

Online Supplements

Metabolic enzyme pyruvate kinase M2 regulates platelet function and arterial thrombosis

Running title: *Nayak et al.: Targeting PKM2 inhibits thrombosis*

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MATERIALS AND METHODS

Materials

ML265 and Glycolysis assay kit was purchased from Cayman. Apyrase, EGTA (ethylene glycol tetraacetic acid), prostaglandin I₂ (PGI₂), thrombin, PAR4, TRAP and protease inhibitors were purchased from Sigma (St. Louis, MO, USA). 14 C-2-deoxy-glucose was purchased from PerkinElmer. Collagen, ADP and chronolume reagent were purchased from Chronolog Corporation (Havertown, PA, USA). Convulxin was purchased from SantaCruz Biotech. Primary antibodies Phospho Akt Ser473 (#9271, 1:1000), total Akt (#4691, 1:1000), Phospho GSK3 β Ser9 (#9322, 1:1000), total GSK3 β (#5676, 1:1000), and goat anti-rabbit IgG, HRP-linked secondary antibody (#7074, 1:2500) were from Cell Signaling Technologies. CD62P-FITC and PAC1 antibody and FITC Annexin V apoptosis detection kit I were purchased from BD Biosciences. Calcein-AM was from Molecular Probes (Invitrogen). Super Signal West Pico

chemiluminescent substrate and PVDF membrane were the products from Thermo Scientific and Millipore, respectively. All other reagents were of analytical grade.

Methods

Human and mouse platelet isolation

Human blood was collected from healthy volunteers free from antiplatelet medication. Informed consent was taken. Human platelets were isolated as described previously¹⁻³. Blood from anesthetized mice was drawn from the retro-orbital plexus and collected in 1.5 mL polypropylene tubes containing enoxaparin (0.3 mg/mL; Sanofi-Aventis, US LLC). Mouse platelets were isolated as described previously³⁻⁶.

In vitro platelet aggregation and ATP secretion

Platelet rich plasma (PRP) or washed platelets (2×10^8 / ml) treated with vehicle (DMSO) or ML265 (50 and 100 μ M) were stirred (1200 rpm) at 37⁰ C for 2 min in a whole blood/optical lumi-aggregometer (Chrono-log; model 700–2) before the addition of agonists (convulxin, collagen, thrombin, PAR4, TRAP (PAR1 agonist) or adenosine diphosphate [ADP]).

Aggregation was measured as percent change in light transmission, where 100% refers to transmittance through the blank sample (PRP/Buffer). Platelet dense granule secretion was determined by measuring the release of ATP using luciferin-luciferase reagent. To examine the effect of ML265 on ATP secretion, washed platelets were pretreated with vehicle (DMSO) or ML265 (50 and 100 μ M) for 10 minutes before stimulation with various agonists. To

determine the total ATP in dense granules, resting platelets were treated with non-ionic detergent digitonin (50 μ M) to lyse the cells. ATP release was performed in a lumi-aggregometer (Chrono-log; model 700–2) at 37°C.

FeCl₃ injury-induced carotid thrombosis

Thrombus formation in the carotid artery after the FeCl₃ injury was assessed by intravital microscopy as described previously³⁻⁶. Briefly, 8-10 weeks old mice were anaesthetized using 100-mg/kg ketamine and 10-mg/kg xylazine. Platelets labelled with calcein green (2.5×10^9 platelets per kg) were infused through the retro-orbital plexus. The common carotid artery was carefully exposed and kept moist by superfusion with warm (~37°C) saline. Whatman paper (0.5 X 1.5 mm) saturated with ferric chloride (5%) solution was applied topically for 1.5 minutes for male and 2 mins for female mice, and thrombus formation in the injured carotid vessel was monitored in real-time using a Nikon upright microscope (Plan Fluor 4X/0.2 objective), and thrombus growth overtime was recorded using a high-speed electron-multiplying camera for 40 minutes or until occlusion occurred. The time to form occlusive thrombus was considered as the time required for blood to stop flowing completely for >1 minute. Videos were evaluated off-line using a Nikon computer-assisted image analysis program.

Laser injury-induced mesenteric artery thrombosis

Micropoint laser ablation system (Andor Technology) was used to make injury in the mesenteric arterioles as described previously^{3,6,7}. Briefly, young mice [3- to 4-weeks (14-16 gm) old male)] were used to minimize fat surrounding the arterioles and facilitate the focusing of the laser.

Fluorescent platelets labelled with calcein green (1.5×10^9 platelets kg^{-1}) were infused in anesthetized mice through the retro-orbital plexus. Infused platelets were isolated from adult (4-5 months) donor mice of the same genotype. Mesenteric arterioles having a diameter of approximately 80-100 μm (with shear rates of ~ 1300 - 1800 s^{-1}) were used for the study. The specific illumination of the area of interest was carried out through the microscope eyepiece. The wavelength of light in the range of 365-400 nm with the maximum output of 50–500 μJ was used for illumination. The power and frequency of pulses were regulated by software and empirically defined. Thrombus growth in the injured vessel was monitored in real-time by using a Nikon upright microscope with a Plan Fluor 10X/0.3 objective, and thrombus formation overtime was recorded using a high-speed EM camera for 3-4 min. In our experimental setup with the laser injury model, the thrombus grows to its maximum size in approximately 1 min, and then gradually disintegrates over time. Videos were evaluated and mean fluorescence intensity was calculated using a Nikon computer-assisted image analysis program.

