

Bz423 promotes procoagulant platelet formation independently of OSCP

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Abstract

Platelet activation is a key driver of in arterial thrombosis and myocardial infarction.

Despite current anti-platelet therapies, mortality and morbidity from myocardial infarction remain high. Alternative anti-platelet therapies are needed that can reduce the risk of myocardial infarction without further increasing bleeding risk.

Activated platelets rapidly diverge into distinct subpopulations. ‘Procoagulant’ platelets form through a mitochondria-dependent pathway. These procoagulant platelets expose phosphatidylserine (PS), which localises and amplifies the coagulation cascade leading to a large increase in thrombin generation and stable thrombosis. Procoagulant platelet formation may be a new target for anti-platelet therapies.

A key regulatory step controlling progression to procoagulant platelets is opening of the mitochondrial permeability transition pore (mPTP). OSCP acts as a regulatory node for mPTP activity. Since mPTP opening regulates procoagulant platelet formation, we hypothesised that OSCP might also be a key regulatory node in platelets.

OSCP is a target of the immunomodulatory benzodiazepine, Bz-423. In this study, we show that Bz-423 increased procoagulant platelet formation. This was associated with

mitochondrial hyperpolarisation prior to platelet activation, and increased mPTP opening after activation. Cellular thermal shift assays (CETSA) showed that both Bz-423 and a structurally similar benzodiazepine, 4-chloro-diazepam, bind OSCP in platelets. Despite this, only Bz-423 increased procoagulant platelet formation. Our findings indicate that Bz-423 promotes procoagulant platelet progression through a target distinct from OSCP.

Keywords

Platelets, thrombosis, pharmacology, mechanism of action, mitochondria

Highlights

- Procoagulant platelets are key drivers of arterial thrombosis
- Benzodiazepine, Bz-423, increases procoagulant platelet formation
- Bz-423 binds to OSCP in platelets
- Action of Bz-423 on procoagulant platelets is likely to be via a different target

1 Introduction

Coronary heart disease is the leading cause of myocardial infarction (heart attack). Rupture of a coronary artery atherosclerotic plaque results in rapid platelet-dependent thrombosis and reduction in blood flow to downstream cardiac muscle [1]. Anti-platelet therapies such as aspirin and clopidogrel are used extensively in at-risk patients to prevent platelet-dependent arterial thrombosis. Despite currently available therapies, mortality and morbidity from myocardial infarction remain high [2–4]. In addition, currently available therapies increase bleeding risk by interfering with hemostasis. Alternative anti-platelet therapies are needed that can reduce the risk of myocardial infarction without further increasing bleeding risk. This requires a better understanding of how platelets are activated during thrombosis.

Activated platelets rapidly diverge into two distinct subpopulations [5,6]. ‘Pro-aggregatory’ platelets activate integrin $\alpha_{IIb}\beta_3$, allowing them to bind to other platelets through plasma fibrinogen. This process is amplified by soluble mediators released by activated platelets. In contrast, ‘procoagulant’ platelets expose phosphatidylserine (PS) in the outer leaflet of their plasma membrane. This PS surface localises and amplifies the coagulation cascade leading to a large increase in thrombin generation [6].

Procoagulant platelet formation is a form of regulated necrosis [7]. A key trigger is high cytosolic Ca^{2+} concentration, usually by coincident stimulation by thrombin and ligands of GPVI, or by Ca^{2+} ionophores or pore-forming toxins [8]. A proportion of the cytosolic Ca^{2+} is sequestered into mitochondria through the mitochondrial Ca^{2+} uniporter (MCU) [9], increasing mitochondrial matrix Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{mito}}$). In some platelets, $[\text{Ca}^{2+}]_{\text{mito}}$ exceeds a threshold for opening of the mitochondrial permeability transition pore (mPTP). mPTP opening triggers a much larger increase in cytosolic Ca^{2+}

1 concentration (which we previously termed ‘supramaximal Ca^{2+} signalling’ [10]) and
2 activation of a phospholipid scramblase, TMEM16F [11,12]. Scramblase activation,
3 combined with Ca^{2+} -dependent inhibition of inward PS translocation [13], results in PS
4 exposure in the outer leaflet of the plasma membrane.

