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RESEARCH ARTICLE

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Dolutegravir potentiates platelet activation by a calcium-dependent, ionophore-like mechanism

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ABSTRACT

Dolutegravir is a highly potent HIV integrase strand transfer inhibitor that is recommended for first-line anti-retroviral treatment in all major treatment guidelines. A recent study has shown that people taking this class of anti-retroviral treatment have a substantially higher risk of early-onset cardiovascular disease, a condition shown previously to be associated with increased platelet reactivity. To date, few studies have explored the effects of dolutegravir on platelet activation. Accordingly, the current study was undertaken with the primary objective of investigating the effects of dolutegravir on the reactivity of human platelets *in vitro*. Platelet-rich plasma, isolated platelets, or buffy coat cell suspensions prepared from the blood of healthy adults were treated with dolutegravir (2.5–10 μg/ml), followed by activation with adenosine 5′-diphosphate (ADP), thrombin, or a thromboxane A_2 receptor agonist U46619. Expression of platelet CD62P (P-selectin), formation of heterotypic neutrophil:platelet aggregates, and calcium (Ca^{2+}) fluxes were measured using flow cytometry and fluorescence spectrometry, respectively. Dolutegravir caused dose-related potentiation of ADP-, thrombin- and U46619-activated expression of CD62P by platelets, as well as a significant increases in formation of neutrophil:platelet aggregates. These effects were paralleled by a spontaneous, receptor-independent elevation in cytosolic Ca^{2+} that appears to underpin the mechanism by which the antiretroviral agent augments the responsiveness of these cells to ADP, thrombin and U46619. The most likely mechanism of dolutegravir-mediated increases in platelet cytosolic Ca^{2+} relates to a combination of lipophilicity and divalent/trivalent metal-binding and/or chelating properties of the anti-retroviral agent. These properties are likely to confer ionophore-type activities on dolutegravir that would promote movement of Ca^{2+} across the plasma membrane, delivering the cation to the cytosol where it would augment Ca^{2+}-dependent intracellular signaling mechanisms. These effects of dolutegravir may lead to hyper-activation of platelets which, if operative *in vivo*, may contribute to an increased risk for cardiometabolic co-morbidities.

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Dolutegravir; platelets; calcium fluxes; CD62P; neutrophil: platelet aggregates

Introduction

Integrase strand transfer inhibitors (INSTI) are a class of anti-retroviral agents that inhibit HIV-1 replication by blocking strand transfer reactions catalyzed by HIV-1 integrase (Smith et al. 2018). The INSTI have shown sustainable virological efficacy (Snedecor et al. 2019; Ambrosioni et al. 2020) with mild (depression, vertigo, and sleep disturbances) or no side-effects reported (Brehm et al. 2019). An improved metabolic profile in lipids (total cholesterol and low-density lipoprotein cholesterol ratio) has also been reported with combined anti-retroviral therapy (cART) regimens that contain INSTI compared to that observed with cART treatments that contain protease inhibitors (PIs) or efavirenz (Taramasso et al. 2018; Bagella et al. 2019). Dolutegravir (GSK-1349572) was the third INSTI approved by the U.S. FDA and regimens containing this drug have been recommended as the preferred first- and second-line anti-retroviral therapies (ART) for all populations (World Health Organization, 2019).

Despite the seemingly satisfactory safety profile of dolutegravir, Neesgaard et al. (2022) recently reported that initiation of anti-retroviral therapy with an INSTI in a large cohort of European and Australian, INSTI-naïve, participants living with HIV was associated with an early onset increased incidence of cardiovascular disease (CVD) - including myocardial infarction and strokes, and a need for invasive cardiovascular procedures - during the first two years of exposure, which declined progressively thereafter. During the first six months of treatment with an INSTI, the adjusted incidence rate ratio for CVD events was 1.85 (CI: 1.44–2.39) compared with no exposure. However, indices of platelet activation and possible relationships with CVD events were not reported.

Platelet markers, such as mean platelet volume, circulating levels of microparticles, oxidation products, platelet-derived

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soluble CD62P (P-selectin), and CD40 ligand have been found to be elevated in people with cardiometabolic risk factors, such as obesity, dyslipidemia, or Type 2 diabetes mellitus (Barale and Russo 2020). Few studies to date have explored the potential effects of dolutegravir on platelet activation. One such study (Taylor et al. 2019) reported that dolutegravir inhibits ADP-mediated platelet aggregation *ex vivo* following oral administration to healthy adult volunteers, as well as collagen-mediated activation of integrin αIIbβ3 and up-regulated expression of CD62P and CD63 following exposure of isolated platelets from healthy volunteers to dolutegravir *in vitro*.

