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Tadalafil impacts the mechanical properties of *Plasmodium falciparum* gametocyte-infected erythrocytes

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Abstract

Plasmodium falciparum gametocytes modify the mechanical properties of their erythrocyte host to persist for several weeks in the blood circulation and to be available for mosquitoes. These changes are tightly regulated by the plasmodial phosphodiesterase delta that decreases both the stiffness and the permeability of the infected host cell. Here, we address the effect of the phosphodiesterase inhibitor tadalafil on deformability and permeability of gametocyte-infected erythrocytes. We show that this inhibitor drastically increases isosmotic lysis of gametocyte-infected erythrocytes and impairs their ability to circulate in an *in vitro* model for splenic retention. These findings indicate that tadalafil represents a novel drug lead potentially capable of blocking malaria parasite transmission by impacting gametocyte circulation.

The clinical symptoms of malaria are attributed to *Plasmodium* asexual stages, whereas parasite transmission from humans to mosquitoes relies on the sexual stages, called gametocytes. For *Plasmodium falciparum*, the causative agent of the most severe form of human malaria, immature gametocytes develop in the bone marrow [1], and appear only as mature stages in the peripheral blood where they are available for uptake by mosquitoes. The persistence of mature gametocytes in the blood circulation is crucial for parasites to be ingested by mosquitoes and thereby to be transmitted to another host. The estimate for the circulating gametocyte lifespan is several weeks during which mature gametocytes are able to avoid splenic retention, immune recognition and hemolysis [2, 3]. Among the different strategies evolved by *P. falciparum* parasites to persist for such a long time in the peripheral blood, gametocytes modify the mechanical properties of their erythrocyte host. As observed in mature asexual stages [4-6], immature gametocytes increase the stiffness and the permeability of their host cell by exporting proteins to the erythrocyte membrane [7, 8]. The increase in stiffness is linked to both the interaction of the parasite proteins STEVOR with one or several erythrocyte membrane skeleton proteins composing the ankyrin complex [9, 10] and to the remodelling of lateral and vertical interactions within the erythrocyte skeleton [11]. The increase in permeability is due to the activation of the new permeability pathways (NPPs) in which the RhopH proteins play an important role [7, 12]. However, unlike in asexual stages, these changes in erythrocyte membrane mechanical properties are reversible in sexual stages. The increase in stiffness observed in immature gametocyte-infected erythrocytes (GIE) is followed by a switch in deformability in mature stages [8, 13, 14]. These observations led to the hypothesis that the important stiffness of the infected erythrocyte may contribute to sequestration of immature stages in the bone marrow parenchyma by mechanical retention, and then the switch in deformability of the infected cell may trigger release of mature stages

in blood circulation. In parallel, gametocytes also regulate the permeability of their host cell: NPPs are activated in stage I GIE at levels similar to that of trophozoites and then this activity declines along gametocyte maturation, leading to undetectable channel activity in stage V GIE comparable to that usually observed in uninfected erythrocytes [7]. The virtual absence of channel activity in mature stages may allow the infected erythrocyte to decrease its osmotic fragility and thus to persist longer in the blood circulation. Interestingly, both deformability and permeability of GIE appear to be tightly regulated by a cyclic AMP (cAMP) signaling pathway. GIE deformability is regulated by protein kinase A (PKA)-mediated phosphorylation of the proteins STEVOR that interact with the erythrocyte skeleton [9]. Likewise, PKA activity contributes to the activation of NPPs in immature GIE, although the phosphorylated substrate has not yet been identified [7]. In mature stages, decreased cAMP levels, due to the high expression of the plasmodial phosphodiesterase (PDE) *PfPDE δ* [15], reduces the phosphorylation of STEVORs and of other proteins potentially involved in channel activity. Consequently, the stiffness and the permeability of infected erythrocytes decrease [7, 9]. Therefore, *PfPDE δ* , which is the only PDE expressed by mature gametocyte stages, appears to be the key regulator of gametocyte persistence in the bloodstream by decreasing GIE osmotic fragility and by increasing GIE deformability. Thus, drugs targeting this PDE are expected to increase the stiffness and the osmotic fragility of mature GIE and, as a result, may interrupt parasite transmission by promoting GIE hemolysis and clearance by the spleen. Here, we address the effect of tadalafil, a marketed PDE inhibitor widely used to treat erectile dysfunction and pulmonary arterial hypertension, on GIE mechanical properties.

