Title

Pharmacologic dissection of the overlapping impact of heat shock protein family members on platelet function

Journal

Journal of Thrombosis and Haemostasis, 18(5)

ISSN

1538-7933

Authors

Jackson, Joseph W
Rivera-Marquez, Genesis M
Beebe, Kristin
et al.

Publication Date

2020-05-01

DOI

10.1111/jth.14758

Peer reviewed

Published in final edited form as:

J Thromb Haemost. 2020 May ; 18(5): 1197–1209. doi:10.1111/jth.14758.

Pharmacologic dissection of the overlapping impact of heat shock protein family members on platelet function

Joseph W. Jackson[*,1], **Genesis M. Rivera-Marquez**[*], **Kristin Beebe**[*], **Andy D. Tran**[¶], **Jane B. Trepel**[†], **Jason E. Gestwicki**[‡], **Brian S.J. Blagg**[§], **Shuichi Ohkubo**[††], **Leonard M. Neckers**[*]

[*]Urologic Oncology Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland [¶]Confocal Microscopy Core Facility, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland [†]Developmental Therapeutics Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland [‡]Department of Pharmaceutical Chemistry and the Institute for Neurodegenerative Disease, University of California at San Francisco, San Francisco, California [§]Department of Chemistry and Biochemistry, The University of Notre Dame, Notre Dame, Illinois [††]Tsukuba Research Center, Taiho Pharmaceutical Co., Ltd., Tsukuba, Ibaraki, Japan

Summary

Background: Platelets play a pivotal role in hemostasis, wound healing, and inflammation, and are thus implicated in a variety of diseases, including cancer. Platelet function is associated with release of granule content, cellular shape change, and upregulation of receptors that promote establishment of a thrombus and maintenance of hemostasis.

Objectives: The role of heat shock proteins (Hsps) in modulating platelet function has been studied for a number of years, but comparative roles of individual Hsps has not been thoroughly examined.

Methods: We utilized a panel of specific inhibitors of Hsp40, Hsp70, Hsp90, and Grp94 (the endoplasmic reticulum homolog of Hsp90) to assess their impact on several aspects of platelet function.

Results: Inhibition of each of the aforementioned Hsps reduced alpha granule release. In contrast, there was some selectivity in impacts on dense granule release. Thromboxane synthesis was impaired after exposure to inhibitors of Hsp40, Hsp90 and Grp94, but not after inhibition of Hsp70. Both expression of active glycoprotein IIb/IIIa (GpIIb/IIIa) and fibrinogen-induced platelet shape change were diminished by our inhibitors. In contrast, aggregation was selectively abrogated

[1]To whom correspondence should be addressed: 9000 Rockville Pike, Bldg. 10/CRC, Room 1-5848 (joe.william.jackson@gmail.com; tel: 240-858-3906).

Addendum

J.W. Jackson designed and performed research, interpreted/analyzed data, and wrote the manuscript. A.D. Tran analyzed and quantified the confocal microscopy data. G. Rivera-Marquez helped perform some of the platelet activation experiments. K. Beebe helped in writing and revising the manuscript. J. B. Trepel, J.E. Gestwicki, B.S.J. Blagg, and S. Ohkubo designed and provided inhibitors used in the study. L.M. Neckers contributed to study design, supervised the project and revised the manuscript.

Declaration of Conflicts of Interest

The authors state that they have no conflicts of interest to declare.

after inhibition of Hsp40 or Hsp90. Lastly, activated platelet – cancer cell interactions were reduced by inhibition of both Hsp70 and Grp94.

Conclusions: These data suggests the importance of Hsp networks in regulating platelet activity.

Keywords

Antiplatelet agents; cancer; heat shock proteins; hemostasis; platelets

Introduction

Platelets are small, anucleate cells derived from precursor megakaryocytes and are essential for wound healing and thrombus formation at the site of vascular injury [1]. After exposure to stimulating mediators such as collagen, thrombin, adenosine diphosphate (ADP), etc., resting platelets undergo an activation process characterized by the release of cellular content mostly contained within α- and dense granules. Additionally, a reorganization of the microtubule ring and platelet cytoskeleton occurs, consequently resulting in drastic shape change from a small, discoid shape to an enlarged, flattened shape. The release of α- and dense granules facilitate hemostatic and inflammatory processes by upregulating prothrombotic factors and promoting the recruitment and activation of neighboring immune cells, such as neutrophils and other platelets, thereby establishing an immune response. The upregulation of adhesion molecules on the platelet surface promotes platelet binding to extracellular constituents, such as fibrinogen, ultimately increasing platelet-platelet aggregation and mitigating bleeding via the formation of a hemostatic plug [2, 3]. Once regarded as “cellular fragments,” it is quite evident that regulated platelet function is pivotal for hemostasis, and the signaling pathways mediating these functions are quite complex [4]. Aberrant, dysregulated platelet activity contributes to a wide variety of pathologies including cardiovascular disease, ischemic and chronic inflammatory events in patients with human immunodeficiency virus infection, and cancer [5–10]. Indeed, platelet binding to and coating of circulating tumor cells not only increases the migratory and extravasation capacity of cancer cells, but also aids in immune cell evasion, ultimately contributing to increased metastatic burden. Although an abundant amount of research has been performed on elucidating the signaling pathways involved in platelet activity, these are not fully understood. Interestingly, several groups have demonstrated that platelets, despite lacking a nucleus, express various heat shock protein family members, and that inhibition of these Hsps results in platelet dysfunction [11–17].

Heat shock proteins serve as molecular chaperones that mediate proper protein folding, stability, and quality control, in addition to facilitating the unfolded protein response [18]. Hsps are expressed under basal conditions, but as their name suggests, are upregulated during heat shock in addition to other cellular stresses such as DNA damage, hypoxia, etc. The most prominently studied member, Hsp90, has been implicated in the folding of a wide array of client proteins including toll-like receptors, kinases, integrins, and transcription factors [19]. Not surprisingly, several disorders have exploited the molecular chaperone service that Hsp90 offers. It is well established that Hsp90 is upregulated in cancer cells, where encouraging *in vitro* and *in vivo* data have led to several clinical trials with various Hsp90 inhibitors [20]. Therapeutically targeting other Hsp members and co-chaperones,

such as Hsp40 and Hsp70, has also been demonstrated to have beneficial effects both *in vitro* and *in vivo* [21]. The large amount of research performed on the basal and pathological functions of Hsps has elucidated their prominence in regulating protein homeostasis, with recent literature suggesting that Hsps may also serve as protein scaffolds for the assembly of multi-protein signaling complexes [22].

Over the last twenty years or more, various Hsps have also been shown to exist in and impact the function of platelets. However, the majority of these studies have focused on an individual Hsp or have used chaperone inhibitors with suboptimal specificity for individual Hsps. In the present study, multiple readouts of platelet activity were measured after treatment with a panel of four distinct and specific inhibitors of Hsp family members: C86 (Hsp40 inhibitor), JG98 (Hsp70 inhibitor), TAS116 (Hsp90 inhibitor), and Kung65 (inhibitor of the Hsp90 homolog GRP94 localized to the endoplasmic reticulum, ER). Inhibition of one or more of these Hsps resulted in a reduction in: both α- and dense granule release, surface expression of P-selectin and glycoprotein IIb/IIIa (IIb/IIIa), spreading on fibrinogen-coated surfaces, and platelet-platelet aggregation. Further, we have demonstrated the therapeutic potential of these inhibitors in reducing platelet-tumor cell interaction *in vitro*. This manuscript not only expands upon the previous work performed by others, but moreover identifies the novel role of Hsp40 in platelet activity. Our findings suggest that most aspects of platelet function rely on a network of two or more Hsps, whereby disruption of one Hsp in the network is sufficient to inhibit activity.