While this seemingly represents a potentially beneficial effect of dolutegravir, further exploration of the effects of this agent on human platelets is clearly needed. Accordingly, the current study aimed to investigate the effects of dolutegravir on platelet activation as measured by CD62P expression and formation of neutrophil:platelet (NP) aggregates following exposure of the cells to several different types of receptor agonists. From a mechanistic perspective, the effects of dolutegravir on alterations in platelet cytosolic Ca^{2+} concentrations were also investigated.

Materials and methods

Ethics statement

Permission to undertake this study, and to draw blood from healthy adult volunteers was granted by the Research Ethics Committee of the Faculty of Health Sciences (University of Pretoria). This study was performed in full compliance with the World Medical Association Declaration of Helsinki 2013 (Approval #116/2017 and 605/2018). Written informed consent was obtained from all blood donors, each of whom underwent a routine health check (including measurement of blood pressure) by an experienced, qualified nurse prior to the blood draw [16 females, 9 males, mean age (± SD) of 31 (±11) years].

Chemicals and reagents

Dolutegravir [(4R,12aS)-N-[(2,4-difluorophenyl)methyl]-3,4,6,8,12,12a-hexahydro-7-hy-droxy-4-methyl-6,8-dioxo-2H-pyrido[1′,2′:4,5]pyrazino[2,1-b][1,3]oxazine-9-carboxamide] was purchased from the Cayman Chemical Company (Ann Arbor, MI) and dissolved in dimethyl sulfoxide (DMSO, 0.1%) at final concentrations of 5 and 10 µg/ml (≈ 10–20 µM). The platelet activators used were adenosine-5′-diphosphate (ADP; Boehringer Mannheim Biochemical, Basel, Switzerland) and thrombin (from human plasma; Sigma, St Louis, MO) and also kindly provided by the South African National Blood Services (Pretoria, SA), used at final concentrations of 100 µM and 0.6 or 1.0 NIH units/ml, respectively. A third activator, U46619 [(Z)-7-[(1*R*,4*S*,5*S*,6*R*)-6-[(*E*,3*S*)-3-hydroxyoct-1-enyl-2-oxabicyclo[2.2.1]heptan-5yl]-hept-5-enoic acid], a thromboxane A_2 surrogate (Tocris Bioscience, Bristol, UK), was used at 5 µM (final concentration). Unless indicated otherwise, all other chemicals and reagents were purchased from Sigma.

Platelet-rich plasma and buffy coat preparations

To prepare platelet-rich plasma (PRP), blood (anti-coagulated with five units of preservative-free heparin/ml blood) was centrifuged at 250 × g for 10 min at room temperature within 15 min of venepuncture as described previously (Nel et al. 2016). The essentially erythrocyte- and leukocyte-free upper layer of PRP was decanted and used in the experiments described below. Buffy coat suspensions prepared by sedimentation of heparinized venous blood were used in assays of NP aggregate formation (Nel et al. 2017).

Purified platelet suspensions

PRP was diluted four-fold with phosphate-buffered saline (PBS, 0.15 M, pH 6.8) containing ethylene glycol-bis(β-aminoethyl ether)-*N*-*N*-*N*′-*N*′-tetraacetic acid (EGTA, 0.6 mM final concentration) to minimize spontaneous platelet activation. Samples were then centrifuged at 250 × g for 10 min at 20 °C to deplete residual erythrocytes and leukocytes. The platelet-enriched supernatants were then centrifuged at 800 × g for 20 min and the cell pellets re-suspended in PBS and counted using a Sysmex XN 20000 Automated Hematology Analyzer (Sysmex Corporation, Kobe, Japan). The median (IQR) platelet count of platelet-enriched supernatants was 1205 (805 − 1505) × 10^9/L.

CD62P expression on platelets

In order to determine the expression of CD62P on platelets, the method of Anderson et al. (2020) was followed. In brief, to PRP (20 µl) in a final volume of 1 ml Hanks' balanced salt solution (HBSS, indicator-free, 1.25 mM calcium Ca^{2+}, pH 7.4), either DMSO (control) or dolutegravir (at final concentrations of 5 and 10 µg/ml) were added. The tubes were then incubated for 10 min at 37 °C after which the platelets were activated with ADP (100 µM) for 5 min. Flow cytometry was then performed to determine the magnitude of expression of CD62P according to the proportion (%) of $CD42a^+$ platelets expressing the adhesion molecule. In an additional series of experiments, the effects of dolutegravir, at a fixed concentration of 10 µg/ml, on the expression of CD62P by platelets activated with thrombin (0.6 NIH units/ml) or U46619 (5 µM) were investigated.

