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ML355 Modulates Platelet Activation and Prevents ABT-737 Induced Apoptosis in Platelets

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ABSTRACT

12-lipoxigenase (12-LOX) is implicated in regulation of platelet activation processes and can be a new promising target for antiplatelet therapy. However, investigations of 12-LOX were restricted by the lack of specific and potent 12-LOX inhibitors and by controversial data concerning the role of 12-LOX metabolites in platelet functions. A novel specific 12-LOX inhibitor ML355 was shown to inhibit platelet aggregation without adverse side effects on hemostasis; however, the molecular mechanisms of its action on platelets are poorly understood. Here, we showed that ML355 inhibited platelet activation induced by thrombin or thromboxane A_2, but not by collagen-related peptide. ML355 blocked protein kinase B, phosphoinositide 3-kinase, and extracellular signal-regulated kinase, but not p38 kinase, spleen tyrosine kinase (Syk), or phospholipase Cγ2 phosphorylation in activated platelets. The main inhibitory effect of low doses of ML355 (1–20 μM) on thrombin activated platelets was mediated by the decrease in reactive oxygen species level, whereas high doses of ML355 (50 μM) caused cyclic adenosine monophosphate activation. ML355 did not affect the activity of nitric oxide-dependent soluble guanylyl cyclase, nor did it affect the relaxation of preconstricted aortic rings in mice. ML355 itself did not affect platelet viability, but at 50 μM dose blocked caspase-dependent apoptosis induced by B-cell lymphoma II inhibitor ABT-737.

SIGNIFICANCE STATEMENT

The current paper provides novel and original data concerning molecular mechanisms of 12-LOX inhibitor ML355 action on platelets. These data reveal antiplatelet and protective effects of ML355 on platelets and may be of importance for both antiplatelet and anticancer therapy.

Introduction

Platelets are anucleate small blood cells, which play a crucial role in hemostasis and maintain blood vessel integrity. Apart from the normal physiologic conditions, platelets are implicated in a wide range of pathologic processes, such as atherosclerosis (Davi and Patrono, 2007), arterial and venous thrombosis (Yeung et al., 2018), cancer metastasis (Plantureux et al., 2018; Foss et al., 2020), and inflammation (Vieira-de-Abreu et al., 2012). Existing antiplatelet therapy targets many proteins involved in platelet activation and pathologic platelet conditions; however, modern antiplatelet therapy often exhibits either drug resistance (Schwartz, 2011) or serious adverse side effects such as bleeding (Jackson, 2011). In this regard, a search of new potential targets for antiplatelet therapy is of great interest.

Lipoxygenase 12 (12-LOX) is an enzyme that catalyzes the oxygenation of arachidonic acid (AA) (Yeung et al., 2016) resulting in generation of a short life metabolite 12(S)-hydroperoxyicosa-5,8,10,14-tetraenoic acid that is quickly reduced by cellular glutathione peroxidase to 12-hydroxyeicosatetraenoic acid (Singh and Rao, 2019). 12-hydroxyeicosatetraenoic acid either acts as a ligand of some G protein coupled receptors (Yokomizo et al., 2001; Guo et al., 2011b) or can undergo further metabolization to epoxins, hepoxillins, eoxins, and other metabolites. Three isoforms of 12-LOX have been described in humans including leukocyte, platelet, and epidermis specific isoforms (Burkhart et al., 2012; Borin et al., 2017). In platelets, 12-LOX is essential for activation of integrins αIIbβ3 (Yeung et al., 2014) and for protease-activated receptor 4 (PAR4)- and glypoprotein VI (GPVI)- mediated platelet

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Informed consent was obtained from all subjects involved in the study.
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ABBREVIATIONS: AA, arachidonic acid; AC, adenylyl cyclase; Akt, protein kinase B; Bcl-2, B-cell lymphoma II; COX, cyclooxygenase; Crp-XL, collagen-related peptide; DCF-DA, 2, 7-dichlorodihydrofluorescein diacetate; Erk1/2, extracellular signal-regulated kinase; GC, guanylyl cyclase; HSD, honestly significant difference; 12-LOX, 12-lipoxygenase; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKG, protein kinase G; PLCγ2, phospholipase Cγ2; PS, phosphatidylserine; ROS, reactive oxygen species; VASP, Vasodilator-stimulated phosphoprotein; WP, washed platelets.

activation (Yeung et al., 2013), whereas 12-LOX inhibition leads to decrease of platelet aggregation (Kim et al., 2014; Svensson Holm et al., 2014).

In addition to platelets, platelet 12-LOX expression is often observed in different cancer cell lines (Singh et al., 2011; Zhong et al., 2018), where 12-LOX expression is associated with tumor progression and angiogenesis (Pidgeon et al., 2002; Matsuyama et al., 2004; Nie et al., 2006). In fact, platelets themselves play an important role in cancer metastasis and angiogenesis (Wojtukiewicz et al., 2017; Plantureux et al., 2018; Schlesinger, 2018; Foss et al., 2020). Hence, 12-LOX inhibition will also have an advantage in cancer therapy due to both platelet inhibition and cancer progression blockage. 12-LOX targeting as a new promising antiplatelet and anticancer therapy was revealed and described in several recent reviews (Yeung and Holinstat, 2011; Tourdot and Holinstat, 2017; Clemente et al., 2020; Zheng et al., 2020).

Despite the fact that 12-LOX and its metabolites are implicated in regulation of platelet and cancer metabolism, the lack of understanding of its physiologic functions restricts therapeutic application of 12-LOX inhibitors. Another essential problem is associated with unspecific action of known 12-LOX inhibitors (Tourdot and Holinstat, 2017). Therefore, the development of new selective and specific 12-LOX inhibitors has a great therapeutic potential. To date, one of the most specific described inhibitors of 12-LOX is ML355 (Luci et al., 2013). ML355 diminishes human platelet aggregation without increased bleeding in mice (Adili et al., 2017), but the molecular mechanisms of its action on platelets are poorly understood.

We investigated the ML355 effect on platelet activation induced by different agonists and showed that ML355 inhibited thrombin or thromboxane A_2, (TxA_2) but not collagen-related peptide (Crp-XL) induced platelet activation. ML355 blocked protein kinase B (Akt), phosphoinositide 3-kinase (PI3K), and extracellular signal-regulated kinase (Erk1/2), but not p38 kinase, Syk kinase , or phospholipase Cγ2 (PLCγ2) phosphorylation. In addition, ML355 significantly decreased reactive oxygen species (ROS) level in thrombin activated platelets. Inhibitory effects of ML355 in high doses was mediated by cyclic adenosine monophosphate (cAMP) activation. ML355 itself did not affect platelet viability, but surprisingly in high doses even blocked caspase-dependent apoptosis induced by ABT-737.

Materials and Methods

Chemicals, Reagents, and Materials

ML355, U46619 (Cayman Chemical, MI); CRP-XL (VCPBIO, Shenzhen, China); Thrombin (Roche, Mannheim, Germany); 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), SQ22563, forskolin, and sodium nitroprusside (Sigma-Aldrich, Munich, Germany); phospho-Akt (# 4060), phospho-PI3K (# 17366), phospho-Erk1/2 (# 4370), phospho-p38 (# 9216), phospho-Syk (# 2710), phospho-PLCγ2 (# 3871), caspase-3 (# 9662), and anti-Actin (# 4970) antibodies (Cell Signaling, Frankfurt, Germany); Phospho-Vasodilator-stimulated phosphoprotein (VASP) S239 (Clone 16c2) and phospho-VASPS159 (clone 5C6) (Nano Tools, Teningen, Germany); Fibrinogen-Alexa-Fluor 647, Calcein-AM (Molecular Probes, Göttingen, Germany); Phycoerythrin conjugated CD62P, Phycoerythrin conjugated Annexin-V (BD Bioscience, Heidelberg, Germany); 2,7-dichlorodihydrofluorescein diacetate (DCF-DA) (Calbiochem, Schwalbach, Germany), ABT-737 (Selleckchem, Munich, Germany); horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG (Amersham, Freiburg, Germany).