Materials and Methods

Reagents

Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), fibrinogen, thrombin, and Tyrode's salt solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Adenosine diphosphate (ADP) was purchased from Chrono-Log (Havertown, PA, USA). Paraformaldehyde (PFA) was purchased from Electron Microscopy Sciences (Hatfield, PA, USA). Prostaglandin I_2 (PGI_2) was purchased from Cayman Chemical (Ann Arbor, MI, USA). The following Hsp inhibitors were kindly provided to us by several collaborators: C86 (Hsp40 inhibitor; Jane Trepel, Developmental Therapeutics Branch, NCI, Bethesda, MD, USA)[21], JG98 (Hsp70 inhibitor; Jason Gestwicki, University of California San Francisco, San Francisco, CA, USA)[23], Kung65 (Grp94 inhibitor; Brian Blagg, University of Notre Dame, Notre Dame, IN, USA), and TAS116 (Hsp90 inhibitor; Taiho Pharmaceutical Co., Tsukuba, Ibaraki, Japan)[24]. Phalloidin AF488, and AlexaFluor AF594 were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The flow cytometry antibodies CD61 AF647, CD62P BV421 (also known as P-selectin), PAC-1 AF647 (the activated form of glycoprotein IIb/IIIa; GPIIb/IIIa), and Annexin V Pacific Blue were purchased from Bio-Rad (Hercules, CA, USA), BD Biosciences (San Jose, CA, USA), BioLegend (San Diego, CA, USA), and Invitrogen Life Technologies (Carlsbad, CA, USA), respectively.

Platelet Isolation

Whole blood was obtained from consented, healthy male and female donor volunteers at the Department of Transfusion Medicine, Clinical Center, NIH, and was collected in acid citrate dextrose (ACD)-buffered vacutainer tubes for subsequent platelet isolation. Briefly, whole blood vacutainers were centrifuged for 15 min at 250 × g at low acceleration and zero deceleration to produce a platelet-rich-plasma (PRP) layer. The PRP layer was carefully collected via transfer pipets (Corning-Falcon, Corning, NY, USA), retaining the buffy coat layer, and centrifuged at 1000 × g for 10 min. The resultant platelet-poor-plasma (PPP) was decanted and the platelet pellet was carefully resuspended in wash buffer (25mL Tyrode's salt solution + 3ml ACD). After centrifuging again for 10 min at 1000 × g, the washed platelet pellet was resuspended in ~3mL Tyrode's salt solution. Unless stated otherwise, all experiments involving platelets and Hsp inhibitors were performed with 2×10^7 platelets per treatment. Additionally, platelets were pre-treated with the indicated Hsp inhibitor for 1 hr prior to exposure to 0.1U/mL thrombin for 15 min. Unless mentioned otherwise, all treatments were performed in PRP for the ADP-mediated activation experiments.

Aggregation Assay

Platelet aggregation in isolated washed platelets was performed using a Model 490 4-channel aggregometer (Chrono-Log, Havertown, PA, USA). Briefly, whole blood was collected from consented human donors in sodium citrate-buffered vacutainers. Isolated, washed platelets were produced as outlined above. Approximately 250ul of Tyrode's buffer served as a blank. Aliquots of platelets (250uL) were treated with 10uM of indicated Hsp inhibitors for 1 hr and aggregation was initiated via addition of 0.1U/mL thrombin or 20uM ADP. Aggregation was observed for 6 minutes per sample.

ATP Luminescence Assay

Dense granules contain a plethora of ions, neurotransmitters, and nucleotides, including ATP, and thus measurement of ATP after stimulation is a means to analyze dense granule release. Therefore, dense granule release was assessed via measurement of ATP in the supernatants of thrombin ± Hsp inhibitor-treated platelets, or ADP ± Hsp inhibitor-treated PRP. ATP was quantified using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 50ul of supernatants from treated platelets or PRP were collected in a 96-well plate and mixed with 50ul ATP-Glo substrate solution for 10 min. Luminescence was subsequently analyzed on an EnSpire Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA).

ELISA

Soluble CD40L (sCD40L), the stable byproduct of thromboxane A2 (TxA2), called thromboxane B2 (TxB2), and serotonin, were measured from the supernatants of treated platelets or PRP using a CD40L ELISA kit (R&D Systems, Minneapolis, MN, USA), a TxB2 ELISA kit (Cayman Chemical, Ann Arbor, MI, USA), and a Serotonin ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA) respectively, following the manufacturer's instructions. sCD40L, TxB2, and Serotonin concentrations are presented as average (± SEM) of the indicated replicates of samples.

Flow Cytometry

Platelet activation and α-granule release were assessed by measuring expression of both CD62P (P-selectin) and the activated conformation of GPIIb/IIIa on platelet surface. Briefly, after treatment with the respective Hsp inhibitors ± thrombin or ADP, 100ul platelet resuspension or PRP was fixed with 4% PFA and subsequently stained with 3ul CD61 AF647 (platelet marker) and 10ul CD62P BV421. Platelets were initially gated on FSC/SSC properties and CD61+ (platelet marker) staining, then assessed for CD62P. For GPIIb/IIIa assessment, isolated, washed platelet resuspensions or PRP were stained directly with PAC-1 AF647 (no fixative). For viability assays, isolated, washed platelets were treated with 0.5uM or 5uM Hsp inhibitors for 1hr and subsequently stained with 5uL Annexin V Pacific Blue to measure Annexin V expression on platelet surface. Data were acquired on a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA).

Platelet Spreading

Isolated, washed platelets (1×10^7 platelets/sample) were pretreated with 10μM indicated Hsp inhibitors for 30 mins at 37 °C and subsequently allowed to spread on glass coverslips previously coated with 150ug/ul fibrinogen for 45min and then blocked with 0.5% BSA. The spread platelets were fixed with 4% PFA and washed with phosphate buffered saline (PBS). Subsequently, the platelets were stained with a 1:200 dilution of Phalloidin AF488 in 0.1% Triton X-100 in PBS for 45 min at room temperature. The coverslips were then mounted onto glass microscope slides and spreading was imaged using a 20x objective lens and a BZ-X710 All-in-One fluorescence microscope (Keyence Corporation of America, Itasca, IL, USA), or with a 100x oil immersion lens using a Zeiss LSM780 confocal microscope. Spreading was assessed by observing platelet morphological changes and quantifying the amount of: 1) fully spread platelets (indicated by the presence of lamellipodia), 2) partially spread platelets (indicated by the presence of filopodia but not lamellipodia), and 3) non-spread platelets (indicated by a rounded, discoid shape) per 20x field.

Platelet-Tumor Binding

Platelet interaction with PC3 prostate cancer cells, which were cultured in Dulbecco's Modified Eagle Media (DMEM, Corning, Manassas, VA, USA) media supplemented with 10% fetal bovine serum (ThermoFisher Scientific, Grand Island, NY, USA), was assessed via immunofluorescence microscopy. Briefly, 5×10^4 PC3 cells were seeded in a 96-well plate or a LabtekII 8-well glass chamber slide for the representative confocal images (ThermoFisher Scientific, Grand Island, NY, USA) and incubated overnight at 37 °C in 5% CO_2. The next day, 100μL (1×10^7) isolated, washed platelet resuspension was treated with 10μM of the indicated Hsp inhibitor for 1 hr with or without subsequent 0.1U/mL thrombin treatment for 15 min. The platelets were then added to plates/chambers containing adherent PC3 cells and allowed to incubate at 37 °C in 5% CO_2 for 1 hr. Subsequently, the wells were washed with PBS, fixed with 4% PFA, washed with PBS again and stained with anti CD41 (platelet marker) overnight at 4°C. The next day, species-specific fluorescently-conjugated secondary antibodies were added for 1 hr, with subsequent DAPI nucleic acid staining (to identify PC3 cells) for 15 min, and images were taken using a 20x objective lens on a BZ-X710 All-in-One fluorescence microscope (n=3, Keyence Corporation of America, Itasca,

IL, USA). Confocal images (n=1 for representative image) were acquired using a Zeiss LSM780 microscope (Carl Zeiss Microscopy, LLC, White Plains, NY, USA) equipped with a 20x plan-apochromat (N.A. 0.8) objective lens and transmitted light detector (T-PMT). Differential interference contrast (DIC) images were collected simultaneously using 0.415 um x-y pixel size and 12-bit data depth. Four to five fields of view for each sample were imaged on the confocal microscope at a 3.5 μm optical thickness and analyzed for total platelet area bound to PC3 cells using FIJI/ImageJ and Weka Trainable Segmentation ML plugin software [25, 26]. Briefly, cell areas were segmented using a combination of a ML algorithm to classify foreground pixels in the DIC channel along with cell separation based on nuclei position. Platelet clusters were segmented based on intensity thresholding of CD41.