First, we assessed the effect of tadalafil on GIE deformability and filterability using a matrix of microspheres [16]. The interstices within this matrix mimic the physical constraints experienced by infected erythrocytes in the splenic microcirculation, permitting passage of

deformable GIE, but impairing filtration of GIE with increased erythrocyte stiffness. Synchronized cultures containing 0.5 to 5% stage V GIE were pre-incubated 30 minutes with 1 nM to 100 μ M tadalafil before filtration through the matrix (Figures 1 A-F). Approximately 40 % of untreated GIE were retained on the microspheres, confirming the retention rates previously observed [9]. Incubation with tadalafil markedly increased the retention rates that reached 82 % at 100 μ M ($P < 0.0001$, Figure 1F). The filterability of GIE was significantly impaired even at low tadalafil concentrations with 59 % retention at 10 nM ($P = 0.0037$, Figure 1B). Importantly, retention rates were already highly increased at 1 μ M tadalafil (77 % vs 41 %, $P < 0.0001$, Figure 1D), which corresponds to peak plasma concentration in humans, measured at 378 ng/ml two hours following a 20 mg oral dose of tadalafil [17]. To validate that increased retention rates in the microsphere matrix correspond to a decrease in deformability of GIE, we visualized the shape of GIE as they flowed through the matrix by adding a paraformaldehyde-fixation step in the filtration experiment. At 100 μ M tadalafil the proportion of paraformaldehyde-fixed GIE that exhibit a twisted and deformed shape decreased to 32%, compared to 59% of untreated cells (Figures 1 G-H). To control that increased retention rates were not the result of gametocyte rounding or egress induced by tadalafil treatment, we stained the membrane of erythrocytes with anti-glycophorin A antibodies after 30 minutes incubation in 30 μ M tadalafil and we analyzed the shape of GIE and the presence of the erythrocyte membrane. The proportion of round parasites and of egressed parasites were similar in tadalafil-treated and untreated cells, indicating that tadalafil did not trigger gamete activation (Figures 1I-K). Finally, to address the specificity of tadalafil on infected cells, a subpopulation of uninfected erythrocytes was labelled and subjected to filtration after incubation with tadalafil. The filterability of uninfected

erythrocytes were not significantly affected at high concentrations of tadalafil, indicating that it specifically affects infected erythrocytes (Figures 1L-M).

To further analyze the effect of tadalafil on GIE permeability, we performed measurements of isosmotic lysis of stage V GIE in sorbitol, a sugar alcohol permeant through NPPs [4]. After 60 minutes in sorbitol, about 10% to 15% of DMSO-treated GIE were lysed, as already described [7]. Incubation with 10 nM tadalafil significantly increased lysis (35% vs 14%, $P = 0.0286$, Figure 2A), and sorbitol uptake gradually increased with tadalafil concentration (Figures 2A-E), reaching 67 % lysis at 30 μ M tadalafil ($P = 0.0286$, Figure 2E). Lysis was totally inhibited when GIE were co-incubated with tadalafil and the anion channel inhibitor 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) that is a well-known NPP inhibitor [18], thus validating that tadalafil-induced GIE lysis corresponds to an activation of NPPs (Figure 2G). The specificity of tadalafil was confirmed as the lysis of labelled uninfected erythrocytes was not significantly increased at high concentrations of tadalafil ($P > 0.999$, Figures 2F).

Altogether, these results show that tadalafil impacts both the deformability and the permeability of mature GIE and therefore represents a novel drug lead potentially capable of blocking malaria parasite transmission by impacting on gametocyte circulation. We had previously observed similar effects with the PDE inhibitor sildenafil that is also used to treat erectile dysfunction [7, 15]. However, the better safety profile and the longer duration of effect of tadalafil (24-36 hours instead of 3-4 hours for sildenafil) make it a more attractive compound [17]. Moreover, the structure of tadalafil is markedly different from that of sildenafil: tadalafil is a non-purine PDE5 inhibitor and displays a novel tetrahydro- β -carboline ring fused to a diketopiperazine scaffold. This structure makes tadalafil more easily modifiable to engineer out its effect on human targets and generate *Plasmodium*-specific analogues [19, 20].