Human Platelet Preparation

The study was performed according to the Declaration of Helsinki and all experimental protocols were submitted and approved by the Ethical Committee of Sechenov Institute of Evolutionary Physiology and Biochemistry of the Russian Academy of Sciences (protocol no. 3-03 from 02.03.20). All donors signed a written consent before the venipuncture. Human platelets were isolated from the whole blood of healthy volunteers free of nonsteroidal anti-inflammatory drugs as described previously (Gambaryan et al., 2010) with small modifications. Briefly, blood was collected into citrate Monovette© with Acid Citrate Dextrose solution (12 mM citric acid, 15 mM sodium citrate, 25 mM D-glucose) and centrifugated (200 × g) for 7 minutes at room temperature. Further, platelet rich plasma was collected and centrifuged (400 × g) for 5 minutes, after which platelet pellets were washed once with CGS buffer (120 mM sodium chloride, 12.9 mM trisodium citrate, 10 mM D-glucose, pH 6.5). Finally, washed platelets (WP) were resuspended in HEPES buffer (150 mM sodium chloride, 3 mM potassium chloride, 1 mM magnesium chloride, 5 mM D-glucose, 10 mM HEPES, pH 7.4). After 15 minutes of platelet rest in water bath (37°C), 1 mM $CaCl_2$ was added and platelets were used for experiments.

Western Blot Analysis

For Western blot analysis, WP (3×10^8 platelets/ml) were stimulated with the indicated compounds and then lysed with Laemmli sample buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were incubated with appropriate primary antibodies overnight at 4°C. To visualize the signal, either goat anti-rabbit or anti-mouse IgG conjugated antibodies with horseradish peroxidase were used. Membranes were scanned using C-DiGit scanner (Li-COR, USA). Chemiluminescence analysis was performed using Image Studio Digits Version 5.2.

Flow Cytometry Analysis

The analysis was performed by CytoFLEX flow cytometer (Beckman Coulter, Inc., USA). Each sample was rated for 15,000 events. The data were analyzed by CytExpert Acquisition and Analysis Software Version 2.4 (Beckman Coulter, Inc., USA).

Analysis of Platelet αIIbβ3 Integrin Activation and α-Granule Secretion. For flow cytometry analysis, WP concentration 1×10^8/ml was used. Platelet αIIbβ3 integrin activation was measured by fibrinogen-Alexa-Fluor 647 binding. Fibrinogen (final concentration 15 μg/ml) was added to WP and platelets were incubated with indicated concentrations of ML355 at 37°C for indicated time. Platelet agonists thrombin, U46619, or CRP-XL were added to the samples and incubated at 37°C for 2 minutes, and the reaction was stopped by the addition of PBS (1:40). For detection of α-granule secretion, CD62P-PE was added to WP (1:10) after the incubation with indicated concentrations of ML355, followed by the addition of thrombin, U46619, or CRP-XL (2 minutes) and additional incubation for 10 minutes in the dark. The reaction was stopped by PBS buffer (1:40).

Analysis of Phosphatidylserine Exposure. Phosphatidylserine (PS) exposure was measured by Annexin-V-PE binding. Platelets were incubated with indicated concentration of ML355 for 30 minutes, then annexin-V-PE (1:10) was added to the samples, and the suspension was immediately dissolved in Annexin-V binding buffer (1:20) (140 mM NaCl, 10 mM HEPES, 2.5 mM $CaCl_2$). After that the samples were incubated for 10 minutes at room temperature in the dark. For positive control, platelets were incubated with apoptosis inducer ABT-737 (1 μM) for 60 minutes.

Analysis of ROS Formation. Analysis of reactive oxygen species (ROS) formation in activated platelets was performed using fluorescent dye DCF-DA. WP were incubated with DCF-DA (10 μM) and indicated concentrations of ML355 for 30 minutes, then thrombin (100 mU/ml) was added for 2 additional minutes, and the reaction was stopped by PBS buffer (1:40).

Analysis of Platelet Viability. To asses platelet viability, cell-permeable Calcein-AM was used. Calcein-AM acquires a green fluorescent signal only after the acetoxymethyl ester hydrolysis by intracellular esterases. WP were incubated with Calcein-AM at a final concentration of 0.2 μM and with indicated concentrations of ML355 for 30 minutes, and then the samples were dissolved by PBS buffer (1:40) and analyzed by Flow Cytometry. For positive control, platelets were incubated with inhibitor of B-cell lymphoma II (Bcl-2) proteins gossypol (30 μM) for 10 minutes.

Preparation of Recombinant Human NO-Stimulated Guanylyl Cyclase GC-1 Enzyme

Full-length NO-stimulated GC-1, also known as soluble guanylyl cyclase, was purified from Sf9 cells as described previously (Sharina et al., 2021). Briefly, 4 l of Sf9 cells were infected with baculoviruses expressing α_1 and β_1 GC-1 subunits. 72 hours later the cells were collected by centrifugation, resuspended, disrupted by sonication, and centrifuged at 100,000 × g. The supernatant was applied onto a 50 ml DEAE-FF Sepharose column. The fraction containing GC-1 was eluted with 350 mM NaCl and was loaded onto a Ni-agarose column. After washes with 40 mM imidazole, GC-1 was eluted with 200 mM imidazole. GC-1-containing fractions were pooled, supplemented with 5 mM dithiothreitol , and concentrated on a 10 kDa centrifugal filter concentrator (Millipore, Bedford, MA). For long-term storage at -80°C, the sample were diluted to 0.25 mg/ml GC-1 using 50 mM triethanolamine pH 7.4, 250 mM NaCl, 1 mM $MgCl_2$ supplemented with 25% glycerol.

Assay of sGC Activity In Vitro

Enzymatic activity of GC-1 was assayed using [α-^{32}P]GTP to [^{32}P] cGMP conversion assay (Schultz, 1974). 0.5 μg GC-1 in 25 mM TEA, pH 7.5, 0.1 mM EGTA, 0.1 mg/ml bovine serum albumin, 1 mM cGMP, 2 mM $MgCl_2$, was incubated with indicated concentrations of ML355 for 10 minutes at room temperature. For experiments with ferric GC-1, 10 μM of GC-1 heme oxidizing agent 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was added at this step. The reaction of cGMP synthesis was initiated by transferring the sample to 37°C and adding 1 mM GTP/[α-^{32}P]GTP (~100,000 cpm). After 10-minute incubation, the reaction was stopped by precipitation of GTP with zinc carbonate and subsequent centrifugation at 10,000xg. The supernatant containing cGMP was passed through 2 ml alumina columns and processed as previously reported (Chauhan et al., 2012).

Aortic Ring Relaxation

All animal experiments were performed in accordance to Public Health Service's Guide for the Care and Use of Laboratory Animals, the Foundation for Biomedical Research's Biomedical Investigator's Handbook for Researchers, and the guidelines of the Animal Welfare Committee of the University of Texas Health Science Center (protocol AWC 18-085). Vasoactivity measurements were performed as described previously (Sharina et al., 2015), with minor modifications. C57Bl6 mice (8–10 weeks old, Jackson laboratories, Ann Harbor, MI) were euthanized by CO_2. After thoracotomy, descending thoracic aorta was dissected, cut into 3–5-mm-long segments and mounted on a four-channel wire Myograph 610 (DMT, Copenhagen, Denmark) under 0.9 g of passive tension. All force measurements were recorded using Powerlab 400 data acquisition system and LabChart software. The rings were equilibrated for the total of 80 minutes in Krebs-Henseleit solution pH 7.4, oxygenated with carbogen (95% O_2, 5% CO_2) with at least three buffer changes every 20 minutes. After equilibration, the rings were contracted with 1μM phenylephrine to achieve submaximal contraction (~1.5–2 g of tension). After stabilization, doses of ML355 were added cumulatively and isometric tension was recorded. To test the effect of ML355 on vasodilation mediated by sGC with oxidized heme, 10 μM ODQ was added to the organ bath containing 300 μM ML355, and isometric tension was recorded. To confirm that the tested aortic ring is capable of vasodilation, 50 μM Gemfibrozil was added at the end to induce relaxation of the smooth muscle.

Data Analysis

All experiments were performed at least from four different donors, and data are presented as means ± S.D. GraphPad Prism 8 (GraphPad Software, San Diego, CA) was used for data analysis. All variables were conformed to a normal distribution by Shapiro–Wilk's test ($P > 0.05$). Differences between groups were analyzed by one-way ANOVA with Tukey honestly significant difference (HSD) post hoc analysis, when the samples were homoscedastic (Levene's test, $P > 0.05$). When the equal variances were not assumed, Tamhane's T2 post hoc analysis was used. For paired groups analysis, Mann-Whitney U test or two-tailed paired t test were used. $P < 0.05$ was considered statistically significant.

