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Full Length Article

The tyrosine kinase inhibitor, nilotinib potentiates a prothrombotic state

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ABSTRACT

Tyrosine kinase inhibitors (TKI) such as imatinib, nilotinib and dasatinib are now established as highly effective frontline therapies for chronic myeloid leukaemia (CML). Disease control is achieved in the majority of patients and survival is excellent such that recent focus has been on toxicities of these agents. Cumulative data have reported an excess of serious vascular complications, including arterial thrombosis and peripheral arterial occlusive disease, in patients receiving nilotinib in comparison with other TKIs, with resultant interest in delineating the pathophysiology and implications for rationale cardiovascular risk modification. To address this issue, we studied the effects of imatinib, nilotinib and dasatinib on platelet function and thrombus formation in human and mouse models using *in vitro, ex vivo* and *in vivo* approaches. *In vitro* studies demonstrated that dasatinib and imatinib but not nilotinib inhibited ADP, CRP, and collagen-induced platelet aggregation and moreover, that nilotinib potentiated PAR-1-mediated alpha granule release. Pretreatment of wild-type C57BL/6 mice with nilotinib but not imatinib or dasatinib, significantly increased thrombus growth and stability, on type I collagen under *ex vivo* arterial flow conditions and increased thrombus growth and stability following $FeCl_3$-induced vascular injury of mesenteric arterioles and carotid artery injury *in vivo*. Whole blood from nilotinib-treated CML patients, demonstrated increased platelet adhesion *ex vivo* under flow, increased plasma soluble P- and E-selectin, sICAM-1, sVCAM-1, TNF-alpha, IL-6 levels and endogenous thrombin potential (ETP) levels *in vivo,* despite being on daily low-dose aspirin. These results demonstrate that nilotinib can potentiate platelet and endothelial activation and platelet thrombus formation *ex vivo* and *in vivo*.

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1. Introduction

The introduction of targeted tyrosine kinase inhibitors (TKIs) including imatinib, nilotinib, dasatinib and ponatinib as treatment of CML has revolutionised management of this disease with deep and durable molecular responses resulting in high survival rates [1–4]. As such, management focus has been toward preventing or minimising important side-effects of these agents to allow consistent longer-term delivery. Of particular relevance, with nilotinib and ponatinib, has been the emergence of clinically significant vascular events, including peripheral arterial occlusive disease (PAOD), myocardial infarction, spinal infarction and cerebrovascular occlusion, with substantial morbidity and even mortality [4–9]. Many of the PAOD events affecting lower limbs, required angioplasty, stent implantation and/or amputation [10]. Recent data from a prospective randomised study demonstrated a cumulative incidence of clinically significant vascular events over 5 years, of 13% and 6% in nilotinib patients receiving 400 mg bd and 300 mg bd respectively, compared with <2% in those receiving imatinib [7,9–11].

The risk of vascular events was increased by concomitant conventional cardiovascular risk factors, such as smoking, hypertension, diabetes mellitus, dyslipidaemia and obesity [12]. Moreover, nilotinib has been shown to be associated with metabolic disturbances, including altered lipid profiles and hyperglycaemia [9]. However, the presence of risk factors and metabolic effects did not fully explain the excess in vascular risk, with events occurring unexpectedly in low risk patients and often after relatively short exposure to drug [9–12]. A number of investigators have attempted to address the issue of why nilotinib may have specific additional prothrombotic/atherogenic effects not observed with other TKIs. One study suggested that nilotinib may induce vasospasm, but it is unclear how this may result in profound atherogenic PAOD [12]. Other recent reports suggest that an imbalance in pro-inflammatory cytokines released from vascular endothelial cells and upregulation of adhesion molecules may contribute to nilotinib-induced vasculopathy [13,14]. However, to our knowledge, the impact of various TKIs on platelet and endothelial function as well as thrombus formation has not yet been comprehensively investigated. In this study, we used

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human and mouse models *in vitro*, *ex vivo* and *in vivo* to evaluate the mechanisms by which nilotinib, in contrast to other TKIs, may contribute to the induction of a prothrombotic state, with the intention of proposing rationale strategies to minimise or ameliorate this effect.