Interfering with mechanical properties to promote GIE clearance by the spleen and hemolysis may be a novel way to target gametocytes and to block malaria parasite transmission. The majority of current first-line antimalarial treatments are curative of asexual parasite stages but they do not eliminate gametocytes and do not effectively interrupt transmission [21]. A key to malaria eradication is therefore the discovery of anti-malarial drugs that target gametocytes in addition to asexual stages. However, the development of gametocyte-targeting drugs is challenging since the metabolic activity is reduced in mature gametocytes compared to asexual stages. Low hemoglobin concentration in the erythrocyte at the end of gametocyte maturation likely also influences the susceptibility of mature gametocytes to different antimalarials whose mechanism of action depends on the degradation of hemoglobin, as is the case with artemisinin. Targeting mechanical properties of infected erythrocytes may overcome these issues. More generally, targeting the non-metabolic pathways of parasites and repositioning PDE inhibitors already used in human pharmacology is a strategy increasingly used in the context of parasitic diseases in an attempt to overcome the problem of resistance or reduced efficacy of current treatments [22].

Declarations of Competing Interest

The authors declare that they have no competing financial interests.

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Author contributions

MEN and CL conceived the project. MEN and LR performed the experiments. MEN, LR and CL designed and interpreted the experiments. CL wrote the article.