Results

1. ML355 Inhibits Activation in Thrombin or TxA2, but Not Crp-XL Induced Platelets. Considering that previous reports demonstrated that ML355 inhibits platelet aggregation induced by thrombin (Adili et al., 2017), we assessed the effect of ML355 on platelet αIIbβ3 integrin activation and α-granule secretion induced by different agonists. Platelet activation was measured by fibrinogen and CD62P (P-selectin) binding in washed platelets. We found that ML355 significantly decreased platelet fibrinogen (Fig. 1, A and C) and CD62P (Fig. 1, B and D) binding after activation induced by thrombin (Fig. 1, A and B) or U46619 (Fig. 1, C and D), although P-selectin binding blockage was less potent for thrombin-induced platelet activation (Fig. 1B). The observed inhibitory effect of ML355 was time-dependent, and significant platelet fibrinogen binding inhibition was exhibited only after 30 minutes of incubation (Fig. 1E). Therefore, the 30-minute incubation was used in the rest of our experiments.

Platelet activation is a complex process that is mediated by a plethora of signal molecules that orchestrate the key activation events in platelets. One of these molecules is phosphoinositide 3-kinase (PI3K), which is activated in response to activation of soluble agonists G-protein-coupled receptors, such as purinergic P_2Y_{12}, PAR1/4, and thromboxane receptor. PI3K plays a crucial role in $\alpha_{IIb}\beta_3$ integrin activation and platelet aggregation (Gratacap et al., 2011). We found that in thrombin-induced platelets ML355 dose-dependently inhibited phosphorylation of PI3K at Tyr199/458 and its downstream effectors protein kinase B (Akt) at Ser473 and extracellular signal-regulated kinase (Erk1/2) at Thr202/Tyr204 (Fig. 2). Another protein expressed in platelets that along with Erk1/2 belongs to mitogen activated protein kinases family and involved in platelet aggregation processes is p38 kinase (Begonja et al., 2007). In our experiments, even high doses of ML355 (50 μM) did not affect p38 Thr180/Tyr182 phosphorylation induced by thrombin (Fig. 2B).

Surprisingly, ML355 did not inhibit fibrinogen or P-selectin binding in Crp-XL activated platelets (Fig. 3, A and B). Cross-linked collagen-related triple-helical peptide (Crp-XL) imitates the triple helical structure of collagen and binds to GPVI collagen receptor in platelets (Asselin et al., 1997; Sang et al., 2019). GPVI binding to collagen leads to activation of Src family kinases Fyn, Lyn, Src, and Syk (Ezumi et al., 1998) that mediate the formation of linker of activated T cells (LAT) signalosome and subsequent phospholipase Cγ2 (PLCγ2) activation. In our

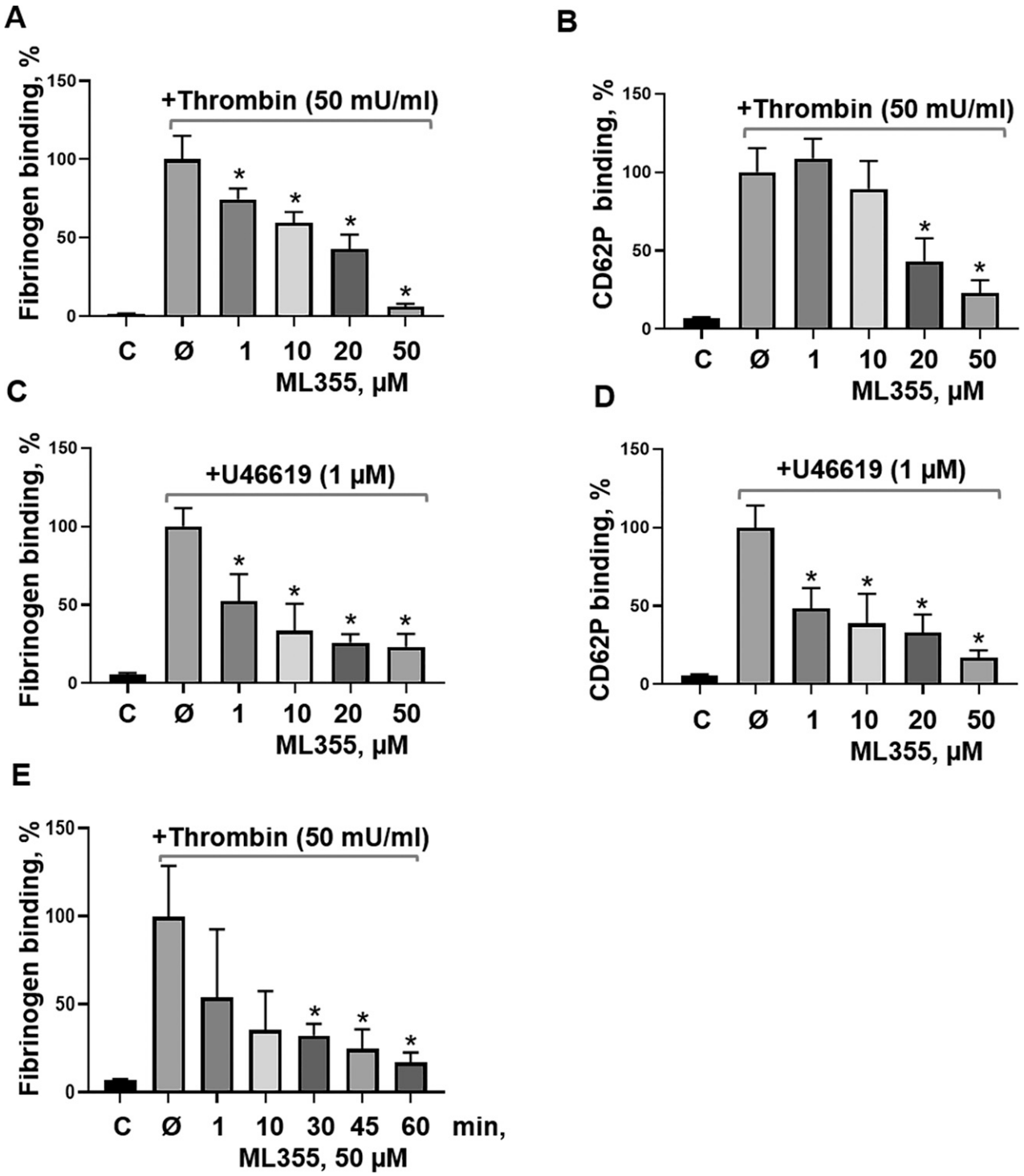

Fig. 1. ML355 inhibits thrombin or U46619 induced platelet αIIbβ3 integrin activation and α-granule secretion. Washed human platelets (1×10^8/ml) were incubated with ML355 (A–D) at indicated concentration for 40 min or (E) during the indicated time (50 μM) and then were activated by Thrombin (50 mU/ml) or U46619 (1 μM). (A, C, E) Flow cytometry analysis of platelet αIIbβ3 integrin activation (Fibrinogen Alexa-647 binding). Fibrinogen (15 μg/ml) was added 15 min before activation. (B, D) Flow cytometry analysis of P-selectin exposure (CD62P-PE binding). CD62P-PE was added (1:10) directly after the activation and incubated with platelets during 10 min before measuring. For both integrin activation and P-selectin externalization analysis, the reaction was stopped by PBS buffer addition (1:40). Data are presented as means ± S.D. Thrombin- or U46619-induced Fibrinogen binding or P-selectin exposure were designated as 100% (one-way ANOVA, Leven's test $p > 0.05$ followed by Tukey's HSD test. *– $p < 0.05$ compared with the activator, $n = 7$).

experiments, ML355 did not inhibit Syk Tyr525/526 or PLCγ2 Tyr1217 phosphorylation in Crp-XL-induced platelets, but high doses of ML355 (50 μM) even potentiated the phosphorylation (Fig. 3, C and D).