Following incubation with the various test agents, the platelet suspensions were stained with 5 µl each of a murine anti-human platelet CD42a-phycoerythrin (PE)-labeled monoclonal antibody (Becton Dickenson, San Jose, CA) and an anti-human CD62P-fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (Beckman Coulter, Miami, FL) to detect total and activated platelet populations, respectively. After 15 min of incubation in the dark, the samples were analyzed on a Gallios flow cytometer (Beckman Coulter) and the results expressed as the percentage of activated platelets, with 50,000 cells interrogated during each measurement. The gating and analytical strategies used in a typical experiment have been described elsewhere (Anderson et al. 2018).

Neutrophil:platelet aggregate formation

Buffy coat suspensions (30 µl) in a final volume of 1 ml HBSS were incubated for 5 min at 37 °C in the presence of either DMSO (control) or dolutegravir (at final concentration of 10 µg/ml) after which the cells were activated with thrombin (0.6 NIH units/ml) for 5 min. The cell suspensions were treated with 5 µl of each of the following murine anti-human fluorochrome-labeled monoclonal antibodies to detect neutrophils, platelets, and total leukocytes, respectively: anti-CD16-PE-cyanin 5 (Beckman Coulter), anti-CD42a-PE (Becton Dickenson, San Jose, CA), and anti-CD45-Krome Orange (Beckman Coulter), and then incubated for 15 min at room temperature in the dark. Cell

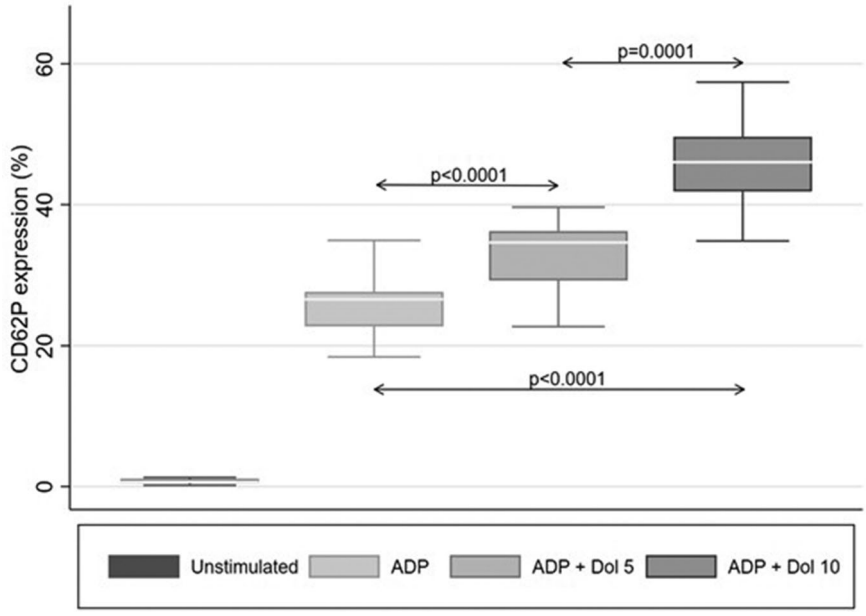

Figure 1. Effects of dolutegravir (Dol, 5 and 10 μg/ml) on the magnitude of CD62P expression on platelets activated with adenosine diphosphate (ADP, 100 μM). Results are expressed as Box and Whisker plots of % CD62P expression for comparison of dolutegravir (5 and 10 μg/ml) with the drug-free control system ($n = 10$ individual donors, with 17 experiments in the series).

suspensions were then analyzed using a Gallios flow cytometer. NP heterotypic aggregate formation was determined as $CD16^+$/$CD45^+$ neutrophils co-expressing CD42a. Results were expressed as the relative mean fluorescence intensity (MFI) of CD42a as emitted by CD16/CD45 neutrophils as an index of the magnitude of the interaction of platelets with individual neutrophils using the gating strategy described previously (Nel et al. 2017).

Platelet cytosolic Ca^{2+} fluxes

Fluo-8 acetoxymethyl ester (Fluo-8-AM) was used as the fluorescent, Ca^{2+}-sensitive indicator for these experiments (Grynkiewicz et al. 1985). In this series of experiments, purified platelets suspended in PBS were incubated at 37 °C for 5 min followed by addition of Fluo-8-AM (2 μM) and a further incubation period of 25 min at 37 °C. The platelets were then pelleted by centrifugation at 500 × g for 15 min, and then re-suspended in PBS. For measurement of cytosolic Ca^{2+} fluxes, 100–300 μl platelet suspension was added to HBSS (final volume of 3 ml containing a maximum of 1×10^8 cells) and incubated for 10 min at 37 °C. The Fluo-8-AM-loaded platelets were then transferred to disposable reaction cuvettes maintained at 37 °C in a Hitachi 650 10S fluorescence spectrometer (Hitachi Ltd., Tokyo, Japan) with excitation and emission wavelengths set at 480 and 525 nm, respectively. Importantly, at these wavelengths, no evidence of dolutegravir-induced autofluorescence was detected. After 1–2 min, dolutegravir (2.5–10 μg/ml) or thrombin (1.0 NIH unit/ml) as a positive control and a check for viability was added to the platelets and alterations in fluorescence intensity measured over a 5 min time course.