Western Blot

After treatment with the indicated inhibitors with or without thrombin-mediated activation, platelet whole cell lysates were generated in TNES buffer (50mM Tris pH7.5, 100mM NaCl, 2mM EDTA, 1% NP-40 supplemented with protease and phosphatase inhibitors (cOmplete and PhosSTOP cocktail tablets, respectively, Sigma-Aldrich, St. Louis, MO, USA). After cellular debris was removed via high speed centrifugation, equal amounts of the lysates were fractionated on gradient 4–20% SDS-PAGE gels and transferred to nitrocellulose membranes (both from Bio-Rad, Hercules, CA, USA). The membranes were analyzed for immunoreactivity to the following antibodies: Hsp40, (Cell Signaling Technology, Danvers, MA, USA), Hsp70, Hsp90, and Grp94 (Enzo Life Sciences, Farmingdale, NY, USA), and α-tubulin (EMD Millipore, Burlington, MA, USA). Bound antibodies were detected via species-specific horseradish peroxidase (HRP)- conjugated secondary antibodies at a 1:000 dilution (GE Healthcare, Pittsburgh, PA, USA), followed by reaction with Pico ECL (Thermo Scientific, Rockford, IL, USA) to produce a chemiluminescent signal, and exposed to x-ray film (Vita Scientific, College Park, MD, USA).

Statistical Analysis

Statistical analysis was performed using one-way and two-way ANOVA followed by Turkey's test for multiple comparisons. In these cases, data are represented as mean ± SEM or SD, and differences were considered to be significant for P values < 0.05.

Results

Hsp expression in platelets and platelet viability after exposure to Hsp inhibitors

Numerous studies have elucidated the role of various heat shock proteins in protein folding, stability and intracellular signaling in nucleated cells. However, a direct comparison of the role(s) of distinct Hsps (Hsp40, Hsp70, Hsp90, Grp94) in anucleate cells such as platelets has not been carried out. Others have demonstrated expression of these Hsps in platelets [11, 13–16], which we confirmed in the present study (Fig.1a). Rigg et. al. has demonstrated the effect of Hsp70 inhibition on platelet activity [14]. Herein, we compared the effects of Hsp70 inhibition with those caused by inhibiting the 3 other Hsps listed above. First, after treatment with C86 (Hsp40 inhibitor), JG98 (Hsp70 inhibitor), Kung65 (Grp94 inhibitor), or TAS116 (Hsp90 inhibitor) at 0.5uM or 5uM for 1 hr, platelet viability was assessed by

Annexin V expression via flow cytometry. The data show that all of our Hsp inhibitors have no detrimental effect on platelet viability (Fig.1b).

Hsp inhibitors reduce α-granule and dense granule release from activated platelets

Platelet activation is often characterized by several factors including receptor-ligand binding of soluble activators such as thrombin, and the subsequent release of both α- and dense granule content. P-selectin is normally stored within the α-granules of resting platelets and is greatly upregulated on the platelet surface upon activation stimulus, making P-selectin an established marker for platelet activation [1].

Therefore, platelets were pretreated with C86 (Hsp40 inhibitor), JG98 (Hsp70 inhibitor), Kung65 (Grp94 inhibitor), or TAS116 (Hsp90 inhibitor) at 0.5uM or 5uM for 1 hr, and then subsequently treated with 0.1U/mL thrombin, or left untreated. The platelet samples were assessed for P-selectin expression via flow cytometry as outlined in Materials and Methods. As demonstrated in Fig.2a–e, pretreatment with the various Hsp inhibitors prior to thrombin exposure significantly reduced P-selectin mean fluorescence intensity (MFI) compared to thrombin only-treated platelets. A similar reduction in CD62P expression is observed in platelet-rich-plasma (PRP) samples treated with Hsp inhibitors ± 20uM ADP (Fig.S1). Of note, Rigg et. al. demonstrated that Hsp70 inhibition-mediated P-selectin expression was only seen in collagen-activated platelets but not in thrombin-activated platelets [14]. In contrast, we demonstrate a significant reduction of P-selectin expression using JG98 in thrombin-activated cells (Fig.2c). This discrepancy may be due to differences in drug potency for Hsp70, as Rigg et al. used the precursor molecule, MKT-077, to JG98, or to the time of exposure to MKT-077, which was 10 minutes as compared to 1 hour treatment used in this work. These data establish that α-granule release and subsequent P-selectin surface expression is dependent upon multiple Hsps. We also tested the role of Hsp inhibition on platelet α-granule release via measurement of soluble CD40L (sCD40L) from platelet supernatant via ELISA. It is thought that the majority of sCD40L originates in the α-granules of platelets [27, 28]. Similar to the data with P-selectin, platelets that were pretreated with indicated Hsp inhibitors prior to thrombin treatment had significantly reduced amounts of sCD40L in their supernatants as compared to thrombin only-treated platelets (Fig.3). This reduction of platelet sCD40L with Hsp inhibition validates the notion that the indicated Hsps are important for α-granule release.

We next evaluated the effect of Hsp inhibition on dense granule release via measurement of ATP from activated platelets. Dense granules contain a variety of neurotransmitters and nucleotides, such as ATP, and thus analyzing ATP levels in platelet supernatant can be used as a surrogate measurement of dense granule release [1]. As demonstrated in (Fig.4a), dense granule release is reduced after exposure to the Hsp40 inhibitor C86 and the Grp94 inhibitor Kung65, but not after exposure to either JG98 or TAS116, Hsp70 and Hsp90 inhibitors, respectively. This suggests that Hsp40 and Grp94 help facilitate dense granule release, whereas Hsp70 and Hsp90 do not, thus identifying a more selective role of Hsps. Interestingly, Hsp inhibitors had no significant impact on dense granule release in ADP-activated PRP via measurement of either ATP (Fig.S2a) or serotonin levels (Fig.S2b). However, there is an encouraging trend towards reduction of serotonin levels following Hsp

inhibition. These data suggest that Hsp involvement in dense granule release may, to some extent, depend on the activation stimulus.

Hsp inhibitors reduce the synthesis of thromboxane A2

In addition to release of granule contents facilitating thrombus formation, several lipid mediators such as thromboxane A2 (TxA2) are synthesized upon platelet activation. TxA2 further stimulates platelets after exposure to initial activators in both a paracrine and autocrine manner. TxA2 is quickly metabolized into its more stable byproduct TxB2; hence, analysis of TxB2 is an indirect measurement of TxA2 synthesis. Shown in Fig.4b, there is a significant decrease in TxB2 in C86-, TAS116-, and Kung65-pretreated platelets, but not in JG98-pretreated platelets, when compared to thrombin only-treated platelets. Of note, the most significant decrease in TxB2 levels occurred in the cells exposed to Kung65, the inhibitor of Grp94 (the Hsp90 homolog of the endoplasmic reticulum) [3]. This is logical, since lipid mediators, such as TxA2, are produced in the dense tubular system (DTS), which is the endoplasmic reticulum of platelets. These data further support the functional diversity of Hsps in platelet signaling.

Hsp inhibitors reduce the expression of active GPIIb/IIIa

As mentioned previously, morphological change and aggregation play a major role in platelets' ability to form a thrombus. This is in part achieved by interaction with other activated platelets via GPIIb/IIIa-fibrinogen-GPIIb/IIIa binding. Upon activation, integrin IIb/IIIa is greatly upregulated in its active form on the surface of platelets, whereupon it attains high affinity for, and subsequently binds to, fibrinogen [29]. We therefore determined the effect of Hsp inhibition on active GPIIb/IIIa expression. After pretreatment with the indicated Hsp inhibitors ± thrombin or ± ADP treatment, active IIb/IIIa expression was assessed via flow cytometry. As demonstrated in Fig.5 (thrombin) and Fig.S3 (ADP), active GPIIb/IIIa MFI is significantly reduced in all of the platelet or PRP samples treated with the indicated Hsp inhibitors as compared to thrombin only- or ADP only-treatment. These data show that multiple Hsps play a role in signaling, and potentially activation, of GPIIb/IIIa in both thrombin and ADP activated settings. Furthermore, the significant inhibition of active IIb/IIIa on the platelet surface suggests that platelet shape change, aggregation, and eventual thrombus formation will also be impacted.