2. ML355 Reduces ROS Formation in Thrombin Activated Platelets. Previous studies demonstrated that ROS have an important role in platelet αIIbβ3 activation, granule secretion, and platelet shape change (Begonja et al.,

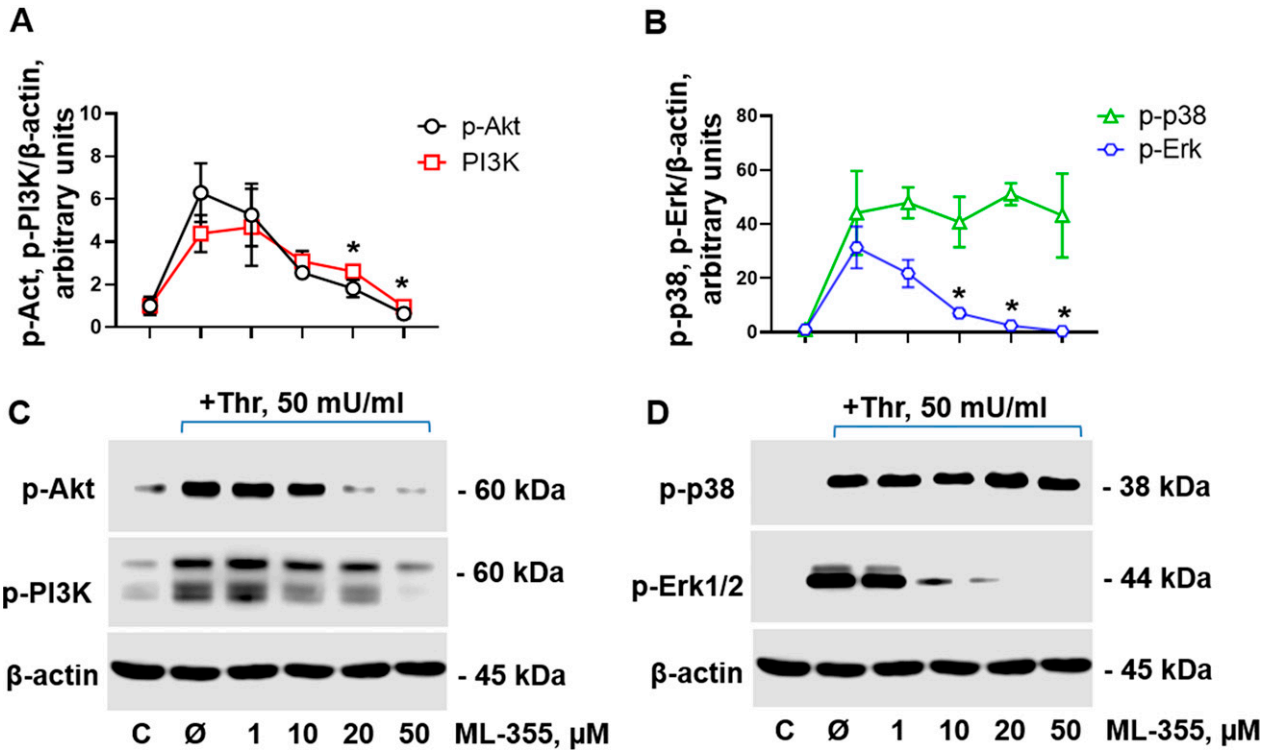

Fig. 2. ML355 blocks Akt, PI3K, and Erk1/2, but not p38 phosphorylation in platelets. Western blot analysis of Akt, PI3K, p38, or Erk1/2 phosphorylation. Washed human platelets (3×10^8/ml) were incubated with ML355 at indicated concentrations for 30 min then activated by Thrombin (Thr, 50 mU/ml, 2 min) and lyzed for Western blotting. Actin blot was used as a loading control. (A, B) Quantitative analysis of Akt (Ser473), PI3K (Tyr199/Tyr458), Erk1/2 (Thr202/Tyr204), and p38 (Thr180/Tyr182) phosphorylation. The intensity of the p-Akt, p-PI3K, p-p38, p-Erk1/2 signal was normalized to the actin signal. For each sample, this ratio is relatively expressed to the ratio of control, which is presented as 1. Data are presented as means ± S.D. (one-way ANOVA, Leven's test $p < 0.05$ followed by Tamhane's T2 test. *–$p < 0.05$ compared with the activator, *– $p < 0.05$, $n = 4$). (C, D) Representative blots from four independent experiments.

2005). Widely used 12-LOX inhibitors Baicalein (Yeh et al., 2015; Tian et al., 2018), nordihydroguaiaretic acid (Guzman-Beltran et al., 2008; Lu et al., 2010), 5,8,11, 14-eicosatetraynoic acid, and 5,8,11-eicosatriynoic acid (Takami et al., 2000) demonstrated antioxidant activity in different cells types. Ipso facto, we tested whether ML355 affects ROS level in activated platelets. For this purpose, we used cell permeable DCF-DA dye that acquire fluorescent signal after oxidation by ROS. We observed that ML355 decreased ROS level in platelets activated by thrombin (0.1 U/ml) in a dose-dependent manner, achieving the reduction of ROS to control levels at 50 μM of ML355 (Fig. 4).

3. ML355 Induces cAMP Dependent VASP Phosphorylation in Platelets. Platelet inhibition is mainly mediated by two inhibitory pathways, acting through the activation of adenylyl (AC) and guanylyl (GC) cyclases and subsequent cyclic adenosine (cAMP) and guanosine (cGMP) monophosphate synthesis. cAMP and cGMP activate effector kinases A (PKA) and G (PKG), respectively, which are responsible for inhibition of the key signaling events in platelet activation (Smolenski, 2012). Activity of AC/cAMP/PKA and GC/cGMP/PKG pathways in platelets can be monitored by the phosphorylation of a major substrate protein Vasodilator-stimulated phosphoprotein (VASP). PKA preferentially phosphorylate the Ser157 position, whereas PKG prefers Ser239. However, strong PKA or PKG activation may lead to phosphorylation of both positions (Butt et al., 1994). As shown in Fig. 5A, high doses of ML355 (50 μM) induced VASP phosphorylation at Ser157 (Fig. 5A). To determine whether the AC/cAMP/PKA pathway is engaged in ML355-induced phosphorylation of VASP at Ser157, platelets were preincubated with AC inhibitor SQ22563 prior to ML355 treatment. Our data demonstrated that SQ22563 strongly inhibited VASP Ser157 phosphorylation induced by ML355 (Fig. 5B), supporting the notion that ML355 affects the AC/cAMP/PKA pathway.

ML355 also resulted in the phosphorylation of VASP at Ser 239 (Fig. 5C), which can be due to the engagement of GC/cGMP/PKG pathway or a potent activation of adenylyl cyclase. To probe for the involvement of GC/cGMP/PKG pathway, we evaluated VASP phosphorylation at Ser 239 after the treatment of platelets with ODQ. ODQ is a heme-oxidizing agent that blocks the activation of GC-1 by its natural activating ligand nitric oxide (NO). This inhibitory action of ODQ on NO-stimulated GC-1 is clearly shown in Fig. 5C, where the strong sodium nitroprusside-induced phosphorylation of Ser239 is significantly prevented by ODQ treatment. Although NO binding to ferrous heme moiety of GC-1 is the main physiologic mean of GC-1 activation, other NO-independent allosteric regulators of GC-1 have also been described. Some of these allosteric regulators increase the activity of GC-1 containing ferrous heme, whereas other compounds activate preferentially GC-1 with oxidized ferric heme (Sandner et al., 2021). As shown in Fig. 5C, the ML355-induced phosphorylation of Ser 239 was substantially enhanced by ODQ. NO- and heme-independent activators of GC-1, such as cinaciguat (Sandner et al., 2021) or gemfibrozil (Sharina et al., 2015) exhibit similar patterns of VASP phosphorylation. Therefore, the possibility that ML355