Glucose uptake Assay

Glucose uptake was determined in activated versus non-activated platelets using 10 mM ^{14}C -2-deoxy-glucose as described ⁸. Washed platelets (2×10^8 cells/mL) treated with vehicle (DMSO) or ML265 (50 and 100 μM) were incubated in 1X Tyrode buffer and stimulated with convulxin or thrombin for 10 minutes, placed on ice and then centrifuged at 4000 rpm for 5 minutes. The pellets were washed with cold 1X PBS and solubilized in 1N NaOH. Radioactive counts were determined using a Perkin Elmer TriCarb 2800TR liquid scintillation counter.

Lactate measurement

Washed platelets (5×10^8 cells/mL) in DMEM (5mM HEPES, 25 mM glucose, 1 mM pyruvate and 2 mM glutamine) were pre-treated with 50 or 100 μ M of ML265 or vehicle (DMSO) for 10 mins and stimulated by convulxin (200 ng/ml) for 1 hr. Cells were centrifuged and supernatant was collected. Levels of L-lactate in supernatant were determined using a glycolysis cell-based assay kit (Cayman; Item No. 600450) according to the manufacturer's instructions.

Integrin α IIb β 3 activation and P-selectin exposure

Mouse platelets (1×10^8 cells in 100 μ l) pre-incubated for 10 min either in presence or absence of ML265 (50 and 100 μ M) were stimulated with convulxin or PAR-4 without stirring. Next, platelets were labeled with FITC conjugated anti-CD62P or PE conjugated JonA antibody as described⁹. Similar protocol was followed for the evaluation of α IIb β 3 activation and P-selectin expression on platelets isolated from PKM2^{fl/fl} and PKM2^{fl/fl}PF4Cre⁺ mice. In case of human, platelets (1×10^8 cells in 100 μ l) were pre-incubated for 10 min either in presence or absence of ML265 (50 and 100 μ M) and stimulated with convulxin or TRAP without stirring. Next, platelets were labeled with FITC conjugated PAC-1 or PE conjugated anti-CD62P antibody. The platelet samples were incubated for 30 min in dark at room temperature and analyzed using flow cytometer (FACS Calibur, Becton Dickinson). An amorphous region (gate) was drawn to differentiate the CD61-positive platelets from the noise and multi-platelet particles. After compensation for FITC and PE, 10,000 events were collected for each sample.

Annexin V-affinity assay

Mouse and human washed platelets (1×10^8 / ml) were pre-incubated with different concentrations of ML265 (10, 25, 50, 150, 200 and 300 mM). Washed platelets activated with thrombin (0.5 U/ml) + convulxin (100 ng/ml) were used as a positive control. The samples were added to 1X Annexin V-binding buffer (BD Biosciences). Following this, annexin V-FITC antibody was added to each sample, gently mixed, and incubated at room temperature for 15 minutes in the dark. After incubation, 1X Annexin-binding buffer was added to each tube and analyzed by a Becton Dickinson FACSCalibur flow cytometer.

Clot retraction

To measure thrombin-stimulated fibrin clot retraction PRP was treated with ML265 (50 and 100 μ M) or vehicle control (DMSO) for 10 minutes. Tyrode buffer was added to test tubes, along with red blood cells, to allow visualization of the clot. This was followed by the addition of PRP. Clot formation was initiated by adding thrombin (final concentration 0.25 U/ml) to the test tubes. Photographs were taken every 15 minutes and the assay was terminated after 60 minutes at which time the clot in the vehicle-treated samples were seen to have retracted completely. Clot area was analyzed using Image J software from NIH (Bethesda, MD, USA).

Bioflux flow chamber assay

In vitro thrombosis assays were performed using BioFluxTM 200 (Fluxion Biosciences, USA) microfluidics flow chamber³. The channels were coated with Type I collagen (100 μ g /ml) for one hour at room temperature and then blocked with 0.5% BSA for 30 minutes. Platelets were isolated from citrated whole blood as described above, washed, labeled with the fluorescent dye

(calcein AM; 2 μ M) and were reconstituted back with PPP and remaining blood containing leukocytes and RBC. The whole blood pretreated with ML265 or vehicle (DMSO) was perfused over the collagen-coated plate at shear stress 1500 s⁻¹ for 5 minutes. Experiments were performed in triplicates using whole blood from human and mice. The fluorescently labeled platelets/thrombi on the collagen-coated surface were analyzed using ImageJ software from NIH (Bethesda, MD, USA).

ML265 preparation and administration for *in vivo* experiments

Initially ML265 dissolved in DMSO was mixed with 200 μ l of deionized water and administered intra-peritoneally to the at a dose of 25 and 50-mg/Kg body weight. *In vivo* thrombosis and tail transection assays were performed 10 minutes after administration of ML265 or vehicle infusion by a person who was blinded to the drug and vehicle infusion.

Tail bleeding assay

Tail-transection bleeding time was measured as described previously¹⁰. Briefly, mice (approximately 8 weeks of age) were anesthetized with 100-mg/kg ketamine and 10-mg/kg xylazine and placed on a heating pad warmed at 37°C, and a 3 mm segment of the tail was amputated with a sharp scalpel blade. The tail was immediately immersed in saline (at 37°C), and the time taken for the stream of blood to stop for more than 30 seconds was defined as the bleeding time. If bleeding did not stop within 10 minutes, hemostasis was achieved by cauterizing the tail.

Immunoblotting

Platelet proteins were separated on 4-20% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gradient gels and electrophoretic transferred to PVDF (polyvinylidene fluoride) membrane by using Bio-Rad western blotting system. For PKM2 dimer and tetramer study 6% native gels are used. Membranes were blocked with 5% BSA or skimmed milk in 10 mM Tris-HCl, 150 mM NaCl, pH 8.0 (TBS) containing 0.05% Tween-20 for one hour at room temperature. Blots were incubated for overnight with primary antibody, followed by horseradish peroxidase-labeled secondary antibody for one hour. Blots were developed using enhanced chemiluminescence and quantified using Image J software from NIH (Bethesda, MD, USA).