Hsp inhibition reduces platelets' ability to spread on fibrinogen

Platelet spreading on extracellular constituents such as fibrinogen and collagen, and morphological change are important parts of the platelet activation process as they are essential for proper thrombus formation. To evaluate the hypothesis that platelet shape change would be abrogated after exposure to Hsp inhibitors (due to a reduction in active IIb/IIIa), we assessed platelet spreading on fibrinogen-coated surfaces. Platelet morphology was characterized as fully spread, partially spread, or non-spread as outlined in Materials and Methods; see Figure 6A, B (top). As shown in Fig.6 (bottom), inhibition of Hsp 40, Hsp 70, and Grp94 had a detrimental effect on platelet spreading as compared to the no inhibitor control group. Exposure to these inhibitors not only significantly reduced the number of fully spread platelets, resulting in a more heterogenous platelet morphology, but also reduced the number of platelets bound to the coated coverslips. Interestingly, the Hsp90

inhibitor TAS116 did not significantly reduce the amount of fully spread platelets when compared to the no inhibitor control. This may be due to binding of fibrinogen to another integrin, α5β1 [30], which may not be dependent upon Hsp90. Ultimately, however, these data suggest that Hsp40, Hsp70, and Grp94, but not Hsp90, interact with key signaling proteins involved in platelet shape change.

Platelet aggregation is disrupted by Hsp inhibition

As mentioned earlier, platelets are known for their traditional role in wound healing by forming a thrombus. Proper thrombus formation is dependent upon the interaction and aggregation of activated platelets. Thus, we sought to determine whether inhibition of Hsps resulted in dysfunction of normal platelet aggregation. As outlined in Materials and Methods, isolated, washed platelets were pretreated with various Hsp inhibitors at 10uM for 1 hr prior to being exposed to 0.1U/mL thrombin (Fig.7) or 20uM ADP (Fig.S4). The Hsp40 inhibitor C86 and the Hsp90 inhibitor TAS116 significantly reduced thrombin-mediated platelet aggregation compared to the vehicle treated group, with C86 being the most potent inhibitor of aggregation (Fig.7a,d,e); this was in contrast to the Hsp70 inhibitor JG98 and the Grp94 inhibitor Kung65 (Fig.7b,c,e). Interestingly, the rate of aggregation (as measured via slope) was markedly decreased with both C86 and TAS116 treatment (Fig.7f), while the initiation of aggregation (as measured via lag time) was decreased with TAS116 treatment only (Fig.7g). This is consistent with a previous study which reported that the pan-Hsp90 inhibitor, geldanamycin, reduced platelet aggregation [17]. In contrast to the requirements of thrombin-mediated platelet aggregation, all Hsp inhibitors caused a significant reduction in ADP-mediated aggregation (Fig.S4), suggesting that general Hsp inhibition may be more detrimental to platelet aggregation induced by ADP compared to thrombin.

Platelet-tumor interaction is reduced by Hsp inhibitor treatment

As mentioned previously, because platelet interaction with cancer cells promotes metastasis by increasing single cell migration and immune cell evasion, disruption of platelet-tumor cell binding may prove beneficial in cancer treatment. As demonstrated in Fig.8a, thrombin-activated platelets demonstrated an increased binding potential in contrast to untreated platelets. Further, an encouraging trend towards reducing thrombin-induced platelet binding to PC3 cells was observed when platelets were treated with the Hsp inhibitors JG98 (Hsp70) and Kung65 (Grp94), suggesting that inhibition of these Hsp may disrupt platelet-cancer cell interactions (Fig.8b).

Discussion

Based on previous studies, it is clear that molecular chaperone activity plays a significant role in platelet function. However, the comparative contributions of each Hsp remain poorly understood. The data presented here demonstrate the consequences of inhibiting a panel of heat shock proteins - Hsp40, Hsp70, Hsp90, and Grp94 - with a set of Hsp-specific, cell-permeable, inhibitors, on various aspects of platelet function. Several studies have demonstrated the selectivity of these inhibitors for their intended targets, with no cross inhibition of the other Hsps used in this work [21, 24, 31]. At the concentrations used, these selective inhibitors did not cause significant changes in platelet viability, suggesting that the

observed effects on platelet activation are not due to increased cell death. As an outcome of this study, we identify the importance of Hsp40 for platelet function, as well as the potential of different Hsp inhibitors in preventing platelet-cancer cell binding, which warrants further exploration as a potential strategy to inhibit metastasis and immune evasion of circulating tumor cells.

Taken together, our data suggest that most platelet functions depend on a network of at least two and sometimes more Hsps. The fact that inhibition of Hsp40, Hsp70, Hsp90 and Grp94 significantly reduces alpha granule release suggests that this consequence of platelet activation depends on a previously uncharacterized network of all 4 Hsps. Since alpha granule release has previously been shown to be regulated by various SNAP Receptor (SNARE) proteins, a role for Hsps in modulating platelet SNAP receptor function is certainly possible [32]. Indeed, consistent with this hypothesis, SNAREs have previously been reported to be highly regulated by certain Hsps [3]. Interestingly, not all Hsp inhibitors affect dense granule release mediated by thrombin, supporting the possibility that a smaller Hsp network is responsible for selective degranulation [33, 34]. Further, lack of significant reduction in dense granule release from ADP-treated platelets as compared to thrombin-treated platelets suggests the occurence of activator-specific dependence on Hsp activity.

It is possible that one or more platelet Hsps interact with G proteins directly downstream of the activator receptors. Several reports have demonstrated that Hsp90 plays a direct role in the stabilization and localization of G proteins $G_{\alpha 12}$ and $G_{\beta/\gamma}$ at lipid rafts and mitochondria [35]. Thus, Hsp chaperoning of G proteins may be necessary for downstream platelet signaling events by virtue of localizing signaling kinases and GTPases. For example, Akt, RAC1, and mitogen activated protein kinases (MAPK) are well established to faciliate granule release and shape change [36], and their stabilization or assembly into signaling nodes by Hsps may be critical for this function.

Calcium (Ca^{2+}) siganling also may be directly or indirectly modulated by Hsp activity. Li et. al. has demonstrated that pan-Hsp90 inhibition (including Grp94) prevents increases in inctracellular [Ca^{2+}] in sperm cells, suggesting that Hsp90 and/or Grp94 is involved in [Ca^{2+}] homeostasis [37]. Considering that signficant Ca^{2+} storage occurs in the platelet dense tubular system (the ER fragments from megakaryocytes), it is reasonable to postulate that the ER-associated Hsp90 isoform, Grp94, may modulate Ca^{2+} signaling in platelets.

Finally, it is likely that Hsps modulate integrin stability, activation, and function. Hsp90 interacts with α_4 integrins in T cell trafficking during fever, and abolishment of this interaction prevented subsequent T cell trafficking and bacterial clearance [38]. Inhibition of Hsps disrupts IIb/IIIa function and platelet spreading. Physical interaction of Hsps with platelet integrins, such as IIb/IIIa, could help stabilize proper integrin conformation in addition to downstream signaling proteins such as Talin.

In conclusion, this study characterizes the potential of inhibiting distinct Hsps to prevent the sequelae of platelet activation. The data are consistent with the importance of multiple Hsp networks in regulating various aspects of platelet function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to thank Thomas Lewis from the Department of Transfusion Medicine at the National Institutes of Health Blood Bank for his organization and recruitment of consented human donors. We also thank Michael Kruhlak of the Confocal Microscopy Core in the National Institutes of Health Center for Cancer Research (CCR) for help in generating confocal images. This work was supported by funding from the Intramural Research Program (L.M.N.) of the National Cancer Institute.