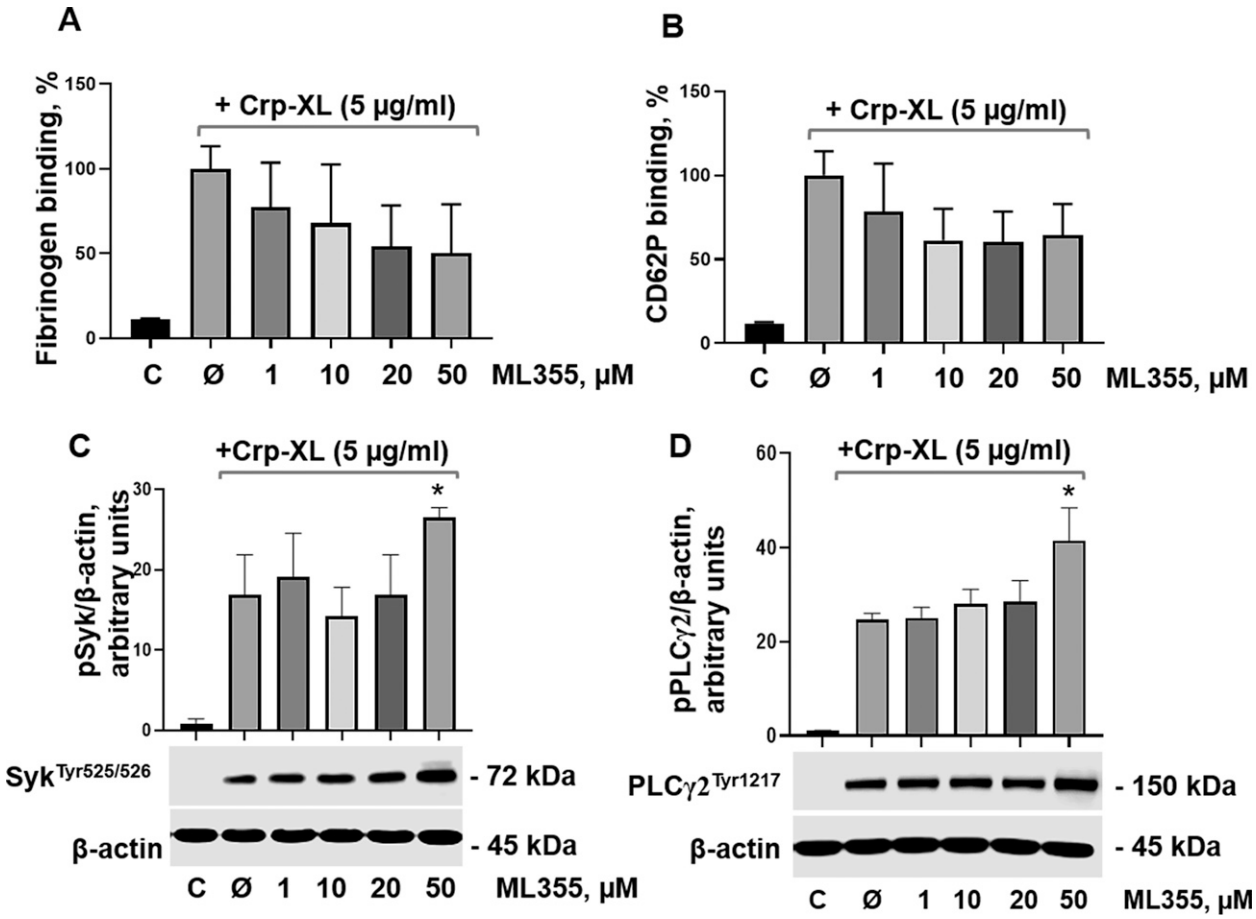

Fig. 3. ML355 does not inhibit Crp-XL induced platelet activation. Washed human platelets (1×10^8/ml for flow cytometry and 6×10^8/ml for Western blotting) were incubated with ML355 for 30 min at indicated concentrations and then activated by Crp-XL. (A, B) Flow cytometry analysis of platelet αIIbβ3 integrin activation (Fibrinogen Alexa-647 binding) and P-selectin exposure (CD62P-PE binding). Fibrinogen (15 μg/ml) was added 15 min before Crp-XL (5 μg/ml, 2 min) administration. CD62P-PE was added (1:10) directly after the activation and incubated with platelets during 10 min before measuring. For both integrin activation and P-selectin externalization analysis the reaction was stopped by dilution in PBS buffer (1:40). Data are presented as means ± S.D., Crp-XL induced Fibrinogen binding was designated as 100% (one-way ANOVA, Leven's test $p > 0.05$ followed by Tukey's HSD test, $n = 6$). (C, D) Western blot analysis of Syk and PLCγ2 phosphorylation. Platelets were incubated with indicated doses of ML355 for 30 min then activated by CRP-XL (5 μg/ml, 2 min) and lyzed for Western blotting. Actin blot was used as a loading control. The intensity of the p-Syk or PLCγ2 signal was normalized to the actin signal. For each sample, this ratio is relatively expressed to the ratio of control, which is designated as 1. Data are presented as means ± S.D. (Mann-Whitney *U* test, *– $p < 0.05$ – compared with Crp-XL (Ø), $n = 3$).

may have some off-target effects and may act as a GC-1 upregulating agent should be considered. To test this possibility, we evaluated the effect of ML355 on the activity of purified GC-1 containing either native ferrous or oxidized ferric forms of heme. As shown in Fig. 6A, even 100 μM ML355 did not affect the activity of either state of GC-1. Moreover, 100 μM ML355 did not affect the activity of GC-1 treated by GC-1 allosteric stimulator BAY41-2272 or the NO donating compound DEA-NO (Fig. 6B). These data clearly demonstrate that ML355 does not affect directly the activity of GC-1. Although the hypothesis of direct GC-1 activation was rejected by biochemical analysis, it remained possible that ML355 undergoes intracellular biotransformation, and the products of such transformation activates the cGMP-forming activity of GC-1. To test this possibility, we evaluated the effect of ML355 on vasorelaxation of preconstricted mouse aortic rings. Relaxation of blood vessels is a well recognized effect of GC-1-mediated increase of intracellular cGMP. As shown in Fig. 6C, doses as high as 300 μM ML355 failed to cause relaxation of phenylephrine-constricted mouse aorta. Moreover, combined treatment with 300 μM ML355 and 10 μM ODQ did not cause any vasorelaxation, just an additional constriction caused by blocked NO-dependent activation of GC-1. In combination, these data strongly support the notion that ML355-induced phosphorylation of VASP is not associated with the GC/cGMP/PKG pathway in platelets.

4. ML355 Does Not Affect Platelet Viability and Inhibits ABT-737 Induced Apoptosis in Platelets. 12-LOX inhibition was shown to be associated with induction of apoptosis in several cancer cell lines (Wong et al., 2001; Pidgeon et al., 2002; Xu et al., 2012). Therefore, we tested whether ML355 affects platelet viability. Platelet viability was assessed using the membrane-permeable dye Calcein-AM. In living cells intracellular esterases cause the cleavage of the acetomethyl ester group of Calcein-AM, resulting in increased green fluorescent signal (De Clerck et al., 1994; Fritzsche and Mandenius, 2010). We found that ML355 did not affect the Calcein-AM signal in platelets (Fig. 7A), nor did it affect caspase-3 cleavage (Fig. 7B), another apoptotic marker. These data indicate that ML355 did not cause any apoptosis-associated events. On the contrary, at higher doses (50 μM), ML355 prevented caspase-3 cleavage induced by the Bcl-2 family

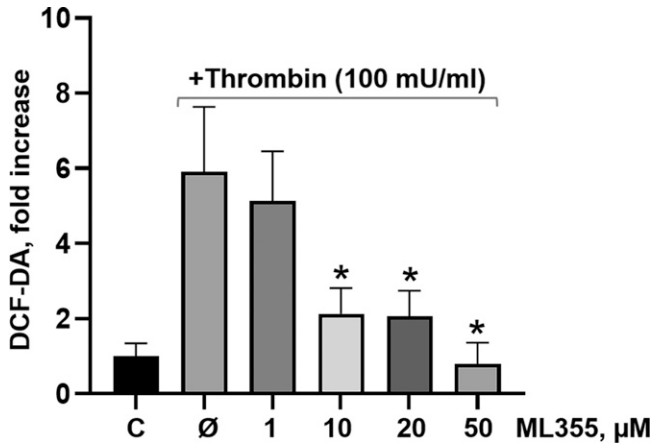

Fig. 4. ML355 inhibits ROS formation in thrombin-activated platelets. Washed human platelets (1×10^8/ml) were incubated with ML355 at indicated concentrations and DCF-DA (10 μM) for 30 min, then Thrombin (100 mU/ml) was added for additional 2 min and the reaction was stopped by dilution in PBS buffer (1:40). Data are presented as means ± S.D., (one-way ANOVA, Leven's test $p > 0.05$ followed by Tukey's HSD test, *– $p < 0.05$ compared with thrombin (Ø), the control mean was designated as 1, n = 6).

protein inhibitor ABT-737 (Fig. 7C). Moreover, ML355 dose-dependently blocked ABT-737 induced PS exposure on platelet surface (Fig. 7D).