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Supplementary Table

	PKM2^{fl/fl}	PKM2^{fl/fl}PF4Cre+	P value
Body weight	25.49 ± 0.46	26.14 ± 0.54	0.2739
WBC (10³/μl)	9.31 ± 1.38	8.94 ± 0.93	0.6617
RBC (10³/μl)	10.01 ± 0.23	10.01 ± 0.26	0.9382
HGB (g/dl)	12.29 ± 0.47	12.11 ± 0.51	0.9808
HCT (%)	50.43 ± 1.08	50.00 ± 1.24	0.9999
Platelets (10³/μl)	1339 ± 107.6	1218 ± 54.44	0.4510
Neutrophil (10³/μl)	0.23 ± 0.03	0.18 ± 0.01	0.1667
Monocytes (10³/μl)	0.07 ± 0.01	0.11 ± 0.04	0.8042

Table S1: Body weight and complete blood counts from 8-10 weeks old mice were obtained using an automated veterinary hematology analyzer (ADVIA-120). Values are expressed as mean ± SEM. N=7-9 mice/group. P=Non-significant versus control PKM2^{fl/fl} mice.

Supplementary Figures

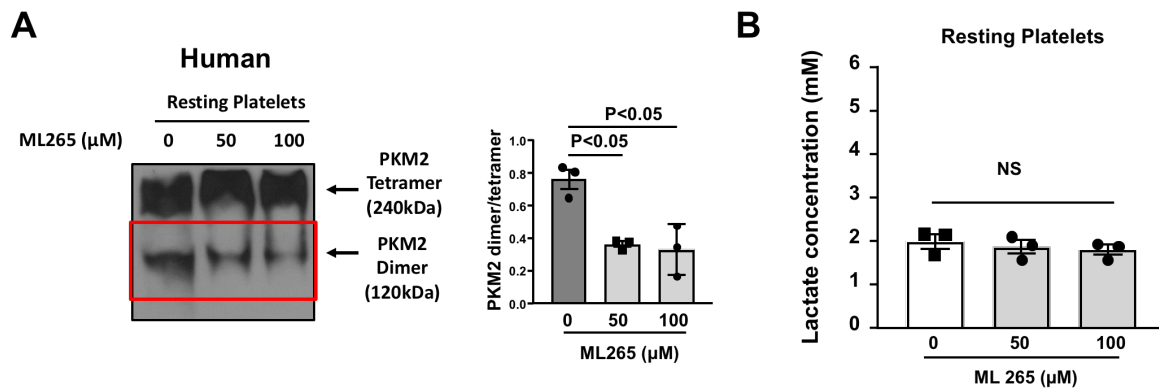


Figure S1. ML265 inhibits PKM2 dimerization in human resting platelets (A) The left panel shows representative Western blot, non-reducing (native), of PKM2 dimer and tetramer expression in human resting platelets pre-treated with vehicle or ML265. Red box denotes the PKM2 dimers. The right panel shows densitometry analysis. Values are mean \pm SEM, $n=3$ donors/group. **(B)** Effect of ML265 on lactate production in resting human platelets. Values are mean \pm SEM, $n=3$ donors/group. One-way ANOVA followed by Tukey's multiple comparisons test.

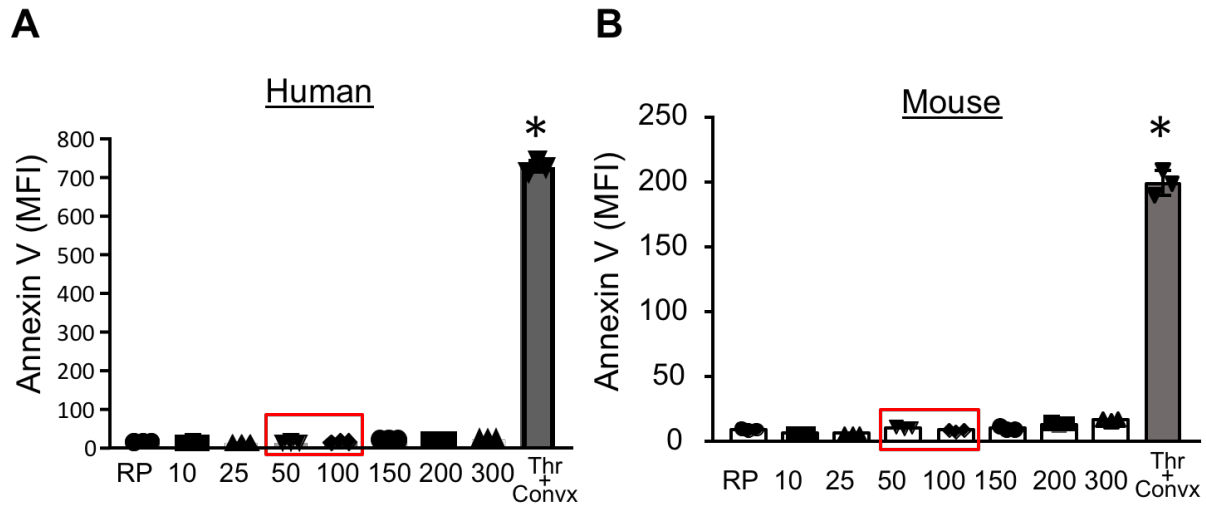


Figure S2. ML265 does not induce apoptosis in resting platelets. Annexin V binding in human (left panel, n=3 individual donors/group) and mouse (right panel, n=3 mice/group) platelets treated with increasing concentrations of ML265. Platelets stimulated with thrombin (0.1U/ml) and convulxin (100 ng/ml) was used as a positive control. Red boxes denote the concentration of ML265 used in most of the *in vitro* studies. *P< 0.05 vs. resting platelets. One-way ANOVA followed by Tukey's multiple comparisons test.

