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Histone deacetylase inhibitors reduce glycoprotein VI expression and platelet responses to collagen related peptide☆

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ABSTRACT

Introduction: Platelet Glycoprotein (GP)VI is a member of the immunoglobulin superfamily expressed only on platelets, and is the major signalling receptor for collagen. Histone deacetylase inhibitors (HDACi) are anti-cancer agents used for the treatment of haematological malignancies, and we examined the effects of administration of HDACi to mice on platelet function including responses to agonists including collagen related peptide (CRP).
Materials and Methods: C57BL/6 mice were injected with two structurally different HDACi, panobinostat and romidepsin, for three days and platelet receptor levels and responses to agonists were assessed by flow cytometry and western blot.
Results: Platelets from mice treated with either HDACi were impaired in their ability to respond to CRP, but not thrombin or adenosine diphosphate (ADP). HDACi treatment increased acetylation of megakaryocytic GPVI, resulting in loss of intact (~60-65-kDa) GPVI and formation of ~10-kDa remnant GPVI. Circulating platelets had reduced surface and total expression of GPVI. Platelets from mice treated with HDACi had impaired GPVI signalling following treatment with CRP, resulting in inhibition of Syk phosphorylation and activation, and the final common pathways of platelet activation.
Conclusions: Administration of HDACi *in vivo* may ablate platelet responses to agonists and platelet function.

Introduction

Platelet Glycoprotein (GP)VI is a member of the immunoglobulin superfamily expressed exclusively on the surface of platelets. GPVI complexes with FcR γ-chain dimers and is the major signalling receptor for collagen on the surface of platelets [1,2]. Interaction of the receptor with sub-endothelial collagen and subsequent clustering results in phosphorylation of the immuno-receptor tyrosine-based activation motif (ITAM) by the Src family kinases Fyn and Lyn [3,4]. Subsequently, spleen tyrosine kinase (Syk) is recruited and is activated by autophosphorylation [5]. Syk downstream signalling involves multiple adaptor proteins, leading ultimately to activation of phospholipase C-γ2, increased intracellular Ca^{2+}, and activation of fibrinogen receptor GPIIbIIIa [6]. GPVI deficiency in humans is rare, and usually manifests with a mild bleeding phenotype [7], while auto-antibody mediated GPVI down-regulation with associated immune thrombocytopenia also results in a mild bleeding disorder [8]. GPVI-deficient mice have few bleeding problems despite a complete absence of platelet activation in response to collagen [9,10]. Conversely, previous reports have demonstrated that antibody-mediated inhibition of GPVI in mice protects against experimentally induced thrombosis [11].

The histone deacetylase inhibitors (HDACi) are a structurally diverse class of targeted anti-cancer agents that have undergone rapid clinical development in recent years. Panobinostat is a pan HDACi that inhibits Class I, II and IV (nuclear and cytoplasmic) HDAC enzymes, while romidepsin (Isodax™) is an iso-selective HDACi which preferentially interacts with class I (nuclear) enzymes. Both drugs have shown marked anti-tumour efficacy in clinical studies [12,13]. We have recently reported that HDACi-induced thrombocytopenia, the most common dose limiting toxicity of this class of drugs, occurs due to reductions

Abbreviations: ADP, adenosine diphosphate; CRP, collagen related peptide; GP, glycoprotein; HDACi, histone deacetylase inhibitor; ITAM, immuno-receptor tyrosine-based activation motif; ITP, immune thrombocytopenia; PBS, phosphate buffered saline; qRT-PCR, quantitive real time PCR; Syk, spleen tyrosine kinase.

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in megakaryocyte pro-platelet numbers, without causing platelet apoptosis or affecting platelet half-life as seen with BH3-mimetics [14]. It was recently reported that HDAC6 may mediate the characteristic shape changes seen in platelet activation and the speed of platelet spreading, via tubulin deacetylation [15].

The effects of administration of HDACi *in vivo* on platelet function and responses to agonists remain unknown. Given the potential effects of HDACi on protein function via lysine acetylation, we considered that effects on platelet function may occur via this mechanism. Platelets from mice treated with HDACi were impaired in their ability to respond to collagen related peptide (CRP), but not thrombin or adenosine diphosphate (ADP). This effect may be due to HDACi increasing the acetylation and cleavage of GPVI within megakaryocytes. Circulating platelets had reduced surface and total expression of GPVI and impaired downstream GPVI signalling, resulting in inhibition of phosphorylation of Syk and the final common pathways of platelet activation.

Materials and Methods

Reagents and Cells

Panobinostat (Novartis Pharmaceuticals) and romidepsin (Celgene Corporation) were provided as indicated. Murine thrombopoietin (TPO) was synthesized at the Walter and Eliza Hall Institute (Parkville, Australia). Thrombin and crosslinked CRP were kind gifts from Carly Simon, St Vincent's Hospital, Melbourne. Flow cytometric beads and anti-CD62P were purchased from Becton Dickinson. Anti-JAQ1, fluorescein isothiocyanate (FITC)-labelled anti-GPVI, anti $\alpha_2\beta_1$ and phycoerythrin (PE)-labelled JON/A antibody were purchased from Emfret Analytics (Eibelstadt, Germany). Monoclonal antibodies (mAb) that recognise phospho-Syk (Tyr525/6), total Syk and an anti-acetylated lysine antibody were from Cell Signalling Technology (Danvers, MA). Murine megakaryocytes were derived from fetal liver cells harvested from E13.5-14.5 pregnant C57BL/6 mice. Cells were plated out at 5×10^5 cells/ml in Stempro 34 media (Invitrogen, Life Technologies, Grand Island, NY) supplemented with 100 ng/ml murine TPO at 37 °C with 5% CO_2 for four days. Following separation by centrifugation on a 3% and 1.5% bovine serum albumin (BSA) gradient at 1 *g* for 40 minutes at room temperature, purified megakaryocytes were harvested from the lower 800 μl-1 ml.

