in the absence (white bars) and presence (black bars) of 2.5 $\mu\text{g}/\text{ml}$ tafenoquine without (resting) and with addition of thrombin (0.01 U/ml) or CRP (2 $\mu\text{g}/\text{ml}$). ### ($p < 0.001$) indicates statistically significant difference from absence of thrombin and CRP, *** ($p < 0.001$) indicates statistically significant difference from absence of tafenoquine.

Fig. 2. Tafenoquine sensitive platelet degranulation. A-C. Original histogram overlays of P-selectin related fluorescence in murine platelets without (A) and with a 15 min treatment with (B) thrombin (0.01 U/ml) or (C) CRP (2 $\mu\text{g}/\text{ml}$) without (grey areas) and with (black lines) presence of tafenoquine (2.5 $\mu\text{g}/\text{ml}$, 30 min). D. Arithmetic means $\pm$ SEM ($n = 6$) of the P-selectin related fluorescence (arbitrary units) in murine platelets in the absence (white bars) and presence (black bars) of 2.5 $\mu\text{g}/\text{ml}$ tafenoquine without (resting) and with a 15 min thrombin (0.01 U/ml) or CRP (2 $\mu\text{g}/\text{ml}$) treatment. ### ($p < 0.001$) indicates statistically significant difference from absence of thrombin and CRP, ** ($p < 0.01$) *** ($p < 0.001$) indicates statistically significant difference from absence of tafenoquine.

increase of $[\text{Ca}^{2+}]_i$. Tafenoquine significantly decreased platelet $[\text{Ca}^{2+}]_i$ both, in the absence and presence of thrombin or CRP. Along those lines, tafenoquine significantly blunted the effect of thrombin and CRP on $[\text{Ca}^{2+}]_i$ (Fig. 1).

Platelet degranulation was estimated from the increase of P-selectin abundance at the platelet surface, which was determined utilizing specific antibodies and flow cytometry. As illustrated in Fig. 2, tafenoquine slightly but significantly increased the P-selectin abundance in the absence of thrombin or CRP. Treatment with either, thrombin or CRP was followed by a sharp increase of P-selectin abundance, an effect significantly blunted in the presence of tafenoquine (Fig. 2).

The abundance of active integrin $\alpha_{\text{IIb}}\beta_3$ was determined utilizing again specific antibodies and flow cytometry. As illustrated in Fig. 3, in the absence of thrombin or CRP, tafenoquine slightly but significantly increased the abundance of active integrin $\alpha_{\text{IIb}}\beta_3$. Treatment with either,

Fig. 3. Tafenoquine sensitive integrin $\alpha_{IIb}\beta_3$ activation. A-C. Original histogram overlays of activated $\alpha_{IIb}\beta_3$ integrin related fluorescence in murine platelets without (A) and with a 15 min treatment with (B) thrombin (0.01 U/ml) or (C) CRP (2 μ g/ml) without (grey areas) and with (black lines) presence of tafenoquine (2.5 μ g/ml, 30 min). D. Arithmetic means $\pm$ SEM (n = 6)

of activated $\alpha_{IIb}\beta_3$ integrin related fluorescence (arbitrary units) in murine platelets in the absence (white bars) and presence (black bars) of 2.5 μ g/ml tafenoquine without (resting) and with a 15 min thrombin (0.01 U/ml) or CRP (2 μ g/ml) treatment. ### ($p < 0.001$) indicates statistically significant difference from absence of thrombin and CRP, *** ($p < 0.001$) indicates statistically significant difference from absence of tafenoquine.

Fig. 4. Tafenoquine sensitive cell membrane scrambling. A-C. Original histogram overlays of annexin-V-binding reflecting phosphatidylserine abundance at the surface of murine platelets without (A) and with a 10 min treatment with (B) thrombin (0.01 U/ml) or (C) CRP (5 μ g/ml) without (grey areas) and with (black lines) presence of tafenoquine (2.5 μ g/ml, 30 min). D. Arithmetic means $\pm$ SEM (n = 4) of the percentage of annexin-V-binding murine platelets in the absence (white bars) and presence (black bars) of 2.5 μ g/ml tafenoquine without (resting) and with a 10 min thrombin (0.01 U/ml) or CRP (5 μ g/ml) treatment. ### ($p < 0.001$) indicates statistically significant difference from absence of thrombin and CRP, *** ($p < 0.001$) indicates statistically significant difference from absence of tafenoquine.