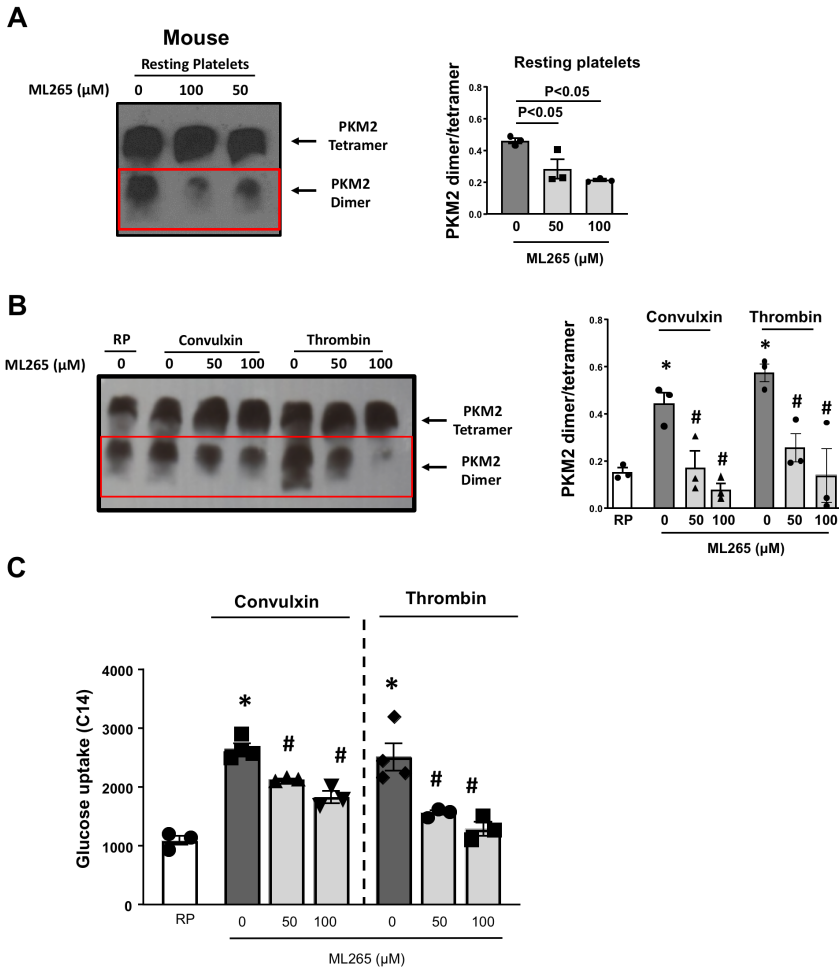


Figure S3. ML265 inhibits PKM2 dimerization and inhibits glucose uptake in activated mouse platelets. (A) The left panel shows representative Western blot, non-reducing (native), of PKM2 dimer and tetramer expression in resting platelets pre-treated with vehicle or ML265. Red box denotes the PKM2 dimers. The right panel shows densitometry analysis of immunoblots. Values are mean \pm SEM, $n=3$ mice/group. One-way ANOVA followed by Tukey's multiple comparisons test. (B) The left panel shows representative Western blot, non-reducing (native), of PKM2 dimer and tetramer expression in platelets pre-treated with vehicle or ML265 and stimulated with agonists including convulxin (100 ng/ml) or thrombin (0.1 U/mL). Red box denotes the PKM2 dimers. The right panel shows the densitometry analysis of immunoblots. Values are mean \pm SEM, $n=3$ mice/group. Two-way ANOVA followed by Tukey's multiple comparisons test. (C) Effect of PKM2 inhibition on glucose uptake in stimulated-platelets with convulxin (100ng/ml) and thrombin (0.1 U/ml). Values are mean \pm SEM, $n=3-4$ mice/group. $*P < 0.05$ vs. resting platelets; $\#P < 0.05$ vs. activated platelets (vehicle). Two-way ANOVA followed by Tukey's multiple comparisons test.