5 mPTP opening is a key regulatory step controlling progression to procoagulant platelets.
6 One regulator of mPTP opening is cyclophilin D (CypD), which lowers the $[\text{Ca}^{2+}]_{\text{mito}}$
7 threshold for mPTP opening [14–16]. CypD-deficient mouse platelets form many fewer
8 procoagulant platelets in response to physiological stimuli, an effect mimicked by the
9 cyclophilin inhibitor, cyclosporin A (CsA) [10,17]. CypD itself binds to a wide range of
10 mitochondrial proteins [18] including a leading candidate for mPTP, the F_0F_1 (F)-ATP
11 synthase [19,20]. The mitochondrial F-ATPase is a large multiprotein complex that
12 catalyses the formation of ATP. CypD interacts with F-ATPase via the OSCP [Oligomycin
13 sensitivity-conferring protein] subunit, which sits at the top of F1 regulating the
14 structural coupling between F0 and F1, and promotes effective ATP synthesis [21,22].
15 OSCP acts as a regulatory node for mPTP activity, and is itself regulated by
16 phosphorylation, acetylation, protons and protein-protein interactions [22]. Since mPTP
17 opening regulates procoagulant platelet formation, we hypothesised that OSCP might
18 also be a key regulatory node in platelets.

19 OSCP is a target of the immunomodulatory benzodiazepine, Bz-423 [23,24]. Bz-423 is
20 reported to compete with and mimic the effects of CypD binding to OSCP, sensitizing
21 the mPTP to opening and promoting apoptosis [22,25].

22 In this study, we show that Bz-423 increases procoagulant platelet formation. We have
23 then investigated its binding to OSCP in platelets and its mode of action compared to

structurally related benzodiazepines. Unexpectedly, our findings indicate that Bz-423 acts through a target distinct from OSCP.

Materials and methods

Reagents and antibodies

Reagents for CETSA and Western blotting and flow cytometry were sourced as follows: Complete Mini protease inhibitors, EDTA free (Roche); NP-40 detergent (Abcam); mouse (A-8) monoclonal IgG1 anti-OSCP (Santa Cruz); Bz-423 (Cambridge Biosciences); alprazolam (Merck); diazepam (BioTechne), 4-chloro-diazepam (a kind gift from Dr Paul Miller, Department of Pharmacology, University of Cambridge, UK); crosslinked collagen-related peptide (CRP-XL) (Triple-Helical Peptides); thrombin (Sigma).

For flow cytometry, FITC-conjugated annexin V was obtained from Invitrogen.

Isolation of human platelets from whole blood

Washed platelets were isolated from blood drawn as previously described [26], from healthy drug-free volunteers who had given fully-informed, written consent, with approval from the Human Biology Research Ethics Committee, University of Cambridge (HBREC.2025.14, 29th May 2025). All experiments were performed in compliance with national and institutional requirements. Briefly, blood was drawn into sodium citrate, supplemented with acid citrate dextrose, and centrifuged to separate platelet-rich plasma. Platelets were pelleted by centrifugation, resuspended in HEPES-buffered saline, and were adjusted to a density of $1 \times 10^9/\text{mL}$ (for CETSA) or $5 \times 10^7/\text{mL}$ (for flow cytometry).

Treatment of platelets with benzodiazepines

Benzodiazepines were pre-incubated with washed platelets at room temperature prior to CETSA (30 minutes) or flow cytometry (15 minutes). Concentrations are as described in the Results & Discussion section. Platelets pre-incubated with 0.1% DMSO were used as an untreated control where appropriate.

Detection of phosphatidylserine (PS) exposure by flow cytometry

Washed platelets supplemented with 2mM CaCl_2 were incubated with platelet activators at the concentrations and times described in the Results & Discussion and then incubated with FITC-conjugated annexin V (to measure PS exposure) for 5 minutes (1:50 dilution in HEPES buffer) prior to analysis by flow cytometry (BD Accuri C6; BD Biosciences). For each experiment, 10,000 platelets were collected for analysis.