To evaluate possible involvement of mobilization of intracellular Ca^{2+} by dolutegravir-treated platelets, the effects of selective inhibitors of phospholipase C (PLC), inositol triphosphate-sensitive Ca^{2+} channels (IP3) and nonselective plasma membrane Ca^{2+} channels (NSCC), on the alterations of cytosolic Ca^{2+}, were investigated. The cells were pretreated for 7 min at 37 °C with the selective inhibitors of each of these mediators of Ca^{2+} mobilization, i.e. U73122 (5 μM), 2-APB (50 μM), and SKF 96365 (50 μM), respectively. Following pre-incubation with U73122, 2-APB or SKF 96365, dolutegravir (10 μg/ml) was added to the cells and alterations in fluorescence intensity monitored over a 5 min time course.

In an additional series of experiments, platelets loaded with Fluo-8-AM were suspended in PBS with EGTA (300 μM), an extracellular Ca^{2+}-chelating agent, to investigate dolutegravir-mediated alterations in platelet cytoplasmic Ca^{2+} concentrations in the absence of extracellular Ca^{2+}. These results, from eight separate experiments using cells from seven different donors, are presented as the basal and peak Ca^{2+} concentrations calculated as described previously (Grynkiewicz et al. 1985) and as representative traces.

Expression and statistical analysis of results

The results of each series of experiments are expressed as the median values and interquartile range (IQR) with the numbers of different donors (n) and replicate experiments clearly indicated. Statistical analyses were performed using Stata 17.0 SE (StataCorp, College Station, TX) software, with levels of statistical significance calculated using the Wilcoxon sign-rank test for comparisons of non-parametric data. A p-value < 0.05 was considered significant.

Results

Effects of dolutegravir on ADP-, thrombin- and U46619-activated expression of CD62P by PRP

The effects of dolutegravir (5 and 10 μg/ml) on expression of CD62P by ADP-activated platelets, relative to those of drug-free control cells, are shown in Figure 1. The CD62P expression levels of the drug-free, unstimulated control system (in the absence of ADP) were minimal, increasing significantly following addition of ADP. Pretreatment of PRP with dolutegravir (5 and 10 μg/ml) resulted in dose-related stimulation of ADP-activated

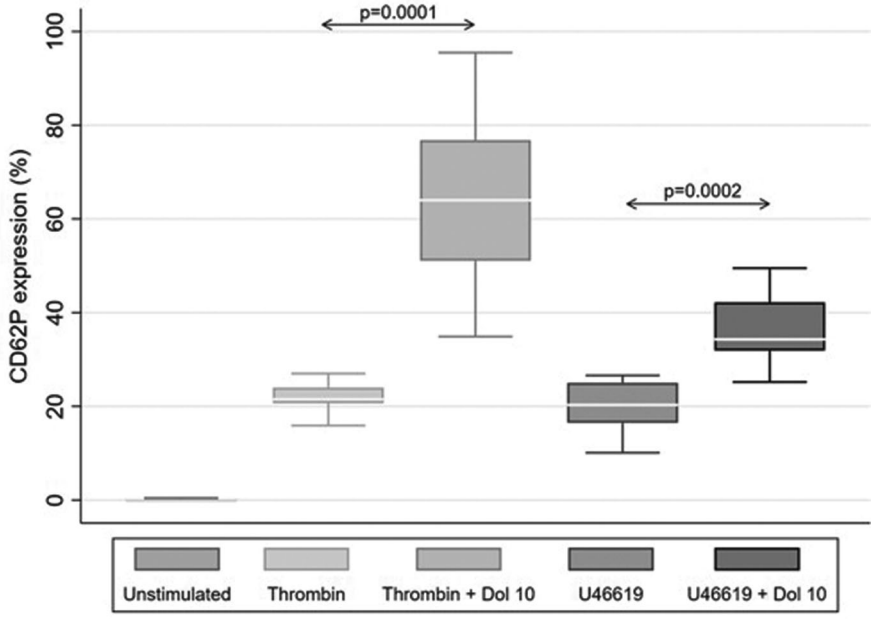

Figure 2. Effects of dolutegravir (Dol, 10 μg/ml) on the magnitude of CD62P expression on platelets activated with thrombin (0.6 NIH units/ml) or U46619 (5 μM). Results are expressed as Box and Whisker plots of % CD62P expression for comparison of dolutegravir with respective drug-free control systems ($n = 4$–6 individual donors, with 13–15 experiments in the series).

up-regula-tion of CD62P expression, with significance levels of $p < 0.0001$ and $p < 0.0001$, respectively.