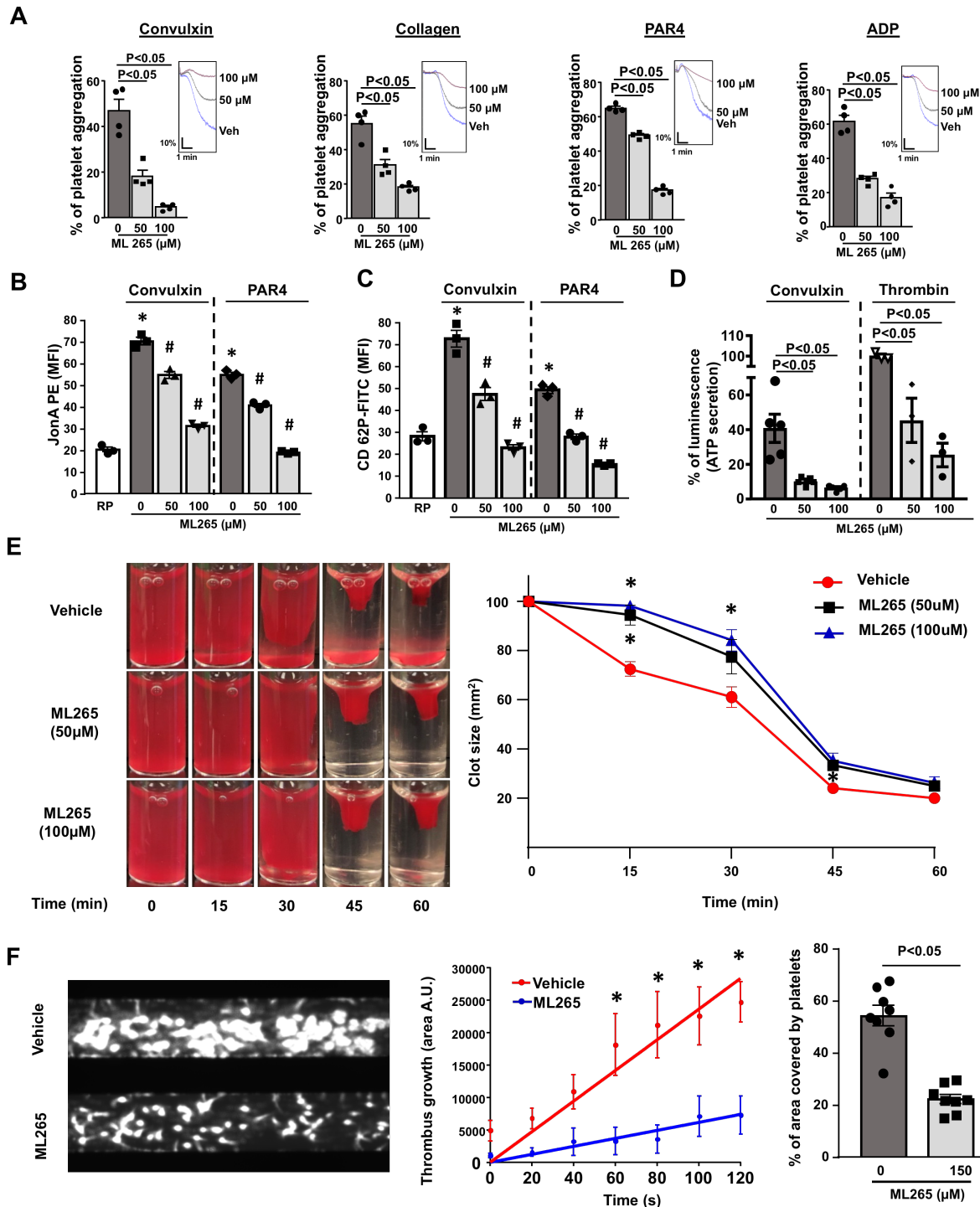


Figure S4. Dimeric PKM2 regulates multiple aspects of platelet function in murine platelets. (A) Mouse platelet-rich plasma pre-treated with vehicle or ML265 and stimulated with agonists including convulxin (50 ng/ml), collagen (1 μ g/ml), PAR4 (75 μ M) or ADP (5 μ M). Results are expressed as the percentage change in light transmission with respect to a blank (platelet-poor plasma/buffer without platelets), set at 100%. The upper panel in each bar graph denotes the representative aggregation curves (blue: control; black: 50 μ M ML265; red: 100 μ M

ML265). Values are mean \pm SEM, with n=4 mice/group. *P< 0.05 vs control. One-way ANOVA followed by Tukey's multiple comparisons test. **(B, C and D)** Effect of dimeric PKM2 inhibition on integrin α IIb β 3 activation, P-selectin surface expression, and ATP secretion from dense granules in platelets stimulated with convulxin (100 ng/ml), PAR4 (75 μ M) or thrombin (0.1 U/ml). Values are mean \pm SEM, n=3-5 mice/group. *P< 0.05 vs. resting platelets, #P< 0.05 vs. vehicle. Two-way ANOVA (B & C) and One-way ANOVA (D) followed by Tukey's multiple comparisons. **(E)** Clot retraction was measured for 1 hr. in platelet-rich plasma, supplemented with RBC, after adding 0.25 U/ml thrombin in the presence of a vehicle or ML265 (50 and 100 μ M). The left panels show representative images at different time points. The right panel shows the quantification of clot size with time. PRP was pooled from 5 mice in each group. Values are mean \pm SEM, n=3 experiments/group. Two-way ANOVA with Tukey's multiple comparison test. **(F)** Mouse whole blood pretreated with vehicle or ML265 (150 μ M) was perfused over a collagen-coated (100 μ g/mL) surface for 5 minutes at a shear rate of 1500 s⁻¹ in a flow chamber system from Bioflux Microfluidics. The left panel shows the representative image at the end of the assay. The middle panel shows the thrombus growth on collagen matrix over time. Slopes over time showed that the rate of thrombus growth in ML265 treated whole blood (slope: 61.65) was decreased when compared with vehicle control (slope: 236.1). Values are mean \pm SEM, with n=3 mice/group. * indicates P<0.05. Two-way ANOVA with Tukey's multiple comparison test. The right panel shows the surface area covered by fluorescent platelets after 5 minutes of perfusion. 3 to 4 areas from different areas of the flow chamber were analyzed from each blood sample, Values are mean \pm SEM, with n=8 areas/group. Mann-Whitney test.