For analysis of mitochondrial membrane potential, TMRM (200nM) was added 30 minutes prior to incubation with benzodiazepines and visualised in the FL-2 channel following excitation at 488nm

Cellular thermal shift assay (CETSA)

Washed platelets were pre-incubated for 30 minutes with drug or corresponding volume of DMSO. 100 μL of treated or control platelets were aliquoted into PCR tubes and CETSA performed as previously described [27]. Heated platelets were equilibrated and lysed with equal volume of lysis buffer containing protease inhibitors (50mM Tris, 150mM NaCl, 1% NP40, pH8.5) and centrifuged at 17,000g for 30 minutes at 4°C. The supernatant was carefully aspirated and subjected to Western blotting under reducing conditions.

Statistical analyses

Mean data are presented $\pm$ standard error of mean. n values in figure legends indicate biological replicates on platelets from different donors. The statistical tests used are described in the figure legends. The thermal aggregation curves (Fig. 2) were fitted to a 4-parameter logistic equation.

Results and Discussion

Bz-423 increases procoagulant platelet formation

Human platelets were incubated with Bz-423 then co-stimulated with thrombin and cross-linked collagen-related peptide (CRP-XL; a GPVI agonist). This co-stimulation is required to trigger a substantial subpopulation of platelets for become procoagulant [28,29]. Platelets were then stained with annexin V-FITC, to detect exposed phosphatidylserine (PS) on the surface of procoagulant platelets. Bz-423 pre-treatment significantly increased the proportion of platelets that exposed PS in a concentration-dependent manner (Fig. 1A-B).

The mitochondrial membrane potential ($\Delta\psi_m$) provides the driving force for Ca^{2+} entry into mitochondria through the MCU. Previous studies have reported that an increase in $\Delta\psi_m$ prior to stimulation correlates with increased procoagulant platelet formation [9]. An increase in $\Delta\psi_m$ can be detected by an increase in fluorescence of TMRM. Treatment with Bz-423 resulted in a concentration-dependent increase in TMRM fluorescence (Fig. 1C), suggesting that Bz-423 acutely hyperpolarises $\Delta\psi_m$ prior to platelet stimulation.

1 Previous studies have demonstrated that procoagulant platelet formation is
2 inhibited by the CypD inhibitor, CsA [10,17]. Consistent with these studies, we also
3 found that CsA (5 μ M) inhibited the proportion of co-stimulated platelets that bound
4 AnV (Fig. 1D).

5 Bz-423 is reported to compete with CypD for binding to OSCP [30] and mimic the
6 effects of CypD binding [31]. Bz-423 can induce mPTP in liver cells treated with CsA or
7 deficient in CypD [25]. We therefore expected that Bz-423 would induce mPTP in
8 platelets treated with CsA, i.e., CsA would no longer block procoagulant platelet
9 formation when platelets were treated with Bz-423. However, CsA significantly inhibited
10 the proportion of platelets that bound AnV even after Bz-423 treatment, and Bz423 did
11 not increase the percentage of CsA-treated platelets that bound AnV (Fig. 1D).

12 Procoagulant platelets lose mitochondrial membrane potential when mPTP opens. This
13 can be detected as a loss of TMRM fluorescence *after* stimulation. We stimulated
14 platelets with Thr + CRP-XL and co-stained with TMRM and annexin V-FITC. In platelets
15 treated with the vehicle control (DMSO), most annexin V-positive platelets had also lost
16 TMRM fluorescence, as expected. Similarly, we observed that in Bz-423-treated
17 platelets, most annexin V-positive platelets were negative for TMRM. In CsA-treated
18 platelets, loss of TMRM fluorescence was largely abolished, consistent with CsA
19 blocking mPTP opening. Bz-423 did not induce further mPTP opening in CsA-treated
20 platelets (Fig. 1E). Bz-423 is therefore not able to mimic or replace the effect of CypD
21 that has been inhibited by CsA.