As shown in Figure 2, augmentation of CD62P expression by dolutegravir (10 μg/ml) was also observed following activation of platelets with either thrombin or U46619 ($p = 0.0001$ and $p = 0.0002$, respectively). The median percentages of stimulation of expression of CD62P by dolutegravir (10 μg/ml)-treated platelets activated with ADP, thrombin or U46619 were 75%, 195% and 69%, respectively. With respect to the effects of dolutegravir alone (in the absence of ADP, thrombin or U46619), a slight augmentation of expression of CD62P was observed at 10 μg/ml of this agent, the median (IQR) values for the untreated control system and system treated with dolutegravir being 0.23 (0.03–0.27) and 0.38 (0.12–0.49), respectively.

Effects of dolutegravir on neutrophil:platelet aggregation

The effects of dolutegravir (at 10 μg/ml) on the formation of NP heterotypic aggregates following addition of thrombin (0.6 NIH units/ml) to buffy coat preparations are shown in Figure 3. Relative to the drug-free, thrombin-activated control system, treatment with dolutegravir resulted in a significant potentiation of the formation of NP aggregates ($p = 0.0005$), representing a mean percentage increase of 22%. Dolutegravir alone, in the absence of thrombin, caused only a slight increase in the formation of NP aggregates, the median (IQR) values for the untreated control system and the system treated with dolutegravir being 5.3 (4.6–5.9) and 5.9 (5.0–6.0), respectively.

Effects of dolutegravir on platelet cytosolic Ca^{2+} fluxes

As shown in Figure 4, addition of dolutegravir (at 2.5, 5. and 10 μg/ml) to plasma-free, isolated pure platelets pre-loaded with Fluo-8-AM resulted in an abrupt concentration-dependent increase in fluorescence intensity that quickly leveled off and remained elevated throughout the time course of each experiment, consistent with an immediate and sustained elevation in the cytosolic Ca^{2+} concentration that was abolished when the cells were suspended in Ca^{2+}-depleted medium. The median (IQR) increases in cytosolic Ca^{2+} concentrations for dolutegravir-treated platelets (2.5, 5, and 10 μg/ml), were 109 (70–233 nM), 408 (179–444 nM), and 458 (356–562 nM), respectively.

The median (IQR) cytosolic Ca^{2+} concentrations for untreated and dolutegravir-treated (10 μg/ml) systems in the presence of extracellular Ca^{2+} were 422 (381–463 nM) and 896 (787–973) nM, respectively, ($p = 0.00009$). In the absence of extracellular Ca^{2+} there was no detectable increase in the cytosolic Ca^{2+} concentrations of dolutegravir-treated platelets and the median (IQR) cytosolic Ca^{2+} concentration in the absence of extracellular Ca^{2+} was 25 (17–39 nM). The dolutegravir-mediated increases in platelet cytosolic Ca^{2+} concentrations were not attenuated in the presence of inhibitors of PLC, IP3 and NSCC (Figure 4). The median (IQR) magnitude of increase in cytosolic Ca^{2+} in the absence or presence of U73122, APB, or SKF 96365 was 458 (356–562 nM), 388 (255–459 nM), 525 (475–581) and 688 (336–800 nM), respectively.

Addition of thrombin to platelets increased the cytosolic Ca^{2+} concentration by 2-fold from 467 (386:602 nM) to 904 (765:950 nM) [$n = 4$], confirming the viability and reactivity of the cells.

Discussion

The findings of the current study showed that dolutegravir, at concentrations of 5–10 μg/ml, caused statistically significant potentiation of several activities of human platelets *in vitro*. These included; (i) up-regulation of expression of the α-granule-derived adhesion molecule CD62P measured in cells activated with ADP, thrombin, or U46619 (a thromboxane A_2 surrogate); (ii) formation of NP aggregates following exposure to thrombin; and, (iii) receptor-independent mobilization of intracellular Ca^{2+}. The concentrations of dolutegravir at which augmentation of receptor-mediated platelet activation were observed are close to those reported to occur in plasma following oral