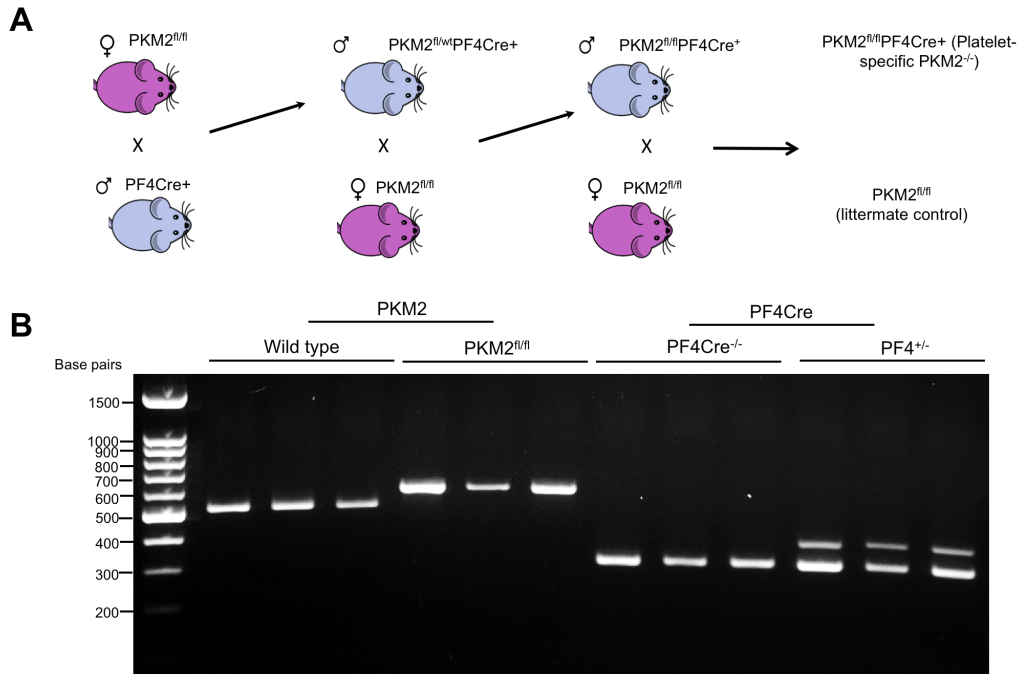


Figure S5. Generation of platelet-specific PKM2 deficient mice.

(A) Schematic showing the strategy to generate PKM2^{fl/fl};PF4Cre⁺ deficient mice .

(B) Genomic PCR confirming the presence of PF4Cre⁺ gene in PKM2^{fl/fl} mice. n=3/group.

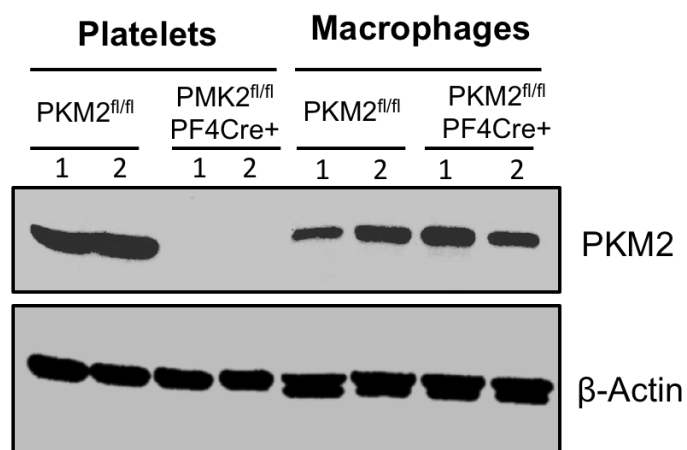


Figure S6. Confirmation of lack of PKM2 in platelets. Western blot confirming the specific deletion of PKM2 from platelets, but not peritoneal monocytes/macrophages. 1 and 2 are samples from individual mouse.

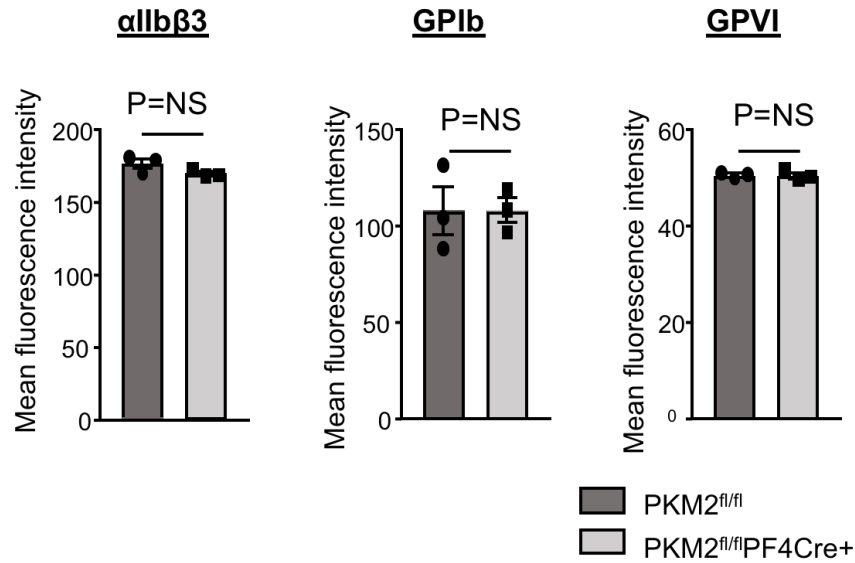


Figure S7. Expression of platelet adhesion receptors. The expression levels of α IIb β 3, GPIb, and GPVI were analyzed in platelets from PKM2^{fl/fl} or PKM2^{fl/fl}PF4Cre⁺ using flow cytometry. The results are expressed as mean fluorescence intensity (MFI). Values are expressed as mean \pm SEM, n=3 mice/group. Mann-Whitney test.