Discussion

The growing body of evidence that 12-LOX is implicated in a wide range of pathologic conditions leads to a necessity of comprehensive analysis and characterization of 12-LOX's role in cellular metabolism. 12-LOX is involved in normal homeostasis and cell migration, but it can also promote cancer cell proliferation, atherosclerosis, and diabetes (Zheng et al., 2020). Several recent reviews addressed the therapeutic potential of 12-LOX in anticancer and antiplatelet therapy (Yeung and Holinstat, 2011; Mashima and Okuyama, 2015; Tourdot and Holinstat, 2017; Clemente et al., 2020). However, the exact mechanisms by which 12-LOX regulate cell metabolism are still poorly understood and remain a matter of debate. The main problem of 12-LOX investigation was associated with poor specificity of existing 12-LOX inhibitors. For example, one of the first described 12-LOX inhibitors, 4,6-10-13-eicosatetrayonic acid, can affect the activity of other LOXs in different cells (Yeung and Holinstat, 2011). Nordihydroguaiaretic acid and baicalein are known not only as 12-LOX inhibitors, but also as strong ROS scavengers (Floriano-Sanchez et al., 2006; Czapski et al., 2012). In addition, baicalein can inhibit reticulocyte 15-LOX with higher efficacy than platelet 12-LOX (Deschamps et al., 2006). Most of these inhibitors can modulate cyclooxygenase-1 (COX-1) activity (Tourdot and Holinstat, 2017), which also participates in AA metabolism and promotes platelet activation (Rukoyatkina et al., 2018).

ML355 was described as a potent and stable 12-LOX inhibitor with excellent selectivity against other related enzymes (Luci et al., 2014; Tsai et al., 2021). In studies performed on purified enzymes, ML355 exhibited a submicromolar IC_{50} (0.34 μM) for 12-LOX. It was reported that ML355 does not affect the activity of COX-1 or COX-2 activity and that ML355 possesses at least 100-fold reduced affinity for 15-LOX-2 or 5-LOX and 28-fold reduced affinity for 15-LOX-1. However, when functional tests were performed with intact cells or platelets, the effective doses of ML355 were higher. E.g., inhibition of 12-hydroxyeicosatetraenoic acid (12-HETE) synthesis in mouse β-cells (BTC3) and human primary donor islets required higher ML355 doses, and 80% inhibition was achieved only at 20 μM (Luci et al., 2014). Experiments performed *in vitro* in human platelets and *in vivo* in mice demonstrated that in the 25–100 μM range ML355 acted as an antiplatelet agent and inhibited thrombin-induced platelet aggregation, attenuated platelet adhesion, and aggregation on collagen matrix and blocked thrombus formation without adverse effects on hemostasis *in vivo* (Adili et al., 2017). Similarly, Luci and colleagues (2014) observed full inhibition of human platelet aggregation only at 50 μM. These previous reports are consistent with our current study, which demonstrates that potent inhibition of αIIbβ3 activity also occurs only at 50 μM of ML355. However, the molecular mechanisms that mediated these effects of ML355 on platelets were not clear. In the current study, we demonstrated that ML355 inhibits thrombin- or TxA_2-induced platelet integrin αIIbβ3 activation and α-granule secretion. The inhibition was mediated by the blockage of the key activation proteins Akt, PI3K, and Erk1/2 phosphorylation, which play an important role in αIIbβ3 activation, Ca^{2+} signaling, and platelet dense granule release (Guidetti et al., 2015). At the same time, ML355 did not affect p38 kinase phosphorylation, which is also involved in platelet activation and is activated in response to a variety of agonists, such as thrombin, TxA2, collagen, ADP, and von Willebrand factor (Adam et al., 2008). p38 kinase was shown to be engaged in platelet adhesion and spreading over collagen or fibrinogen matrix under blood flow conditions (Mazharian et al., 2005, 2007). However, according to Adili et al. (2017), ML355 impairs platelet adhesion to collagen matrix, suggesting that ML355 inhibits platelet adhesion via mechanisms unrelated to p38 kinase activity.

Surprisingly, ML355 did not inhibit platelet αIIbβ3 activation and α-granule secretion induced by Crp-XL. Crp-XL binds to and activates the GPVI receptor, which is a member of the Ig receptor superfamily. It consists of two extracellular Ig chains, both of which are associated with two fragment crystallizable region receptor (FcR) γ-chains containing an immunoreceptor tyrosine-based activation motif (Moroi and Jung, 2004; Clark et al., 2021). GPVI signaling is mediated by recruitment to the intracellular domain and activation of Src family kinases that phosphorylate immunoreceptor tyrosine-based activation motif domain in the GPVI receptor (Watson et al., 2010). Syk phosphorylation leads to PLCγ2 activation, which is indispensable for Ca^{2+} mobilization, cytoskeletal rearrangement, and PS externalization in activated platelets (Dutting et al., 2012). Previously, it was shown that some 12-LOX inhibitors (baicalein, NCTT-956, and 694) prevent GPVI-mediated platelet activation (Yeung et al., 2013). ML355 also was reported to inhibit platelet reactivity induced by FcγRIIa activation (Yeung et al., 2014). However, the effect of ML355 on GPVI-mediated platelet activation has not yet been described. Therefore, we evaluated ML355 effects on well established GPVI signaling events, including Syk and PLCγ2 phosphorylation. In our experiments, ML355 did not inhibit Syk (Tyr525/526) or PLCγ2 (Tyr1217) phosphorylation induced by Crp-XL, and at high doses (50 μM) even potentiated the phosphorylation. All these data indicate that 12-LOX signaling in platelets is very