Mice

All animal studies were approved by the Peter MacCallum Cancer Centre Animal Experiment Ethics Committee. C57BL/6 mice (8 to 12-weeks-old) were purchased from the Walter and Eliza Hall Institute (Parkville, Australia) and were injected with either panobinostat 10 mg/kg or romidepsin 1 mg/kg intra-peritoneally (IP) daily for up to 4 days as indicated.

Isolation of Murine Platelets

Following HDACi treatment, mice were culled and immediately bled via cardiac puncture into aster-jandl anticoagulant (85 mM sodium citrate dihydrate, 69 mM citric acid anhydrans, 10 mM glucose). After centrifugation at 125 *g* for eight minutes, the supernatant and approximately 200 μL of red cells were removed, and 300 μl of wash buffer (140 mM NaCl, 5 mM KCl, 12 mM sodium citrate, 10 mM glucose, 12.5 mM sucrose, pH 6.0) added before centrifugation at 125 *g* for eight minutes. The supernatant was removed and 300 μl of wash buffer was added and samples were spun at 860 *g* for five minutes, and resuspended in wash buffer. Samples were then spun at 860 *g* for five minutes then mixed with 100 μl of resuspension buffer (10 mM HEPES, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2.6H_2O$, 0.5 mM $NaHCO_3$ 10 mM glucose, pH 7.4) and a platelet count was made using flow cytometric beads as a reference concentration.

Flow Cytometry and Platelet Function Testing

For assessment of surface GPVI and $\alpha_2\beta_1$, either primary cultured megakaryocytes or washed platelets were washed with ice cold phosphate-buffered saline (PBS; 10 mM NaH_2PO_4, 0.15 M NaCl, pH 7.4), and pre-incubated on ice with an Fc-blocking mAb, 2.4 G2 (Peter MacCallum Cancer Centre), at 1 in 100 dilution for 15 minutes. Cells were then incubated with either FITC-labelled anti-GPVI or anti-$\alpha_2\beta_1$ mAbs at a 1 in six dilution for 15 minutes. For platelet function testing, washed platelets from each HDACi-treated mouse were mixed with either PE-labelled JON/A (detects only active GPIIbIIIa) or anti-CD62P (P-selectin) antibodies at a final concentration of 1×10^7 platelets/ml in 30 or 50 μl, and either thrombin, ADP or CRP was added at the indicated concentrations for 20 minutes. Reactions were terminated by the addition of 300 μl of resuspension buffer. All samples were analysed by a Canto II flow cytometer using FCS express version 3 (BD Biosciences).

qRT PCR Assay

Following megakaryocyte purification as described above, $20\text{-}40\times10^5$ cells per well were resuspended in Stempro 34 media supplemented with 100 ng/ml murine TPO and either vehicle, 10 nM panobinostat or 1 nM romidepsin. At the indicated time points, RNA was extracted from cells (Qiagen RNA plus, Qiagen, Chadstone, Australia) following the manufacturer's instructions. RNA was reverse transcribed into cDNA, and quantification of GPVI was performed using the SYBR green dye detection system (Applied Biosciences, Warrington, UK) and mouse *GP6* primers (5′-TGCTGGACGGTATCGATGCTCTTA-3′and 5′-AACTGTATG GGCTCTGGCACTTCA-3′) or the non-HDACi modulated mouse L32 primers (5′-3′and 5′-3′) as a reference gene. The fold change in GPVI was calculated against L32 using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

Following the isolation of either washed platelets or cultured megakaryocytes, cells were lysed with Triton X lysis buffer (20 mM Tris-pH 7.4, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% (v/v) glycerol and 1% (v/v) Triton X-100) with protease and phosphatase inhibitors (Complete™, Roche, Basel Switzerland). Samples were boiled for five minutes in non-reducing loading buffer and equal amounts of extracts were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Protein was transferred to nitrocellulose membranes and blocked for 60 minutes at room temperature in PBS containing 5% skim milk. Membranes were incubated with one of 1/500 monoclonal rat anti-mouse GPVI ectodomain antibody (JAQ1), or 1/500 of a rabbit antibody directed against the C-terminus of mouse GPVI or rabbit anti-acetylated lysine antibody. Primary antibodies were detected with secondary antibodies conjugated with horseradish peroxidise (HRP), and filters were developed with enhanced chemiluminescense (ECL; Amersham Biosciences). Western blot densitometry was performed using ImageJ software (National Institute of Health).

Immunoprecipitation

Following their isolation, washed platelets was lysed using an immunoprecipitation (IP) lysis buffer (50 mM Tris pH 8, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100). Immunoprecipitation was performed with sepharose protein A beads (Zymed Laboratories, San Francisco, CA, USA.) following protein quantification and equalisation of the protein concentration and volume of all samples. A one hour pre-clear to reduce non-specific protein binding was performed on the lysates by the addition of the appropriate sepharose beads with an isotype control