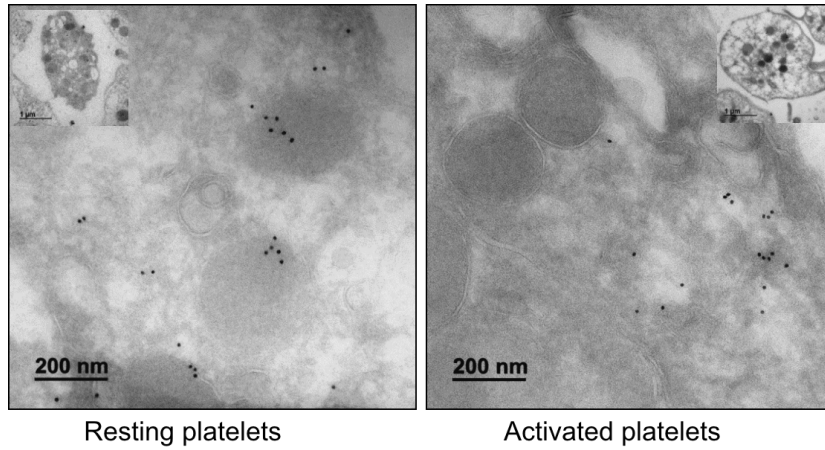


Figure S8. Immunogold staining of PKM2 in platelets. PKM2 was predominantly localized in the α -granules in resting platelets, whereas in the cytoplasm of activated platelets as determined by immunogold staining using transmission electron microscopy.

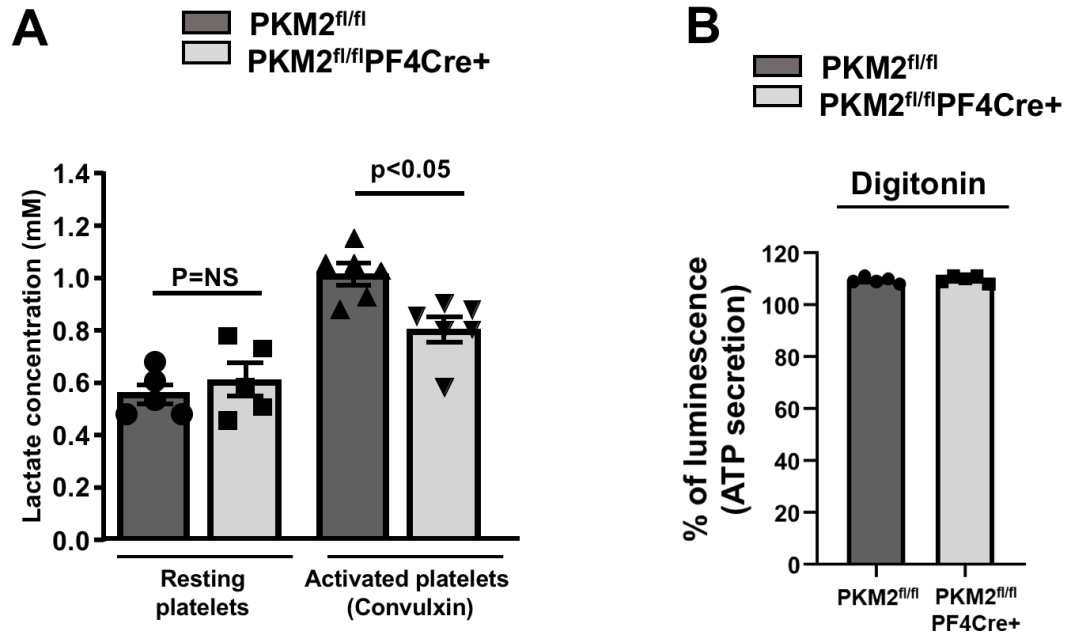


Figure S9. Lack of PKM2 in murine platelets reduces lactate production but do not affect ATP production. (A) Effect of lack of PKM2 on lactate production in mice platelets stimulated with convulxin (200 ng/ml) Values are mean \pm SEM, n=5-6 mice/group. One-way ANOVA with Tukey's multiple comparisons test. (B) Using Lumi-aggregometry, total ATP content was measured in digitonin-treated (50 μ M) resting platelets from PKM2^{fl/fl} and PKM2^{fl/fl}PF4Cre+ mice. Values are mean \pm SEM, n=5 mice/group. Mann Whitney test.

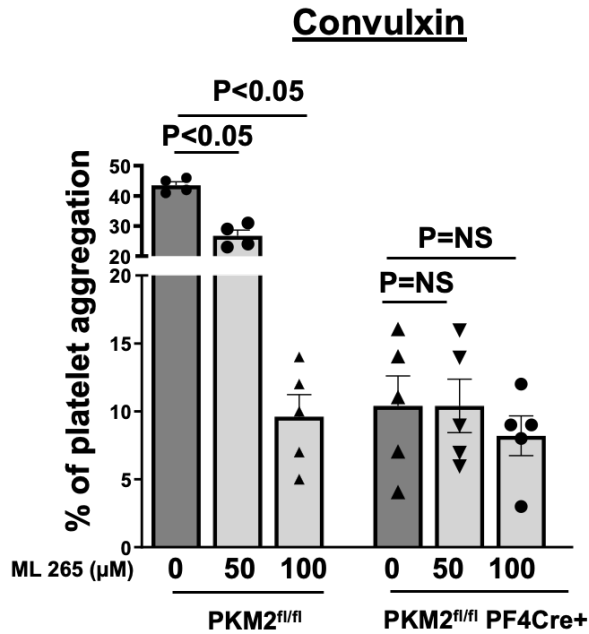


Figure S10. Treatment of PKM2^{fl/fl} platelets with ML265 caused a significant reduction in aggregation, whereas, no additional inhibitory effect PKM2-deficient platelets. Light-transmission aggregometry was used to evaluate the specificity of ML265 using platelet rich plasma from PKM2^{fl/fl} and PKM2^{fl/fl}PF4Cre⁺ mice that were stimulated with convulxin (25 ng/ml). Values are mean \pm SEM, n=4-5 mice/group. One-way ANOVA with Tukey's multiple comparison test. NS denotes non-significant.