D. Arithmetic means $\pm$ SEM (n = 4) of the percentage of annexin-V-binding murine platelets in the absence (white bars) and presence (black bars) of 2.5 μ g/ml tafenoquine without (resting) and with a 10 min thrombin (0.01 U/ml) or CRP (5 μ g/ml) treatment. ### ($p < 0.001$) indicates statistically significant difference from absence of thrombin and CRP, *** ($p < 0.001$) indicates statistically significant difference from absence of tafenoquine.

thrombin or CRP was followed by a sharp increase of active integrin $\alpha_{IIb}\beta_3$, an effect significantly blunted in the presence of tafenoquine (Fig. 3).

Phosphatidylserine abundance was estimated from annexin-V-binding. As illustrated in Fig. 4, the percentage of annexin-V positive platelets was markedly and significantly enhanced by treatment of the platelets with thrombin or CRP. In the absence of thrombin and CRP, treatment with tafenoquine was followed by a slight but significant increase of the percentage of annexin-V

Fig. 5. Tafenoquine sensitive platelet forward scatter. A-C. Original histogram overlays of forward scatter reflecting cell volume of murine platelets without (A) and with a 10 min treatment with (B) thrombin (0.01 U/ml) or (C) CRP (5 µg/ml) without (grey areas) and with (black lines) presence of tafenoquine (2.5 µg/ml, 30 min). D. Arithmetic means ± SEM (n = 4) of forward scatter reflecting cell volume of murine platelets in the absence (white bars) and presence (black bars) of 2.5 µg/ml tafenoquine without (resting) and with a 10 min thrombin (0.01 U/ml) or CRP (5 µg/ml) treatment. ### (p<0.001) indicates statistically significant difference from absence of thrombin and CRP, *** (p<0.001) indicates statistically significant difference from absence of tafenoquine.

binding platelets. Tafenoquine further significantly augmented the effect of thrombin and CRP on the percentage of annexin-V binding platelets (Fig. 4).

Platelet volume was estimated from forward scatter, which was determined by flow cytometry. As illustrated in Fig. 5, both, thrombin and CRP, significantly decreased the forward scatter pointing to cell shrinkage. Tafenoquine treatment did not significantly modify forward scatter in the absence of thrombin or CRP and did not significantly modify the effect of CRP on forward scatter, but significantly augmented the effect of thrombin on the forward scatter (Fig. 5).

The abundance of reactive oxygen species (ROS) was quantified utilizing DCF fluorescence. As illustrated in Fig. 6, the ROS abundance was markedly and significantly enhanced by treatment of the platelets with thrombin or CRP. In the absence of thrombin and CRP, treatment with tafenoquine was followed by a slight but significant increase of the ROS abundance. Tafenoquine further significantly augmented the effect of thrombin and CRP on the ROS abundance (Fig. 6).

A kit has been used for the detection of activated caspase 3. As illustrated in Fig. 7, both, thrombin and CRP, significantly increased the caspase activity. Tafenoquine treatment did not significantly modify caspase activity in the absence of thrombin or CRP and did not significantly modify the effect of CRP on caspase activity, but significantly augmented the effect of thrombin on caspase activity (Fig. 7).

To elucidate the effect of tafenoquine on platelet aggregation, platelets were labeled with two distinct dyes and the coincidence of the two dyes estimated by flow cytometry. As illustrated in Fig. 8, aggregation was markedly and significantly enhanced by treatment of the platelets with thrombin or CRP. In the absence of thrombin and CRP, treatment with tafenoquine was followed by a slight but significant increase of aggregation. Tafenoquine further significantly augmented the effect of thrombin and CRP on aggregation (Fig. 8).