References

1. Michelson AD. Platelets. 3rd ed. London ; Waltham, MA: Academic Press; 2013 xliv, 1353 p. p.
2. Italiano JE Jr., Hartwig JH. Megakaryoctye and platelet structure Hematology: basic priniciples and practice. 7th edition ed. Philadelphia, PA: Elsevier; 2018 p. p. 1857–69.
3. Rand ML, Israels SJ. Molecular basis of platelet function Hematology: basic priniciples and practice. 7th edition ed. Philadelphia, PA: Elsevier; 2018 p. p. 1870–84.e2.
4. Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. Arterioscler Thromb Vasc Biol. 2010;30(12):2341–9. [PubMed: 21071698]
5. Davidson DC, Jackson JW, Maggirwar SB. Targeting platelet-derived soluble CD40 ligand: a new treatment strategy for HIV-associated neuroinflammation? J Neuroinflammation. 2013;10:144. [PubMed: 24289660]
6. Huong PT, Nguyen LT, Nguyen XB, Lee SK, Bach DH. The Role of Platelets in the Tumor-Microenvironment and the Drug Resistance of Cancer Cells. Cancers (Basel). 2019;11(2).
7. McManus DD, Freedman JE. MicroRNAs in platelet function and cardiovascular disease. Nat Rev Cardiol. 2015;12(12):711–7. [PubMed: 26149483]
8. Metcalf Pate KA, Mankowski JL. HIV and SIV Associated Thrombocytopenia: An Expanding Role for Platelets in the Pathogenesis of HIV. Drug Discov Today Dis Mech. 2011;8(1–2):e25–e32. [PubMed: 22577463]
9. Pan V, Siva PN, Modery-Pawlowski CL, Sekhon UDS, Sen Gupta A. Targeted killing of metastatic cells using a platelet-inspired drug delivery system. Rsc Adv. 2015;5(57):46218–28.
10. Willoughby S, Holmes A, Loscalzo J. Platelets and cardiovascular disease. Eur J Cardiovasc Nurs. 2002;1(4):273–88. [PubMed: 14622657]
11. Goh YC, Yap CT, Huang BH, Cronshaw AD, Leung BP, Lai PB, et al. Heat-shock protein 60 translocates to the surface of apoptotic cells and differentiated megakaryocytes and stimulates phagocytosis. Cell Mol Life Sci. 2011;68(9):1581–92. [PubMed: 20953657]
12. Ji Y, Ferracci G, Warley A, Ward M, Leung KY, Samsuddin S, et al. beta-Actin regulates platelet nitric oxide synthase 3 activity through interaction with heat shock protein 90. Proc Natl Acad Sci U S A. 2007;104(21):8839–44. [PubMed: 17502619]
13. Lalo U, Jones S, Roberts JA, Mahaut-Smith MP, Evans RJ. Heat shock protein 90 inhibitors reduce trafficking of ATP-gated P2X1 receptors and human platelet responsiveness. J Biol Chem. 2012;287(39):32747–54. [PubMed: 22851178]
14. Rigg RA, Healy LD, Nowak MS, Mallet J, Thierheimer ML, Pang J, et al. Heat shock protein 70 regulates platelet integrin activation, granule secretion and aggregation. Am J Physiol Cell Physiol. 2016;310(7):C568–75. [PubMed: 26764050]
15. Staron M, Wu S, Hong F, Stojanovic A, Du X, Bona R, et al. Heat-shock protein gp96/grp94 is an essential chaperone for the platelet glycoprotein Ib-IX-V complex. Blood. 2011;117(26):7136–44. [PubMed: 21576699]
16. Suttitanamongkol S, Polanowska-Grabowska R, Gear AR. Heat-shock protein 90 complexes in resting and thrombin-activated platelets. Biochem Biophys Res Commun. 2002;297(1):129–33. [PubMed: 12220519]

17. Suttitanamongkol S, Gear AR, Polanowska-Grabowska R. Geldanamycin disrupts platelet-membrane structure, leading to membrane permeabilization and inhibition of platelet aggregation. Biochem J. 2000;345 Pt 2:307–14. [PubMed: 10620508]
18. Schopf FH, Biebl MM, Buchner J. The HSP90 chaperone machinery. Nat Rev Mol Cell Biol. 2017;18(6):345–60. [PubMed: 28429788]
19. Echeverria PC, Bernthaler A, Dupuis P, Mayer B, Picard D. An interaction network predicted from public data as a discovery tool: application to the Hsp90 molecular chaperone machine. PLoS One. 2011;6(10):e26044. [PubMed: 22022502]
20. Trepel J, Mollapour M, Giaccone G, Neckers L. Targeting the dynamic HSP90 complex in cancer. Nat Rev Cancer. 2010;10(8):537–49. [PubMed: 20651736]
21. Moses MA, Kim YS, Rivera-Marquez GM, Oshima N, Watson MJ, Beebe KE, et al. Targeting the Hsp40/Hsp70 Chaperone Axis as a Novel Strategy to Treat Castration-Resistant Prostate Cancer. Cancer Res. 2018;78(14):4022–35. [PubMed: 29764864]
22. Stetler RA, Gan Y, Zhang W, Liou AK, Gao Y, Cao G, et al. Heat shock proteins: cellular and molecular mechanisms in the central nervous system. Prog Neurobiol. 2010;92(2):184–211. [PubMed: 20685377]
23. Li X, Srinivasan SR, Connarn J, Ahmad A, Young ZT, Kabza AM, et al. Analogs of the Allosteric Heat Shock Protein 70 (Hsp70) Inhibitor, MKT-077, as Anti-Cancer Agents. ACS Med Chem Lett. 2013;4(11).
24. Ohkubo S, Kodama Y, Muraoka H, Hitotsumachi H, Yoshimura C, Kitade M, et al. TAS-116, a highly selective inhibitor of heat shock protein 90alpha and beta, demonstrates potent antitumor activity and minimal ocular toxicity in preclinical models. Mol Cancer Ther. 2015;14(1):14–22. [PubMed: 25416789]
25. Arganda-Carreras I, Kaynig V, Rueden C, Eliceiri KW, Schindelin J, Cardona A, et al. Trainable Weka Segmentation: a machine learning tool for microscopy pixel classification. Bioinformatics. 2017;33(15):2424–6. [PubMed: 28369169]
26. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012;9(7):676–82. [PubMed: 22743772]
27. Andre P, Nannizzi-Alaimo L, Prasad SK, Phillips DR. Platelet-derived CD40L: the switch-hitting player of cardiovascular disease. Circulation. 2002;106(8):896–9. [PubMed: 12186789]
28. Sahler J, Spinelli S, Phipps R, Blumberg N. CD40 ligand (CD154) involvement in platelet transfusion reactions. Transfus Clin Biol. 2012;19(3):98–103. [PubMed: 22703674]
29. Nieswandt B, Varga-Szabo D, Elvers M. Integrins in platelet activation. J Thromb Haemost. 2009;7 Suppl 1:206–9.
30. Podolnikova NP, Yakubenko VP, Volkov GL, Plow EF, Ugarova TP. Identification of a novel binding site for platelet integrins alpha IIb beta 3 (GPIIbIIIa) and alpha 5 beta 1 in the gamma C-domain of fibrinogen. J Biol Chem. 2003;278(34):32251–8. [PubMed: 12799374]
31. Li X, Colvin T, Rauch JN, Acosta-Alvear D, Kampmann M, Dunyak B, et al. Validation of the Hsp70-Bag3 protein-protein interaction as a potential therapeutic target in cancer. Mol Cancer Ther. 2015;14(3):642–8. [PubMed: 25564440]
32. Sharda A, Flaumenhaft R. The life cycle of platelet granules. F1000Res. 2018;7:236. [PubMed: 29560259]
33. Golebiewska EM, Poole AW. Platelet secretion: From haemostasis to wound healing and beyond. Blood Rev. 2015;29(3):153–62. [PubMed: 25468720]
34. Jonnalagadda D, Izu LT, Whiteheart SW. Platelet secretion is kinetically heterogeneous in an agonist-responsive manner. Blood. 2012;120(26):5209–16. [PubMed: 23086755]
35. Streicher JM. The Role of Heat Shock Proteins in Regulating Receptor Signal Transduction. Mol Pharmacol. 2019;95(5):468–74. [PubMed: 30670482]
36. Estevez B, Du X. New Concepts and Mechanisms of Platelet Activation Signaling. Physiology (Bethesda). 2017;32(2):162–77. [PubMed: 28228483]
37. Li K, Xue Y, Chen A, Jiang Y, Xie H, Shi Q, et al. Heat shock protein 90 has roles in intracellular calcium homeostasis, protein tyrosine phosphorylation regulation, and progesterone-responsive sperm function in human sperm. PLoS One. 2014;9(12):e115841. [PubMed: 25541943]

38. Lin C, Zhang Y, Zhang K, Zheng Y, Lu L, Chang H, et al. Fever Promotes T Lymphocyte Trafficking via a Thermal Sensory Pathway Involving Heat Shock Protein 90 and alpha4 Integrins. Immunity. 2019;50(1):137–51 e6. [PubMed: 30650373]