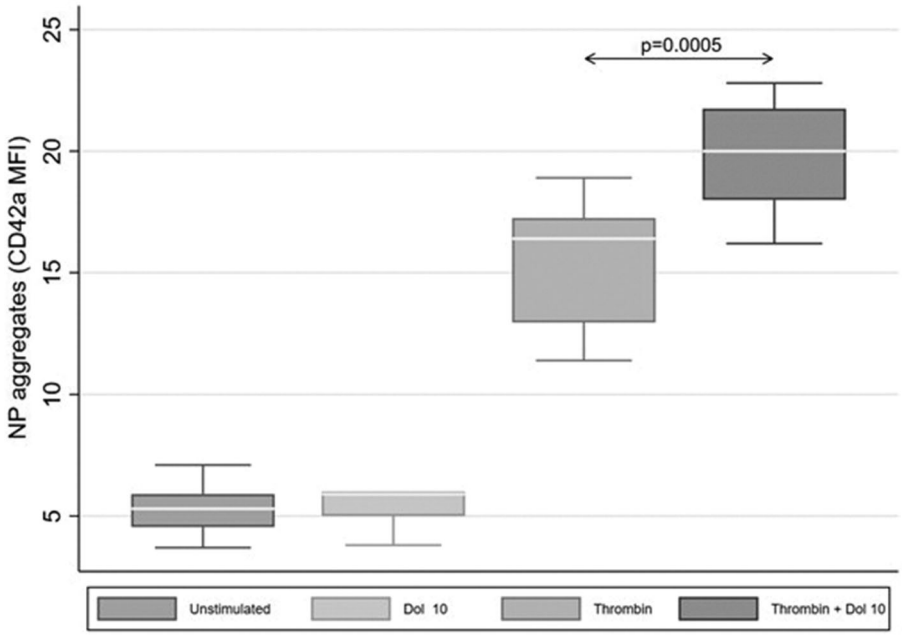

Figure 3. Effects of dolutegravir (Dol) at 10 μg/ml on the formation of neutrophil platelet (NP) heterotypic aggregates following addition of thrombin (0.6 NIH units/ml). Results are expressed as Box and Whisker plots of mean fluorescence intensity (MFI) of CD42a-PE emitted by $CD16^+/CD45^+$ neutrophils ($n = 6$ individual donors, with 12 experiments in the series).

administration of this anti-retroviral agent. These range from 3.34–6.16 μg/ml, depending on whether tablets or suspensions at a daily dose of 50 mg were administered (Cottrell et al. 2013).

Platelet CD62P (P-selectin) is widely recognized as being a mediator of platelet pro-inflammatory and pro-thrombotic activities that drive heterotypic aggregation and activation of neutrophils, monocytes, and endothelial cells (Théorêt et al. 2011; Cerletti et al. 2012). CD62P *via* binding to its ligand, P-selectin glycoprotein ligand-1 (PSGL-1), plays a central role in the interactions between platelets, leukocytes, and endothelial cells (Yun et al. 2016). From a mechanistic perspective, dolutegravir appears to prime (sensitize) platelets to hyper-respond to receptor-mediated activation, irrespective of receptor type, including those activated by ADP (P2Y1/P2Y12), thrombin (PAR-1/PAR-4) and thromboxane A2 (TXA2). Mechanistically, elevations in cytosolic Ca^{2+} concentrations represent a critical, early, and common event in platelet activation initiated by all of these receptor types (Varga-Szabo et al. 2009). In this context, dolutegravir-mediated spontaneous, receptor-independent increases in platelet cytosolic Ca^{2+} concentrations appear to underpin the mechanism by which the anti-retroviral agent augments the responsiveness of these cells to ADP, thrombin, and U46619. Observations that dolutegravir at the concentrations used did not induce spontaneous, as opposed to receptor-mediated, activation of platelet CD62P expression may indicate that threshold levels of cytosolic Ca^{2+} were not exceeded in this setting (Mazzucato et al. 2002).

Although prior literature on the effects of Ca^{2+} mobilization in eukaryotic cells is extremely limited, two studies are, however, noteworthy. In the first of these, Al Mamun Bhuyan et al. (2016) reported that exposure of isolated human erythrocytes to dolutegravir, albeit for 48 h, at concentrations comparable to those used in the current study (4.77–19.08 μM equivalent to ≈ 2–8 μg/ml), resulted in an influx of extracellular Ca^{2+} that triggered oxidative stress, eryptosis, and hemolysis. The authors did not speculate on the mechanisms of dolutegravir-mediated Ca^{2+} influx. More recently, Wang et al. (2020) investigated the effects of prolonged exposure (24 h) of a human non-small cell lung cancer cell line to a novel derivative of dolutegravir at concentrations of 5–20 μM on cell viability and alterations in cytosolic Ca^{2+} concentrations. Those authors observed that exposure of the cancer cell line, but not a control non-cancerous cell line, to the dolutegravir derivative caused dose-related induction of cell death *via* a pro-apoptotic mechanism associated with both elevated concentrations of cytosolic Ca^{2+} and cellular oxidative stress that was linked to inhibition of the Ca^{2+}-sequestering endo-membrane Ca^{2+}-ATPase (Al Mamun Bhuyan et al. 2016). These effects were not, however, observed following exposure of the cancer cell line to dolutegravir *per se* at a fixed concentration of 10 μM, possibly due to this being a threshold concentration for detection of effects on cytosolic Ca^{2+}.