Discussion

The present observations demonstrate that tafenoquine significantly decreases $[Ca^{2+}]$, and significantly interferes with the strong up-regulating effect of thrombin and collagen related

Fig. 6. Tafenoquine sensitive reactive oxygen species (ROS). A-C. Original histogram overlays of DCF fluorescence reflecting ROS abundance (arbitrary units) in murine platelets without (A) and with a 10 min treatment with (B) thrombin (0.01 U/ml) or (C) CRP (2 µg/ml) without (grey areas) and with (black lines) presence of tafenoquine (2.5 µg/ml, 30 min). D. Arithmetic means ± SEM (n = 4) of DCF fluorescence reflecting ROS abundance (arbitrary units) in murine platelets in the absence (white bars) and presence (black bars) of 2.5 µg/ml tafenoquine without (resting) and with a 10 min thrombin (0.01 U/ml) or CRP (2 µg/ml) treatment. ## (p<0.01) indicates statistically significant difference from absence of thrombin and CRP, * (p<0.05) ** (p<0.01) indicates statistically significant difference from absence of tafenoquine.

Fig. 7. Tafenoquine sensitive caspase-3 activity. A-C. Original histogram overlays of fluorescence reflecting caspase-3 activity in murine platelets without (A) and with a 10 min treatment with (B) thrombin (0.01 U/ml) or (C) CRP (5 µg/ml) without (grey areas) and with (black lines) presence of tafenoquine (2.5 µg/ml, 30 min). D. Arithmetic means ± SEM (n = 4) of the percentage of caspase-3-FITC positive murine platelets in the absence (white bars) and presence (black bars) of 2.5 µg/ml tafenoquine without (resting) and with a 10 min thrombin (0.01 U/ml) or CRP (5 µg/ml) treatment. ### (p<0.001) indicates statistically significant difference from absence of thrombin and CRP, *** (p<0.001) indicates statistically significant difference from absence of tafenoquine.

peptide (CRP) on $[Ca^{2+}]_i$. Moreover, tafenoquine slightly increased the P-selectin abundance reflecting platelet degranulation and slightly increased $\alpha_{IIb}\beta_3$ integrin activity in the absence of thrombin and CRP, but significantly blunted the stimulating effect of thrombin and CRP on platelet degranulation and $\alpha_{IIb}\beta_3$ integrin activity. In contrast, tafenoquine increased cell

membrane scrambling apparent from annexin-V-binding both, in the absence and presence of thrombin and CRP. Thus, tafenoquine augmented the effect of thrombin and CRP on annexin-V-binding. Similarly, tafenoquine augmented the effect of thrombin on forward scatter.

The observed effects of tafenoquine on platelet degranulation and $\alpha_{IIb}\beta_3$ integrin activity may be secondary to its effect on cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) [39, 48]. An increase of $[Ca^{2+}]_i$ is a powerful mechanism triggering platelet activation and may thus lead to development of arterial thrombosis [37]. Increased $[Ca^{2+}]_i$ is further a well-known stimulator of cell membrane phospholipid scrambling with translocation of phosphatidylserine to the platelet surface [49-52]. However, tafenoquine stimulates cell membrane scrambling and augments the stimulating effect of thrombin and CRP on cell membrane scrambling, an effect not explained by the observed effects on $[Ca^{2+}]_i$. Triggering of platelet apoptosis may involve several additional mechanisms including depolarization of the mitochondrial inner membrane with subsequent cytochrome c release and caspase activation [43]. The mitochondria may be influenced by either pro- or anti-apoptotic members of the Bcl-2 family of proteins [43]. The present observations do not allow any safe conclusions as to the mechanisms underlying the effect of tafenoquine on platelet apoptosis. It is noteworthy, however, that stimulation of Trypanosoma and Leishmania death by tafenoquine is preceded by depolarization of the mitochondrial membrane and production of reactive oxygen species [28, 30, 32]. Tafenoquine triggers, however, the suicidal death of erythrocytes, i.e. of cells

lacking mitochondria [34]. Thus, mitochondria are not required for the effect of tafenoquine on cell death.

Whatever mechanism involved, phosphatidylserine translocation to the platelet surface supports the procoagulant function of platelets and thus contributes to stimulation of hemostasis [53]. Eventually, phosphatidylserine exposing platelets are further bound to and engulfed by macrophages [54].

In conclusion, treatment of platelets with tafenoquine decreases cytosolic Ca^{2+} activity and blunts the effect of thrombin and CRP on degranulation and $\alpha_{\text{IIb}}\beta_3$ integrin activity. By the same token, tafenoquine augments the effect of thrombin and CRP on cell membrane scrambling and aggregation. Whether or not those effects modify platelet activation and aggregation *in vivo*, remains to be shown.

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Disclosure Statement

All authors declare that there are no conflicts of interest.

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