Essentials

- Heat shock proteins (Hsps) promote protein folding, stability and serve as signaling scaffolds
- This study sought to elucidate the comparative role of Hsps in platelet biology
- New pharmacological tools are available to study the roles of distinct Hsps in platelet function
- Our findings show that networks of Hsps participate in regulating platelet function

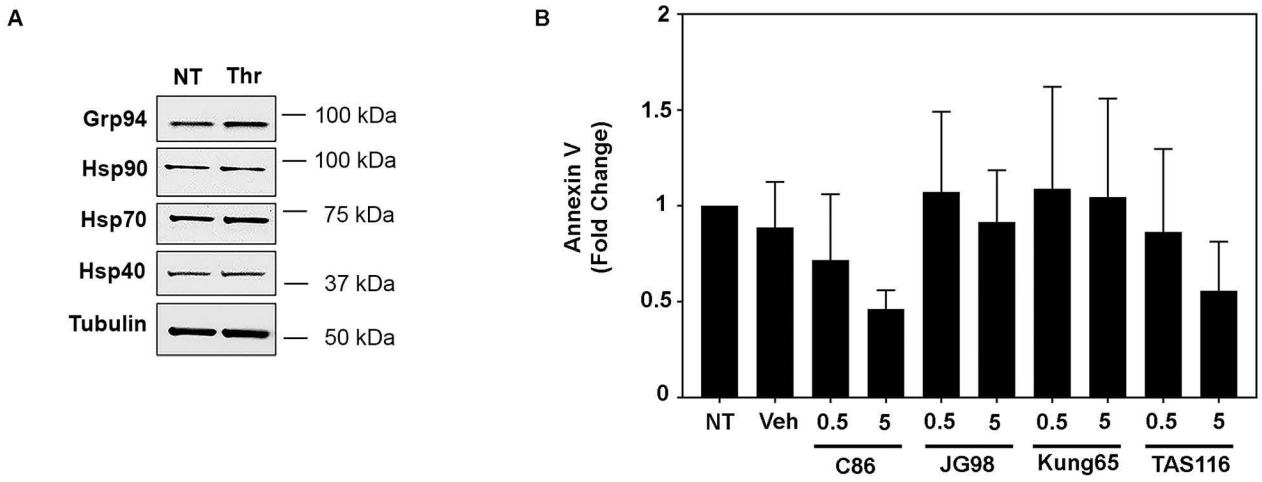

Figure 1. Hsp expression in quiescent and activated platelets.

A) Hsp expression was analyzed in both non-treated (NT) and thrombin-treated (Thr) platelets. The western blot shows similar Hsp expression amongst NT and Thr platelets and is representative of three independent experiments. The blots were cropped and boxed for visual clarity. Tubulin serves as a loading control. *B)* Platelet viability was assessed in the presence of the indicated Hsp inhibitors via Annexin V expression on the platelet surface, as measured by flow cytometry. Data reflect the mean of three replicates per group ± SD.

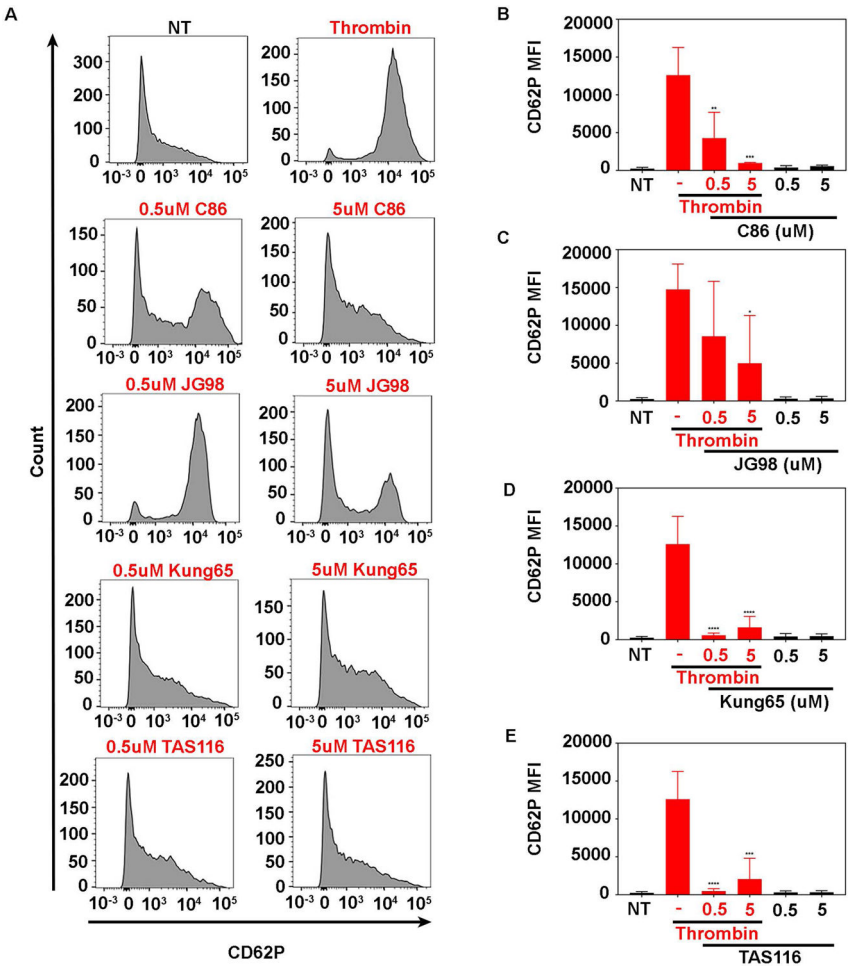

Figure 2. Hsp inhibitors reduce P-selectin expression on the platelet surface.

After treatment with indicated Hsp inhibitors ± thrombin (as outlined in Materials and Methods), platelet P-selectin (CD62P) surface expression was assessed via flow cytometry. *A)* A representative image illustrating the histogram plots of CD62P expression after treatment with Hsp inhibitors and thrombin. *B-E)* Quantification of compiled CD62P MFI average, where data reflect the mean of 3–7 replicates per group ± SD. Statistical significance compared to thrombin-only treated platelets was performed via one-way ANOVA with Turkey's test for multiple comparisons, where *denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$, and **** denotes $p<0.0001$..

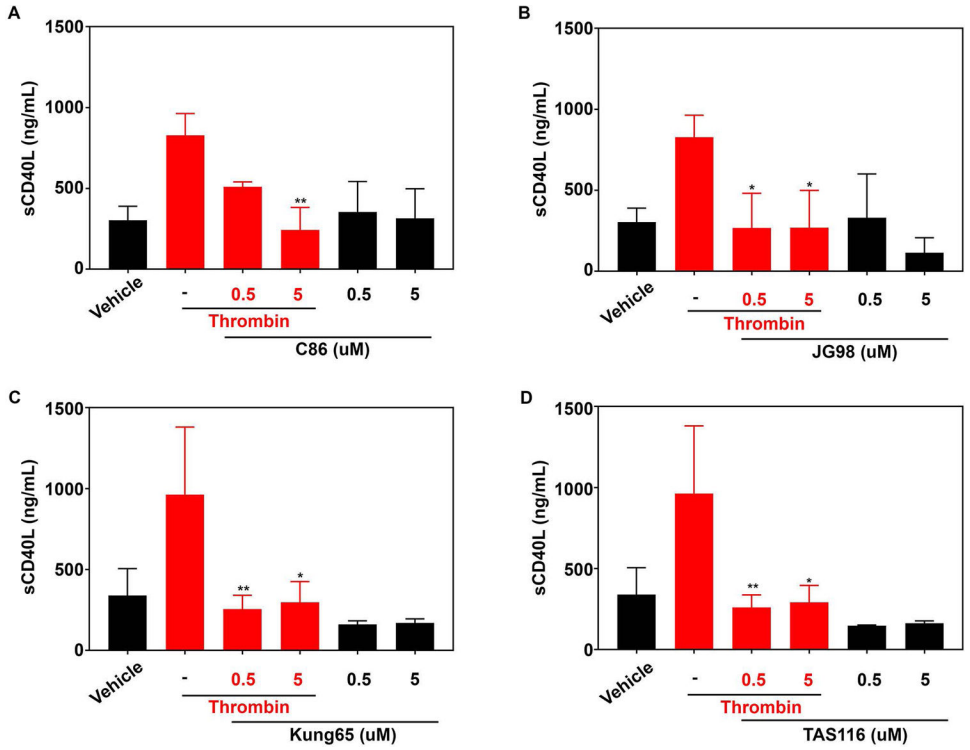

Figure 3. Hsp inhibitors reduce sCD40L release from activated platelets.