22 These data suggest that the action of Bz-423 is more complex than acting as a
23 mimic of CypD binding to OSCP.

Bz-423 binds to OSCP in platelets

To determine whether Bz-423 can bind to OSCP in platelets, we used cellular thermal shift assays (CETSA) [32]. Drug binding to protein targets may alter the thermal stability of the protein. CETSA is a powerful tool to detect such changes in intact cells, including platelets [27]. The use of intact cells is important because drugs that bind proteins with high affinity *in vitro* may nonetheless be unable to extensively bind the same target inside a cell, for example due to inability to cross the plasma membrane or because the binding site is concealed.

Washed platelets were treated with Bz-423 (or DMSO, as vehicle control) then briefly subjected to various temperatures. Aggregated protein was removed by centrifugation, soluble (non-aggregated) protein separated by SDS-PAGE, and OSCP detected by immunoblotting. In DMSO-treated platelets, OSCP showed high stability, with the temperature required for 50% aggregation (T_{agg}) of 87.4 °C (Fig. 2). This high stability may be a consequence of being in a large, multi-protein complex with the mitochondrial F-ATPase [21]. Bz-423 treatment shifted the thermal aggregation curve to the left (i.e. stabilizing; T_{agg} 82.8 °C) and up, suggesting that Bz-423 binds to OSCP but has complex effects on its stability in intact platelets.

Although ligand binding often increases protein thermal stability, resulting in a rightward shift of the thermal aggregation curve, there are previous examples of proteins that are destabilised by ligand binding [33,34], for example when the target protein is part of a complex of protein-protein interactions (as expected here for OSCP), or by competing with a native ligand.

Since CypD and Bz-423 are believed to compete for binding at OSCP, we treated platelets with CsA to displace CypD from OSCP [35], which we speculated might increase access for Bz-423 and remove any effect of CypD on OSCP thermal stability. CsA treatment itself had no effect on the OSCP thermal stability curve (T_{agg} 87.0 °C). In the presence of CsA, Bz-423 shifted the OSCP thermal aggregation curve further to the left (T_{agg} , 80.5 °C). Notably, Bz-423 in the presence of CsA no longer shifted the OSCP thermal stability curve upwards, indicating that CypD increases the complexity of OSCP thermal stability. These data suggest that CsA treatment increases the ability to detect Bz-423 binding to OSCP in platelets, likely because Bz-423 can more readily access OSCP and confounding effects of CypD binding are no longer present.

Benzodiazepines show differential binding to OSCP and effects on procoagulant platelet formation

To further understand the action of Bz-423 on procoagulant platelet formation, we compared the effect of various structurally-related benzodiazepines (Fig. 3; structures in Supplementary Fig. 1) on thermal shifts of OSCP at a single temperature, and their effect on PS exposure. Interestingly, although 10 μ M Bz-423 destabilized OSCP, there was no consistent effect at higher or lower concentrations. This is likely to be due to the complex effect of Bz-423, shifting the OSCP curve to the left and upwards, which would result in opposite effects on the percentage of soluble OSCP at a single temperature. In contrast, Bz-423 destabilized OSCP in a concentration-dependent manner when CypD was inhibited by CsA. This was consistent with Fig. 2, where the effect of Bz-423 was clearer when platelets had been also treated with CsA.

Similarly, 4-chloro-diazepam (Ro 5 4864) showed little effect on OSCP stability in the absence of CsA, but consistently destabilized OSCP to the same extent as Bz-423 when platelets were also treated with CsA. In contrast, diazepam had no effect on OSCP stability even in the presence of CsA. Finally, alprazolam had trend towards a small stabilizing effect on OSCP when added to CsA-treated platelets (Fig. 3). These data show that small differences in ligand structure affect binding to OSCP in platelets. Interestingly, only Bz-423 increased the percentage of stimulated platelets that exposed PS (Fig. 4). Despite 4-chloro-diazepam affecting OSCP stability in the same manner as Bz-423, it had no effect on the percentage of platelets that bound annexin V. Similarly, neither diazepam nor alprazolam affected platelets PS exposure. These data, notably the lack of effect of 4-chloro-diazepam, further suggest that the procoagulant effect of Bz-423 is not through its binding to OSCP.