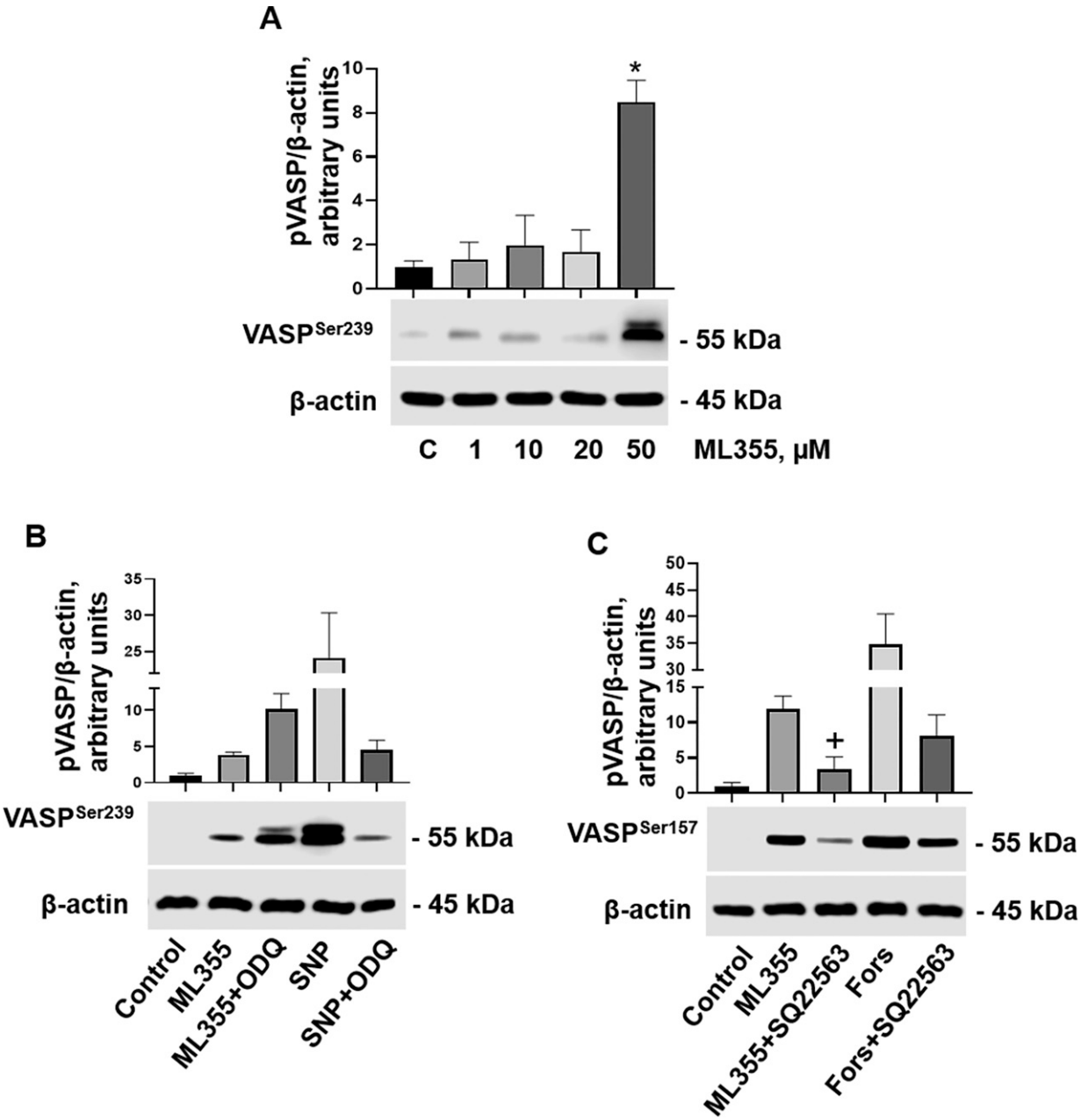

Fig. 5. ML355 activates AC/cAMP/PKA inhibitory pathway in platelets. Western blot analysis of VASP phosphorylation. (A) Washed human platelets (3×10^8/ml) were incubated with ML355 for 30 min at indicated concentrations and lyzed for Western blotting. (B, C) Washed platelets (3×10^8/ml) were preincubated with ODQ (20 μM) or SQ22563 (100 μM) during 10 min and then ML355 (50 μM, 30 min) was added. Sodium nitroprusside (SNP,1 μM) and Forskolin (1 μM) were used as positive controls. For Western blotting analysis, platelets were lyzed and analyzed for Ser157 or Ser239 VASP phosphorylation. Actin blot was used as a loading control. The intensity of VASP signal was normalized to the actin signal. For each sample, this ratio is relatively expressed to the ratio of control, which is designated as 1. Data are presented as means ± S.D. (Mann-Whitney *U* test, *– $p < 0.05$ – compared with control, + - $p < 0.05$ – compared with ML355, (A) n = 4, (B, C) n = 5).

complex and needs future investigations. At the same time, 50 μM of ML355 caused strong VASP (Ser157/239) phosphorylation in platelets, which was blocked by AC inhibitor SQ22563, but not GC-1 inhibitor ODQ. In addition, experiments with purified GC-1 and vasorelaxation of preconstricted mouse aortic rings showed no ML355 effect on GC/cGMP/PKG pathway activity. These data clearly indicate AC/cAMP/PKA activation in ML355-treated platelets. A recent report demonstrated the dual role of cyclic nucleotides and corresponding protein kinases in echicetin mediated glypoprotein GPIbα signaling (Makhoul et al., 2019). In echicetin stimulated platelets, PKA and PKG activation led to inhibition of platelet aggregation, but strongly potentiated Syk (Tyr525/526) phosphorylation. According to these data, the increase of Syk phosphorylation observed in our current study can be associated with ML355 induced PKA activation. It should be mentioned, that the mechanism of PKA activation by high doses of ML355 remains unclear and should be further investigated. During AA metabolism, COX-1 contributes to the synthesis of TxA_2 and E_2, $F_{2\alpha}$, I_2, D_2, E_2 prostaglandins (Braune et al., 2020). Activation of some prostaglandin receptors on platelets was shown to cause elevated cAMP and subsequent PKA activation in platelets

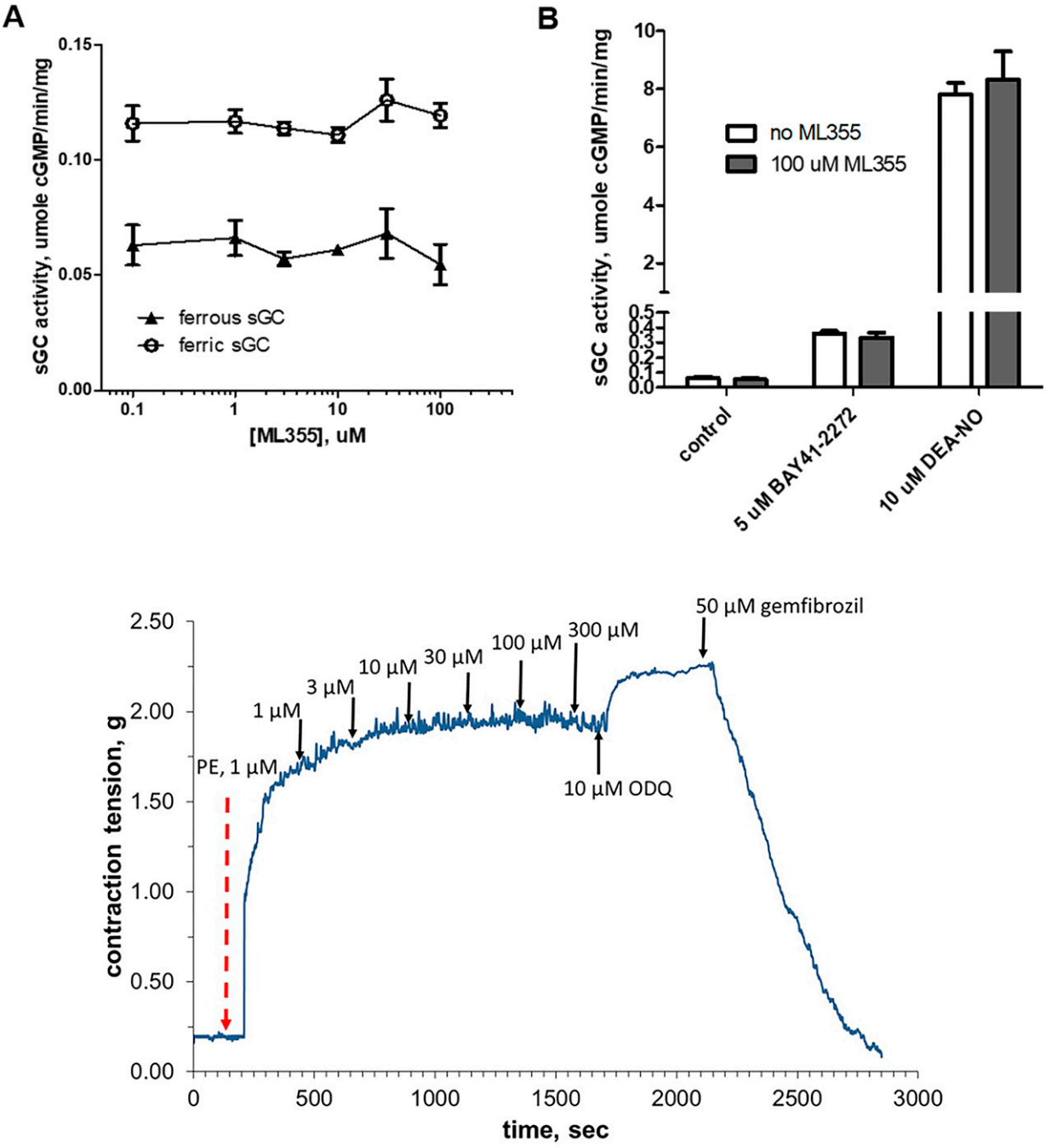

Fig. 6. ML355 does not activate recombinant GC-1 enzyme and lacks vasodilatory properties. (A) Purified human GC-1 (sGC) containing ferrous or ferric heme was preincubated with different concentrations of ML355 and then tested for cGMP-forming activity using the [^{32}P]GTP assay. Data are mean ± S.D. (n = 6). (B) The effect of 100 µM ML355 on the cGMP-forming activity of recombinant ferrous GC-1 (sGC) activated by indicated doses of BAY41-2272 or DEA/NO. Data are mean ± S.D. (n = 6). ns – indicates the difference is not significant. (C) Changes in isometric tension of isolated mouse aortic rings in response to ML355. Mouse aortic rings were isolated and prepared as described in *Methods*. After the contraction induced by 1 µM phenylephrine stabilized, cumulative doses of ML355 were added to the organ bath and changes in isometric tension were recorded. Data are mean ± S.D. (n = 4). *- $P < 0.05$ versus control (*t* test). (D) A representative wire myograph showing changes in isometric tension in response to phenylephrine, ML355, ODQ, and gemfibrozil. Arrows point to when indicated amounts of phenylephrine (PE), ML355, or ODQ were added. 10 µM ODQ was added to test the effect of ML355 on ferric GC-1-dependent vasodilation. Gemfibrozil was added to confirm vessel's ability to relax.