The findings of the current study differ from those reported by Taylor et al. (2019) who observed that collagen-mediated activation of platelets and expression of CD62P was inhibited in the presence of dolutegravir. These differences likely relate to the different types of activating stimuli used in both studies.

With respect to potential mechanisms of dolutegravir-mediated alterations in cytosolic Ca^{2+} concentrations and cell viability, the current study differs in a number of aspects from those performed by Al Mamun Bhuylan et al. and Wang et al. These include differences in cell type, duration of exposure to dolutegravir, and apparent lack of cytotoxicity. The latter contention is based on our findings that rather than inhibiting platelet activation, which would have been the case in the event of cytotoxicity, the opposite was observed. Based on the results of our intracellular Ca^{2+}-focused experiments, we believe that the most likely mechanism of dolutegravir-mediated increases in platelet cytosolic Ca^{2+} relates to the combination of lipophilicity and divalent/trivalent metal-binding/chelating properties of this anti-retroviral agent (Song et al. 2015; Grießinger et al. 2016; Thierry et al. 2017). These properties are likely to confer ionophore-type properties on dolutegravir that would promote movement of Ca^{2+} across the plasma membrane, delivering the cation to the cytosol where it is likely to augment Ca^{2+}-dependent intracellular signaling mechanisms. This contention is supported by the