Together these data suggest that the enhancing effect of Bz-423 on procoagulant platelet formation is not due to binding to OSCP. Instead, Bz-423 is likely to bind a further, unknown target. Benzodiazepine is a privileged scaffold, that is, one that can provide a framework for ligands of diverse receptors [36,37], perhaps due to structural similarity to peptide β turns [38]. Identification of the target of Bz-423 in platelets may further reveal further the molecular regulation of procoagulant platelets.

Conflict of interest

There are no conflicts of interest to declare.

Availability of data and materials

Data are available from the corresponding author on reasonable request.

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Figure legends

Figure 1: Bz423 increase procoagulant platelet formation. Human platelets were isolated from whole blood. Platelets were treated with Bz423 (10 μ M in **A-B**, **E-F**; concentration indicated in **C-D**; 15 min) or equivalent volume of DMSO as control. Platelets were co-stimulated with thrombin (Thr, 1 U/ml) and CRP-XL (0.5 μ g/ml) for 10 min where indicated. **A-B**: PS exposure was detected by FITC-conjugated Annexin V (AnV) binding and flow cytometry. **A**: Representative flow cytometry histogram of FITC-AnV fluorescence, indicating AnV positive (+ive) and negative (-ive) populations. **B**: Percent AnV positive platelets. Linked points indicate donor matched experiments. **C**: Platelets were incubated with indicated concentration of Bz423 then stained with TMRM to measure mitochondrial membrane potential. TMRM fluorescence is normalised to unstimulated samples (n = 3). **D-E**: Platelets were treated with Bz-423 and/or cyclosporine A (CsA), as indicated, prior to stimulated with Thr + CRP-XL and staining with FITC-AnV (**D**) or with both FITC-AnV and TMRM (**E**) (n=4). Note that TMRM positive (+) / AnV negative (-) platelets (white) are the state of unstimulated platelets, whereas TMRM negative (-) / AnV positive (+) platelets (black) are the expected state of procoagulant platelets. Mean data are shown $\pm$ stand error of mean. ** p < 0.01; *** p < 0.01.

Figure 2: Bz-423 binds to OSCP in platelets. Washed platelets were treated with Bz-423 and/or CsA (or DMSO as vehicle control) then briefly heated to the temperature indicated (8 min) then lysed. Aggregated protein was removed by centrifugation. Soluble protein was separated by SDS-PAGE and OSCP identified by western blotting. The amount of soluble OSCP in each sample is expressed as a percentage of the soluble OSCP in an unheated sample. **A:** mean data $\pm$ standard error of mean (n = 3). **B:** Representative blots from one experiment. * indicates sample unheated prior to lysis.

Figure 3: Benzodiazepines show differential binding to OSCP. Washed platelets were treated with the benzodiazepine indicated and/or CsA prior to CETSA. (4-Cl-D: 4-chloro-diazepam; Dia: diazepam; Alp: alprazolam). **A:** mean data $\pm$ standard error of mean (n = 3), normalized to untreated control (100%, indicated by dotted line). Data were analysed by 2-way ANOVA with Dunnett's post-hoc test (compared to untreated control). * p < 0.05; ** p < 0.01; *** p < 0.001. **B:** Representative blots from one experiment.

Figure 4: Benzodiazepines show differential effects of platelet PS exposure. Washed platelets were treated with the benzodiazepine indicated. Platelets were co-stimulated with thrombin (Thr) and CRP-XL for 10 min (blue) or left unstimulated (black). PS exposure was detected by FITC-conjugated Annexin V (AnV) binding and flow cytometry. Data were analysed by 2-way ANOVA with Dunnett's post-hoc test (compared to untreated control). ** p < 0.01. (n = 5).

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