Supernatant from *A)* C86-, *B)* JG98-, *C)* Kung65-, and *D)* TAS116-treated platelets were collected and assessed for expression of sCD40L via ELISA. Data reflect the mean of three replicates per group ± SD. Statistical significance was analyzed via one-way ANOVA with Turkey's test for multiple comparisons, where * denotes $p<0.05$ and ** denotes $p<0.01$.

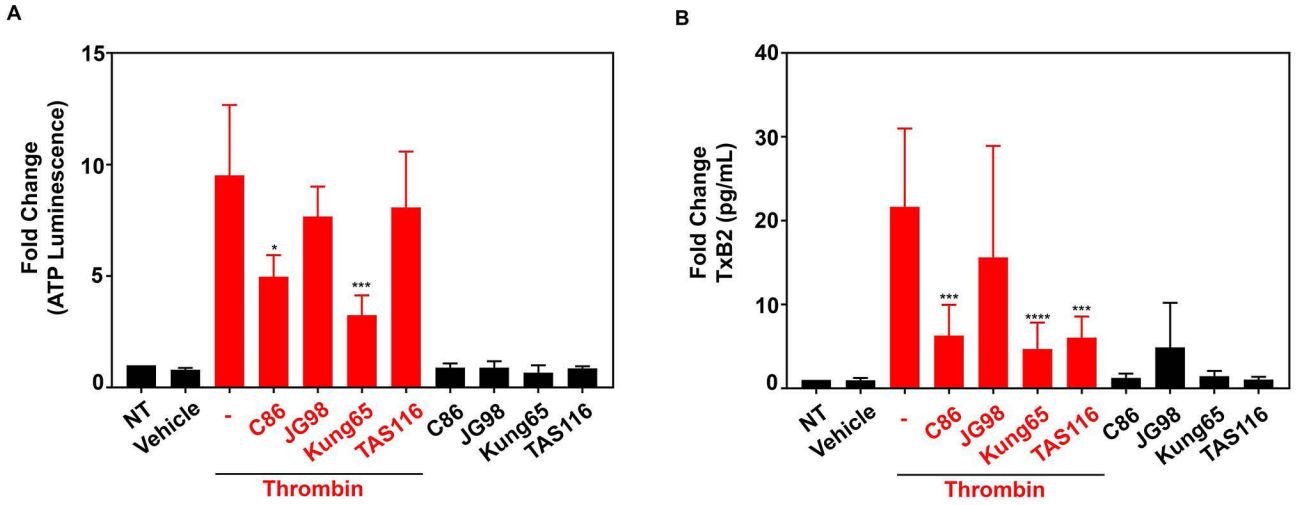

Figure 4. Dense granule release and thromboxane synthesis antagonism by Hsp inhibition.

A) Dense granule release was assessed via measurement of ATP from the supernatants from 10uM Hsp inhibitor-treated, thrombin-activated platelets via ATP CellTiter-Glo assay. 10uM C86 and 10uM Grp94 treatment significantly reduce ATP luminescence as compared to thrombin-only treated platelets (in contrast to JG98 and TAS116 treatment). Data reflect the mean of three replicates per group ± SD. *B)* Levels of thromboxane B2 (TxB2), the stable metabolite of TxA2, from 10uM Hsp inhibitor-treated, activated platelets was analyzed via ELISA. Inhibition of Hsp40, Grp94, and Hsp90 cause a significant reduction in of TxB2 compared to thrombin-only treated cells, in contrast to Hsp70-inhibited platelets. Data reflect the mean of 5–6 replicates per group ± SD. Statistical significance was analyzed via one-way ANOVA with Turkey's test for multiple comparisons, where * denotes $p<0.05$, *** denotes $p<0.001$, and **** denotes $p<0.0001$.

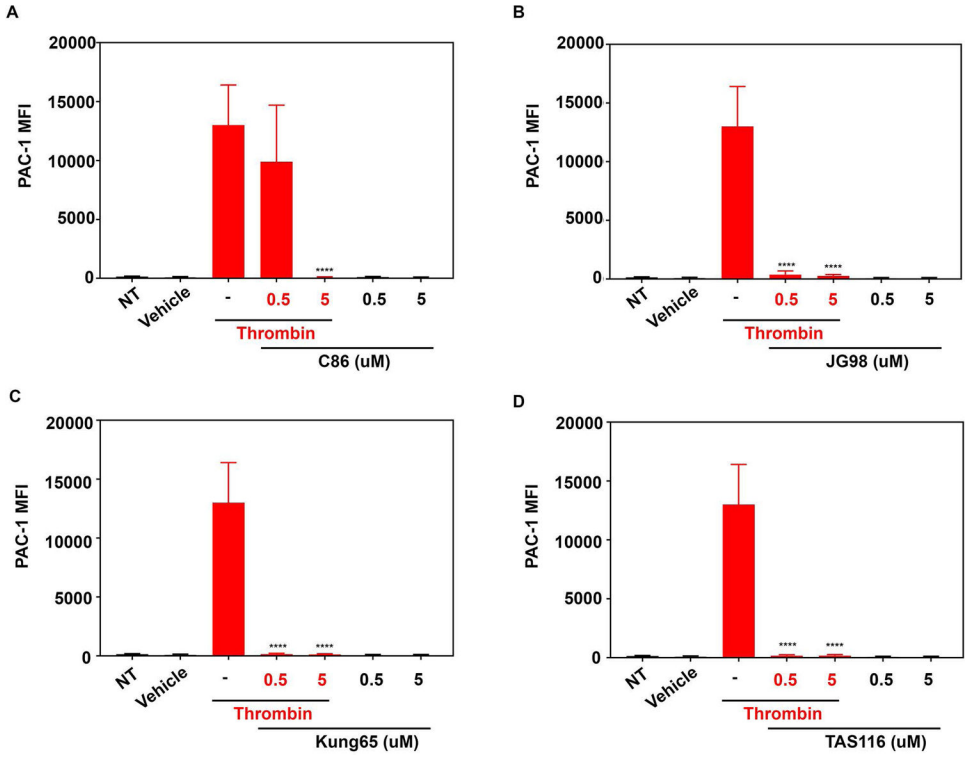

Figure 5. Hsp inhibitors reduce expression of active IIb/IIIa on the surface of thrombin-treated platelets.

After treatment with the indicated Hsp inhibitors ± thrombin: *A)* C86, *B)* JG98, *C)* Kung65, and *D)* TAS116, platelet IIb/IIIa surface MFI was assessed via flow cytometry. Data reflect the mean of four replicates per group ± SD. Statistical significance was analyzed via one-way ANOVA with Turkey's test for multiple comparisons, where **** denotes $p<0.0001$.

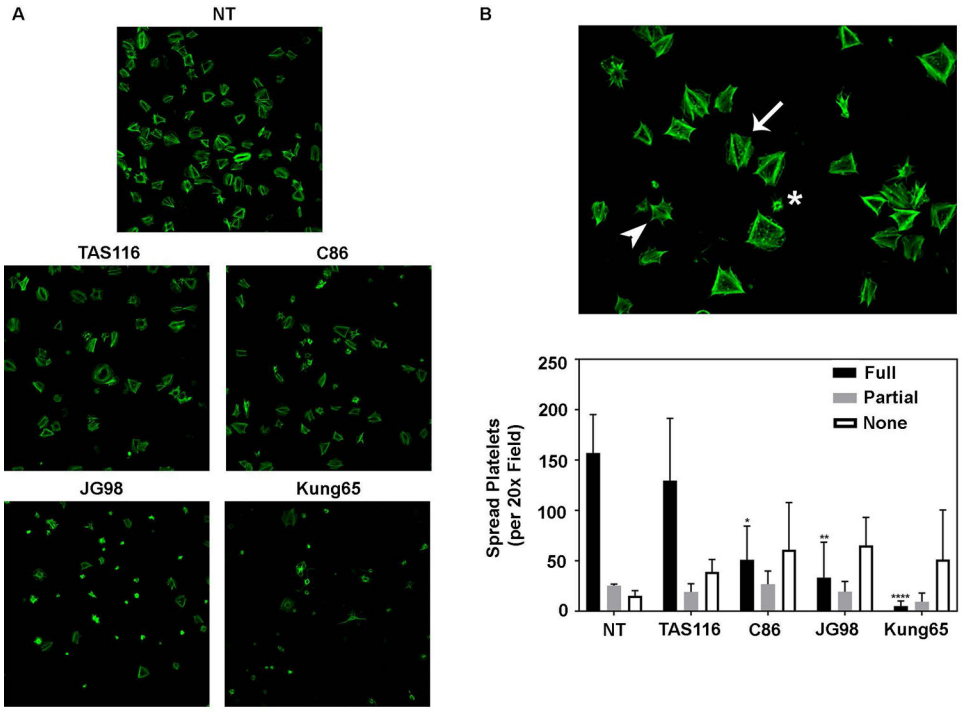

Figure 6. Platelet spreading on fibrinogen is reduced in the presence of Hsp inhibitors.