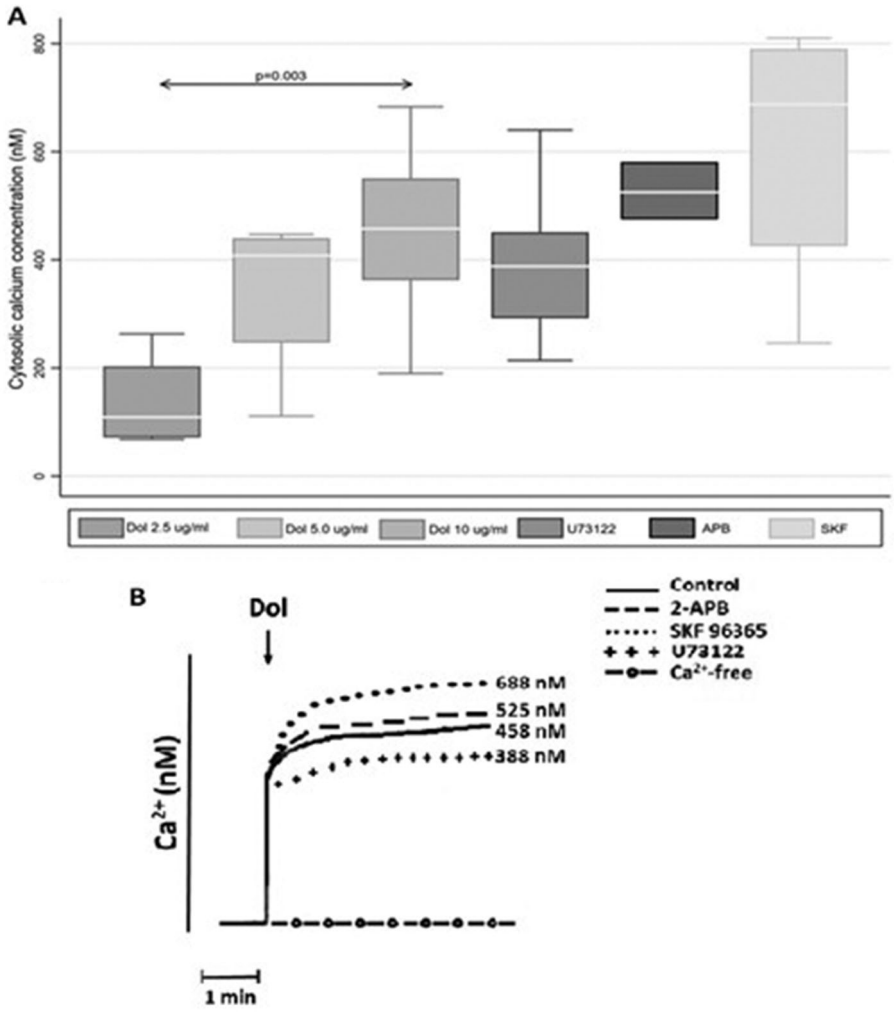

Figure 4. Effects of dolutegravir on platelet cytosolic Ca^{2+}concentrations. Results of the entire series of experiments (20 separate experiments; $n = 7$ individual donors) are shown in the upper panel A as the magnitude of increase in cytosolic Ca^{2+} following addition of dolutegravir (2.5, 5, and 10 μg/ml) to platelets suspended in Ca^{2+}-replete medium and on the alterations of dolutegravir (10 μg/ml)-mediated Ca^{2+} mobilization in the presence of selective inhibitors of PLC, IP3 and NSCC, namely U73122 (5 μM), 2-APB (50 μM) and SKF 96365 (50 μM), respectively. Representative traces from a single experiment are shown in the lower panel B. Dolutegravir (Dol, 10 μg/ml) as denoted by the arrow (↓) was added to platelets suspended in Ca^{2+} -replete (——) and -depleted (- - -) medium. An abrupt and sustained increase in cytosolic Ca^{2+} was observed following addition of dolutegravir to platelets suspended in Ca^{2+}-replete HBSS which was abolished in Ca^{2+}-free medium.

observation that the abrupt dolutegravir-mediated increase in platelet cytosolic Ca^{2+} was markedly attenuated following depletion of extracellular Ca^{2+}. Furthermore, selective inhibitors of PLC, IP3 channels, and NSCC did not attenuate the dolutegravir-mediated increases in platelet cytosolic Ca^{2+} concentrations which supports the contention that dolutegravir acts *via* an ionophore-like mechanism independent of receptor-mediated Ca^{2+} signaling pathways. This observation is seemingly in contrast to the effects of ionomycin and A23187 on cell types (that did not include platelets) (Dedkova et al. 2000), indicating that dolutegravir at the concentrations used may act in a manner distinct from that of commonly-used ionophores.

Importantly, obesity and metabolic syndrome are associated with an accelerated atherothrombotic process, resulting from a combination of factors that includes platelet hyper-activity (Santilli et al. 2012). In the context of cART, associations with dyslipidemia (Tsai et al. 2017) and obesity (Bakal et al. 2018) have been described that were linked to an increase in platelet aggregation and reactivity, thus placing HIV-infected individuals at increased risk of CVD (Nkambule et al. 2017; Mesquita et al. 2018). As documented in recent reports, excess weight gain during therapy with dolutegravir (as well as some other INSTI) might therefore pose a serious risk for development of CVD and other cardiometabolic co-morbidities, such as insulin resistance and diabetes mellitus (Eckard and McComsey 2020; Gorwood et al. 2020; Taramasso et al. 2020; Hindley et al. 2021; Lahiri et al. 2021).

In addition, ongoing systemic inflammation observed in HIV-infected individuals, despite cART, has been identified as a risk factor for CVD that is associated with higher levels of D-dimer, as well as those of the inflammatory markers, interleukin (IL)-6 and C-reactive protein (CRP), as reported by the Strategies for the Management of Anti-retroviral Therapy (SMART) study (Borges et al. 2016).

Activated platelets also play a fundamental role in both inflammation and thrombosis that is associated with the release of granule-derived, pro-inflammatory mediators (Koupenova et al. 2018). Notably, HIV-infected individuals have impaired platelet function resulting from increased platelet activation, possibly as a result of microbial translocation and sustained exposure to microbial components in the circulation (Brenchley et al. 2006; Cassol et al. 2010). Although cART has been shown to

effectively increase platelet counts following three months of treatment, platelet activation and dysfunction persist (Hottz et al. 2018; Mesquita et al. 2018; Madzime et al. 2021). Clearly, further activation of platelets by dolutegravir could have clinical consequences for cardiometabolic health, which should be explored in future studies.

Informed consent statement

Written informed consent was obtained from all blood donors who partook in the study.

Institutional review board statement

As noted, permission to undertake this study and to draw blood from healthy adult human volunteers was granted by the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria (Approval #116/2017 and 605/2018).

Author contributions

All authors contributed significantly to the conceptualization and planning of the study. MM, AJT, RA, GRT, HCS and JGN performed laboratory investigations; AJT, TMR, PWAM, RA and GRT contributed to analysis of the data and preparation of the figures, while all authors contributed to interpretation of data and compilation of the manuscript. All authors have approved this submitted version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

Data can be made available upon reasonable request.

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