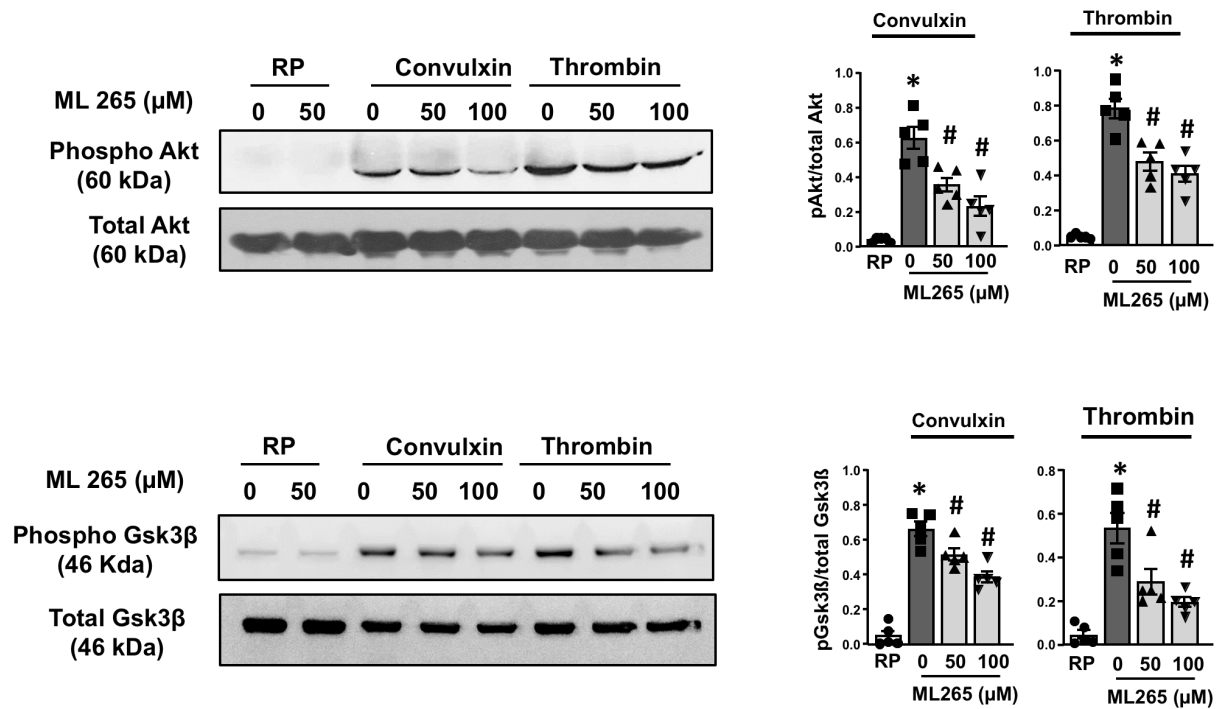


Figure S11. PKM2 regulates PI3 kinase-mediated Akt/GSK3 β signaling in mouse platelets. Mouse platelets were pretreated with vehicle or ML265 (50 and 100 μ M) for 10 minutes at room temperature before stimulation with agonists, including convulxin (100 ng/mL) or thrombin (0.1 U/ml) for 10 minutes. The left panels show representative Western blots for phospho-Akt (Ser 473), total Akt, phospho-GSK3 β , and total GSK3 β . The middle and right panels show densitometry analysis of immunoblots. Values are mean \pm SEM, $n=5$ /group. * $P < 0.05$ vs. resting platelets; # $P < 0.05$ vs. activated platelets (vehicle). One-way ANOVA with Tukey's multiple comparison test.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-PKM2 (D78A4), Rabbit	Cell Signaling Technology	Cat #4053
Anti- Phospho Akt Ser473	Cell Signaling Technology	Cat #9271
Anti-Akt (pan) (40D4) Mouse	Cell Signaling Technology	Cat #2920
Anti-Phospho GSK3 β Ser9, (D3A4) Rabbit	Cell Signaling Technology	Cat #9322
Anti- GSK3 β (D75D3) Rabbit	Cell Signaling Technology	Cat #5676
Goat- Anti-rabbit IgG HRP	Dako	Cat #P0448
Goat- Anti-mouse IgG HRP	Dako	Cat #P0447
Anti-Human PAC1 FITC	BD Biosciences	Cat #340507
Anti-Human CD62P-PE	BD Biosciences	Cat #551142
Anti-mouse CD62P-FITC	BD Biosciences	Cat #553744
Isotype Rat IgG	BD Biosciences	Cat #553995
Anti-JonA-PE	Emfret analytics	Cat #M023-2
Anti-CD61-FITC	Emfret analytics	Cat #M025-1
ML265	Cayman Chemical	Cat #13942
Glycolysis assay kit (L- Lactate)	Cayman Chemical	Cat #600450
Apyrase	Millipore Sigma	Cat # A6535
EGTA	Millipore Sigma	Cat # E3889
prostaglandin I ₂ (PGI ₂)	Millipore Sigma	Cat # P6188
Thrombin	Millipore Sigma	Cat # T6884
PAR4 agonist peptide	Millipore Sigma	Cat # A3227
TRAP-6 (PAR-1 agonist peptide)	Millipore Sigma	Cat # T1573
14C-2-deoxy-glucose	Perkin Elmer	Cat #NEC720A050UC
Collagen	Chronolog	Cat #P/N 385
ADP	Chronolog	Cat # P/N 384
CHRONO-LUME	Chronolog	Cat #P/N 395
ATP Standard	Chronolog	Cat # P/N 387
Convulxin	Santa Cruz	Cat #CAS 37206-04-5
FITC Annexin V apoptosis detection kit I	BD Biosciences	Cat #556547
Calcein-AM	Molecular Probes (Invitrogen)	Cat # C3100MP
Super Signal West Pico chemiluminescent substrate	Thermo Scientific	Cat # PI34080
PVDF membrane	Millipore	Cat # IPVH00010