The effect of Hsp inhibition on platelet spreading and shape change was assessed on fibrinogen-coated surfaces. Phalloidin AF488 was used to aid in visualization of platelet shape change (see Materials and Methods). *A)* Representative images of non-treated or 5 uM inhibitor-treated platelet spreading on fibrinogen coverslips imaged at 100x oil immersion. *B, top)* Representative, enlarged image of platelets stained to depict the three distinct stages of spreading morphology: 1) fully spread denoted by lamellipodia (arrow), 2) partially spread denoted by filopodia but not lamellipodia (arrowhead), and 3) non-spread denoted by rounded shape (asterisk). *B, bottom)* Quantification of characterized spread platelets as treated in *(A)*, per 20x field. Data are representative of four replicates ± SD. Statistical significance compared to non-treated (NT) fully spread platelets was analyzed via two-way ANOVA with Turkey's test for multiple comparisons, where * denotes $p<0.05$, ** denotes $p<0.01$, and **** denotes $p<0.0001$.

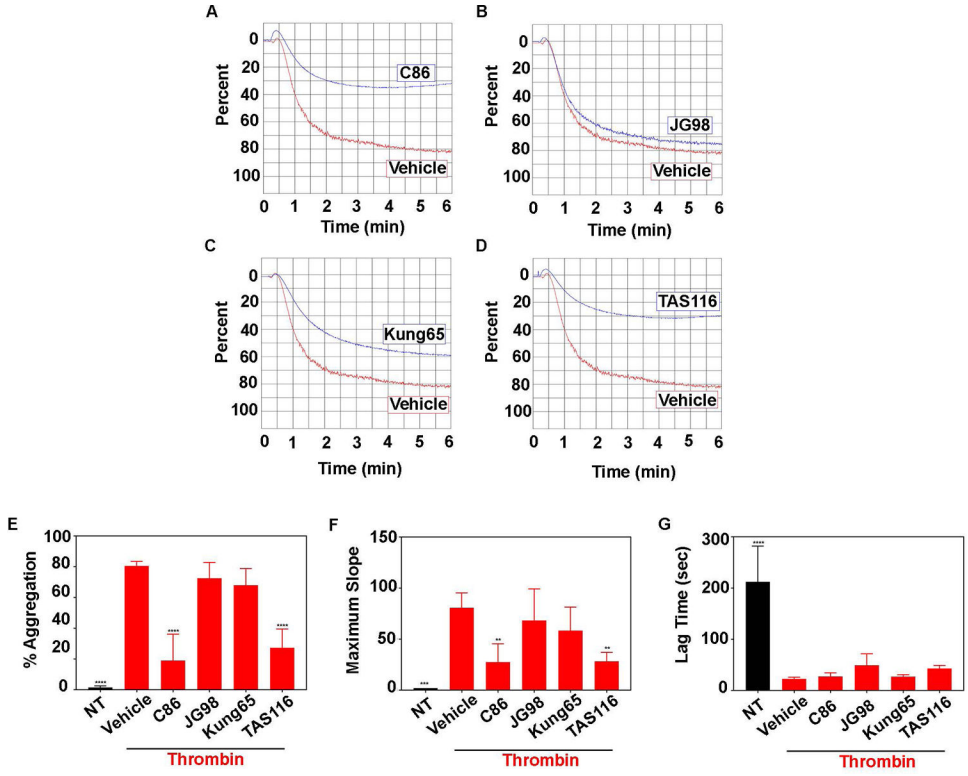

Figure 7. Hsp40 and Hsp90 inhibition attenuate platelet-platelet aggregation.

Platelet aggregation was assessed on washed platelets with or without exposure to 10 uM of *A)* C86, *B)* JG98, *C)* Kung65, and *D)* TAS116; *A-D)* representative aggregation traces, *E)* quantification of percent aggregation, *F)* maximum slope (rate of aggregation), and *G)* lag time (time for aggregation to begin). Data reflect the mean of four separate experiments ± SD. Individual treatment of C86 and TAS116 greatly reduces percent aggregation, the initialization of aggregation (represented via Lag Time) and aggregation rate (represented via Slope). Statistical significance compared to DMSO + thrombin group was analyzed via one-way ANOVA with Turkey's test for multiple comparisons, where ** denotes $p<0.01$, *** denotes $p<0.001$, and **** denotes $p<0.0001$.

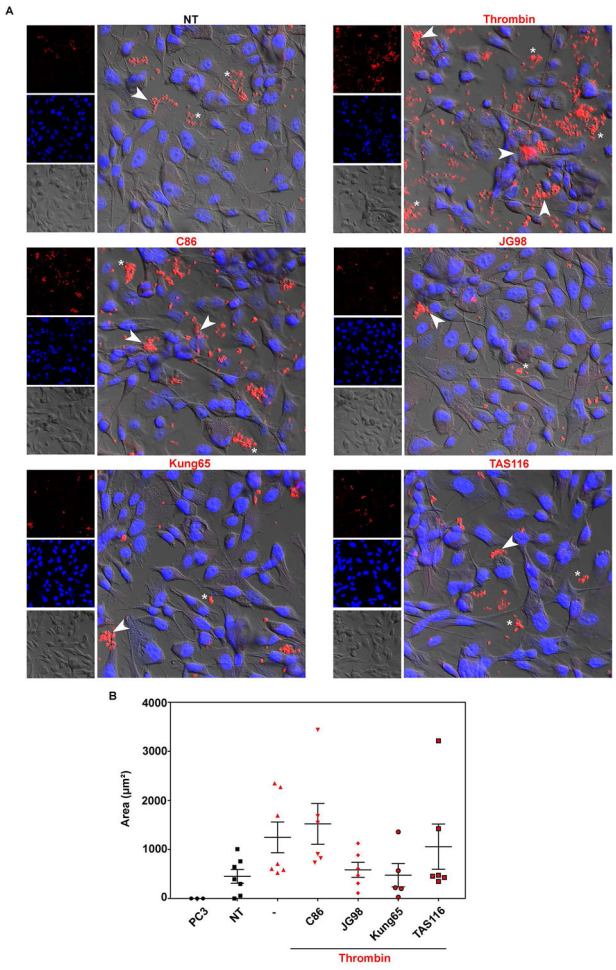

Figure 8. Hsp inhibitors reduce platelet-tumor binding.

Platelet interaction with PC3 prostate cancer cells was assessed via confocal immunofluorescence microscopy after pretreatment with 10 μM Hsp inhibitor as outlined in Material and Methods. *A)* Merged images demonstrate that thrombin treatment markedly increases platelet binding to the PC3 monolayer, which appears attenuated in the presence of the indicated Hsp inhibitors. Image is representative of four separate experiments and 4–5 randomized fields of view per sample. Images were captured at 20x using anti-CD41 (red, platelet marker), DAPI (blue, stains nucleus of PC3 cells), and differential interference contrast (DIC) microscopy. Arrowheads depict platelet-PC3 cell interaction, whereas an asterisk depicts platelet binding to plastic dish. *B)* Quantification of total platelet area bound to PC3 cells as described in Methods. Image analysis was performed on 4–5 fields of view per sample performed once and plotted as Area (μm^2 ± SEM.)