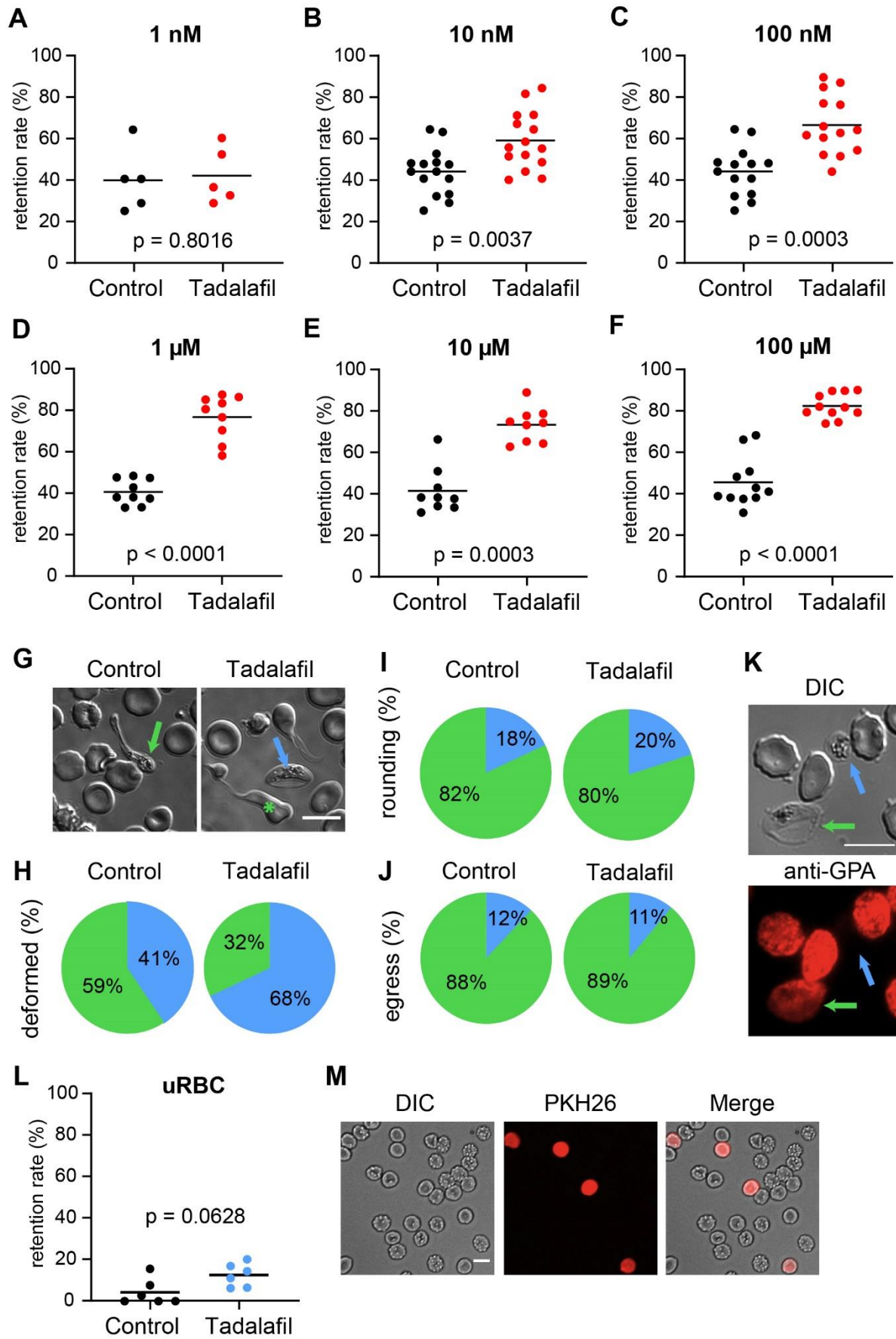


Figure 1. Tadalafil impacts stage V GIE deformability. A-F. Retention in microspheres of stage V GIE. Synchronized cultures from the NF54 strain containing 0.5 to 5 % GIE were pre-incubated 30 minutes at 37° C with 1 nM to 100 µM tadalafil (Selleckchem or Sigma) and were perfused through a microsphere matrix as described [16]. The upstream and downstream samples were smeared, stained with Giemsa reagent, and parasitaemia was assayed by counting at least 2000 erythrocytes to determine parasite retention. Circles indicate the number of independent experiments. P indicates the statistical significance determined by a Mann Whitney test using GraphPad Prism 9. **G-H.** To visualize GIE shape during their flowing through the matrix, 1 mL of PBS/4% paraformaldehyde was added after perfusion of the GIE-containing culture on the microsphere matrix. After 5 min of incubation, fixed GIE were separated from the microspheres by a 3-step decantation procedure, and GIE morphology was observed on a glass slide by light microscopy using a Leica DM 5000 B at 100X magnification. **G.** Differential interference contrast images of paraformaldehyde-fixed GIE, as they flow through the matrix. Green arrow shows untreated GIE with a deformed shape (left panel), blue arrow shows 100 µM tadalafil-treated GIE that keeps a regular shape (right panel), green star shows tadalafil-treated uninfected erythrocyte with a deformed shape (right panel). **H.** Graphical representation for the proportion of GIE showing a regular (blue) or deformed (green) shape in a population of paraformaldehyde-fixed GIE. Experiments were performed in triplicate and 100 cells were counted per experiment. **I-K.** Immunofluorescence analysis was performed on untreated and 30 µM tadalafil-treated GIE using mouse anti-glycophorin A (GPA) antibody (1/1,000, Santa Cruz Biotechnology) for staining the erythrocyte membrane. Samples were observed at 100X magnification using a Leica DMI8. Percent rounding was determined by calculating the % of round parasites and percent egress was determined by calculating the % of GPA-negative parasites in the total GIE population. **I.** Graphical

representation for the proportion of GIE showing a round (blue) or elongated (green) shape. **J.** Graphical representation for the proportion of GPA-positive (green) or GPA-negative (blue) GIE. **K.** Representative images showing an elongated stage V GIE inside an erythrocyte (green arrow) and a round, egressed gamete (blue arrow). **L-M.** Retention rates of uninfected erythrocytes (uRBC) pre-incubated 30 minutes at 37°C with or without 100 µM tadalafil were monitored after labelling a subpopulation of erythrocytes with PKH26 (Sigma-Aldrich) according to manufacturer's instructions. The proportion of labelled erythrocytes in upstream and downstream samples was determined by fluorescence microscopy using a Leica DM 5000 B at 40X magnification. At least 1500 erythrocytes per experiments were counted. DIC: Differential Interference Contrast. Bars represent 5 µm.