(Hubertus et al., 2014). ML355-induced PKA activation can be mediated by an unspecific action of high doses of ML355 or it can be related to strong 12-LOX inhibition in platelets. In turn, this could lead to an increase of processing of AA by COX-1 and subsequent prostaglandins formation, leading to PKA activation.

Another mechanism involved in platelet inhibition by ML355 was ROS formation blockage. In our experiments, ML355 strongly decreased the level of ROS in platelets activated by thrombin. As mentioned above, some of the potent 12-LOX inhibitors are also efficient ROS scavengers (Florian-Sanchez et al., 2006; Czapski et al., 2012). The strong decrease

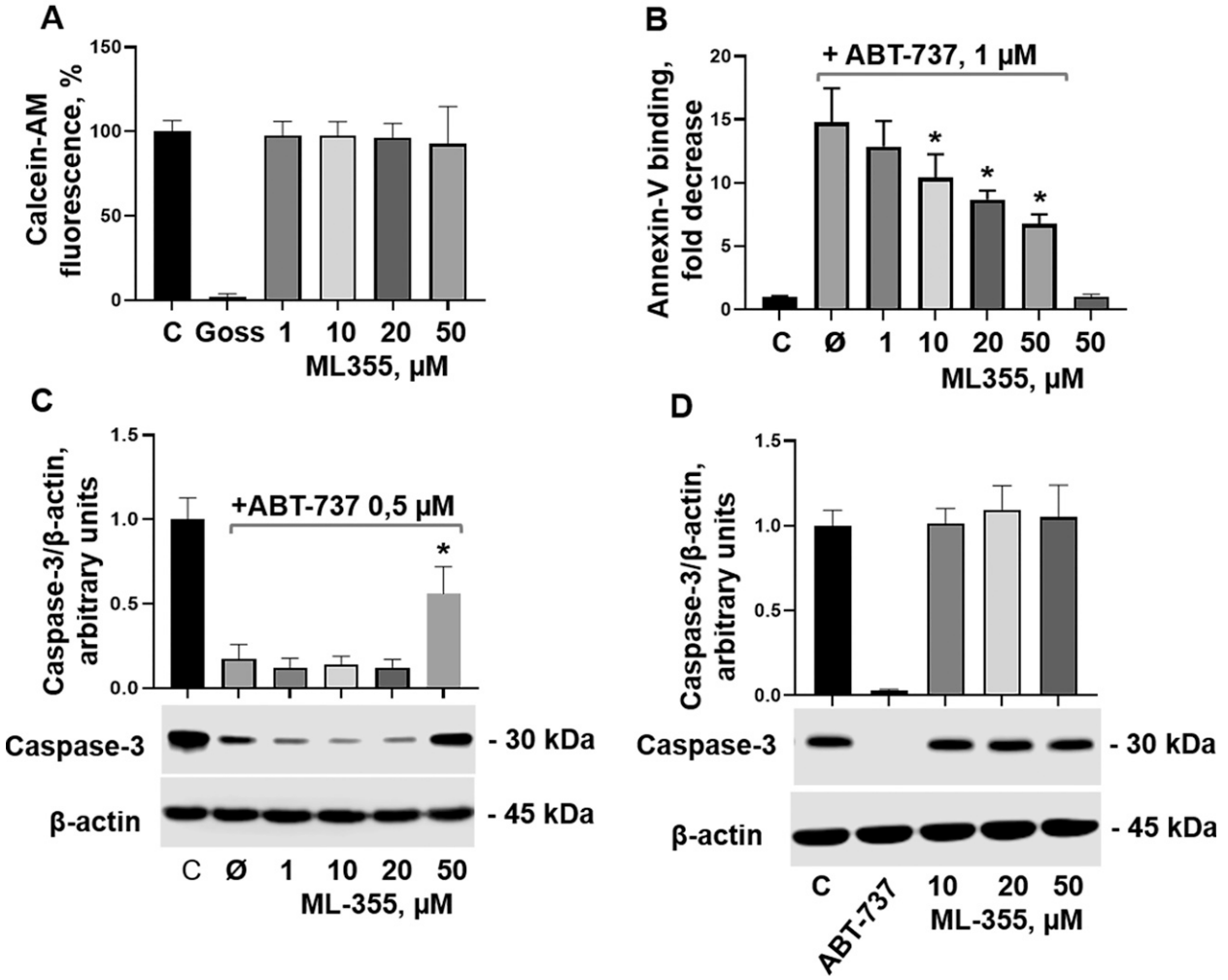

Fig. 7. ML355 inhibits ABT-737 induced platelet apoptosis. Flow cytometry analysis of (A) calcein-AM fluorescence, (B) PS externalization (Annexin-V-PE binding) and (C, D) Western blot analysis of caspase 3 activation. Washed human platelets (1×10^8/ml for flow cytometry and 3×10^8/ml for Western blotting) were incubated with the indicated doses of ML355 during 30 min. (A) Platelets were incubated with calcein-AM (1 μM) during 30 min before the measuring. Gossypol (30 μM, 10 min) was used as a positive control. (B) ML355 and ABT-737 (0.5 μM) were added simultaneously and after 60 min of incubation Annexin-V-PE (1:10) and Annexin-binding buffer (1:20) were added, then platelets were incubated during 5 min and analyzed by flow cytometry. Data are presented as means ± S.D., (one-way ANOVA, Leven's test $p > 0.05$ followed by Tukey's HSD test. *– $p < 0.05$ compared with ABT-737 (Ø), the control meaning was designated as 100% in (A) and as 1 in (B), n = 6). (C, D) ML355 (50 μM) and ABT-737 (0.5 μM) were added simultaneously and after 60 min of incubation platelets were lyzed for Western blot analysis. The intensity of caspase 3 signal was normalized to the actin signal. For each sample, this ratio is relatively expressed to the ratio of control, which is taken as 1. Data are presented as means ± S.D. (Mann-Whitney *U* test. *– $p < 0.05$ – compared with ABT-737 (Ø), in (C) n = 3, in (D) n = 4).

in ROS due to 12-LOX inhibitors can also be related to the fact that the processing of AA by 12-LOX results in metabolites that may activate NADPH oxidase (Cho et al., 2011).

Although platelets are anucleated, they contain a whole range of proteins controlling the mitochondrial apoptosis pathway (Lebois and Josefsson, 2016). Therefore, we tested whether ML355 can induce platelet apoptosis or decrease their viability. Data presented in this report demonstrated that ML355 does not affect platelet viability. Furthermore, we demonstrated that ML355 inhibits apoptotic processes induced in platelets by Bcl-2 family protein inhibitor ABT-737. Unraveling the exact mechanism(s) underlying these anti-apoptotic effects will require a separate investigation in the future.

The overexpression of ALOX12 gene, which codes for the arachidonate 12-lipoxygenase, is often a feature of cancer cells (Tomita et al., 2019). ALOX12 overexpression was shown to promote cancer metastasis (Zhong et al., 2018; Mi et al., 2020), epithelial-mesenchymal transition (Zhong et al., 2018; Yang et al., 2019), tumor growth, and cancer cell proliferation (Guo et al., 2011a). Conversely, the inhibition of 12-LOX decreases cancer cell proliferation and induces apoptosis of different cancer cell lines (Wong et al., 2001; Xu et al., 2012). Considering the anti-apoptotic effect of ML355 observed in anucleated platelets, further studies are needed to determine the contribution of ML355 to the apoptotic processes in nucleated primary or cancer cell lines.

In conclusion, our data demonstrated that prevention of platelet activation by ML355 is mediated by inhibition of several pathways involved in platelet activation. In addition, ML355 can modulate platelet apoptosis induced by Bcl-2

inhibitor. These findings may be of great benefit for the design of optimized antiplatelet and anticancer treatments.

Authorship Contributions

Participated in research design: Shpakova, Rukoyatkina, Kharazova, Gambaryan, Martin.

Conducted experiments: Shpakova, Al Arawe, Prilepskaya, Sharina.

Performed data analysis: Shpakova, Kharazova, Sharina, Martin.

Wrote or contributed to the writing of the manuscript: Shpakova, Rukoyatkina, Gambaryan, Martin.

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