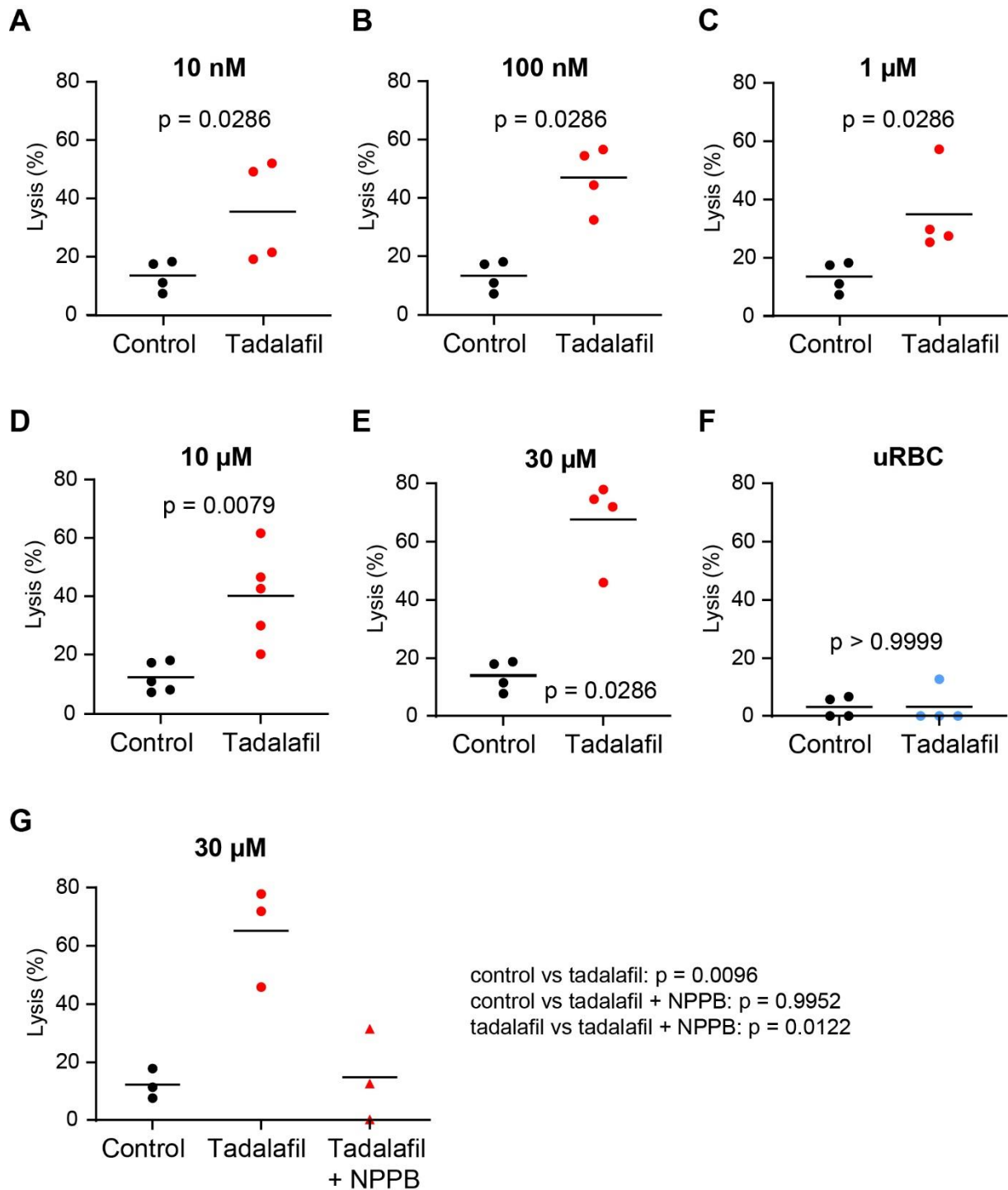


Figure 2. Tadalafil impacts stage V GIE permeability

A-E. Sorbitol-induced isosmotic lysis of stage V GIE. Synchronized cultures from the NF54 strain containing 0.5 to 3 % GIE were pre-incubated 30 minutes at 37° C with 10 nM (A), 100 nM (B), 1 μM (C), 10 μM (D), 30 μM (E) tadalafil or 0,5 % DMSO (Control) and were subjected to isosmotic lysis for 60 minutes at 37° C in a solution containing 300 mM sorbitol

supplemented with 10 mM Hepes, 5 mM glucose and with a pH adjusted to 7,4. After 60 minutes, cultures were smeared onto glass slides for staining with Giemsa reagent, and parasitaemia was assayed by counting at least 2000 erythrocytes to determine lysis percentage using the formula: % lysis (t60) = $[1-(\text{parasitemia (t60)}/\text{parasitemia (t0)})]*100$. Only the cells present in the smears (ie the non-lysed cells) were counted. **F.** Sorbitol-induced isosmotic lysis of uninfected erythrocytes (uRBC) pre-incubated 30 minutes at 37°C with 30 µM tadalafil or 0,5% DMSO (Control) was monitored after labelling a subpopulation of erythrocytes with PKH26. The proportion of labelled erythrocytes at T = 0 minute and at T = 60 minutes was determined by fluorescence microscopy using a Leica DM 5000 B at 100X magnification. At least 2000 erythrocytes per experiments were counted. **G.** Sorbitol-induced isosmotic lysis of stage V GIE pre-incubated 30 minutes at 37° C with 0,5 % DMSO (Control) or 30 µM tadalafil in presence or absence of 100 µM NPPB. In all panels, circles indicate the number of independent experiments and P indicates the statistical significance determined by a Mann Whitney test (A-F) or by one-way ANOVA with Sidak correction (G) using GraphPad Prism 9.

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