2. Materials and methods

2.1. Chemicals

Sodium chloride (NaCl), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), glucose, tri-sodium citrate, magnesium chloride hexahydrate ($MgCl_2$-$6H_2O$), disodium hydrogen phosphate (Na_2HPO_4), and potassium dihydrogen orthophosphate (KH_2PO_4) were purchased from Merck, Kilsyth (Victoria, Australia). HEPES and prostaglandin E_1 (PGE_1), bovine serum albumin (BSA), paraformaldehyde, iron (III) chloride ($FeCl_3$), quinacrine, dimethyl sulfoxide (DMSO), Rhodamine G6 dye (Rh6G), acetylsalicylic acid (aspirin), thrombin and Protease activated receptor-1 (PAR-1, SFLLRN) were purchased from Sigma-Aldrich (St Louis, MO). Protease activated receptor-4 (PAR-4) agonist peptide (H-Ala-Tyr-Pro-Gly-Lys-Phe-NH_2 (AYPGKF-NH2) was purchased from GL Biochem Ltd. (Shanghai, China). Adenosine diphosphate (ADP) and acid-soluble collagen were purchased from Chrono-log Co (Havertown, PA). Type I collagen fibrils was purchased from Nycomed (Linz, Austria). Type I collagen-related peptide (CRP) was purchased from Dr. Richard Farndale (Cambridge University, Cambridge, UK). Ketamine was purchased from Pfizer, (Auckland, New Zealand) and xylazine was purchased from Ilium, Troy Laboratories Pty Ltd. (Smithfield, NSW, Australia).

2.2. TKI doses and preparation

Imatinib (STI-571) and nilotinib (AMN-107) were purchased from Novartis Pharmaceuticals (North Ryde, NSW) and dasatinib (BMS-354,825) from Bristol Myers Squibb (BMS; Mulgrave, Victoria) as a pure powder. TKI doses for murine testing was imatinib 25 mg/kg, nilotinib 25 mg/kg and dasatinib 5 mg/kg (equivalent animal dosing to that used in human CML patients), unless otherwise stated. All TKI's were dissolved in DMSO to a concentration of 10 mM. Stocks were diluted in phosphate buffered saline (PBS) pH 7.2 to give a final concentration of DMSO $< 0.02\%$ (v/v). PBS pH 7.2 was used as the sham control.

2.3. Antibodies

Anti-human CD63 antibody-unconjugated was purchased from Abcam (Redfern, NSW, Australia). Rabbit anti-mouse phycoerythrin (PE), and anti-rabbit fluorescein isothiocyanate (FITC) were purchased from Dako (Botany, NSW, Australia). Antibodies including FITC-conjugated anti-mouse CD62P, PE-mouse anti-human CD41a, PE-labelled mouse IgG isotype control, anti-mouse integrin β3 (CD62) and anti-mouse CD3e were purchased from BD Biosciences Pharmingen (Franklin Lakes, NJ). The mouse anti-human CD14-FITC antibody was purchased from Beckman Coulter (NSW, Australia). Anti-mouse PECAM-1 antibody (390) was obtained from Dr. Steve Albelda (University of Pennsylvania, Pennsylvania, USA). PE-conjugated JON/A monoclonal antibodies, anti-mouse integrin $\alpha_2\beta_1$ (CD49b), anti-mouse GPIbα-IX-V complex (CD42b), anti-mouse GPVI (JAQ1) and anti-mouse CD9 antibodies were purchased from Emfret Analytics (Würzburg, Germany).

2.4. Mice

Wild type C57BL/6 mice were purchased from ARC animal facility (Perth, WA). These mice were housed at RMIT University animal facility. All procedures were approved by the RMIT University animal ethics committee AEC1333.

2.5. Human blood samples

Whole blood was collected *via* venipuncture into 3.2% (w/v) trisodium citrate tubes from healthy donors from RMIT University and TKI-treated CML patients from the Department of Haematology, Austin Health (Heidelberg, Victoria), after informed consent. The Austin Health and RMIT University human ethics approved the study (HREC/14/Austin/404 and HREC/RMIT/18713). Samples were processed within 3 h of collection.

2.6. Haematological parameters

Haematological parameters of whole blood derived from nilotinib-treated mice *versus* sham control were determined as previously described using the Cell Dyn Emerald Haematology analyser (Abbott, Macquarie Park, Australia) [15].

2.7. Platelet preparation

Platelet rich plasma and washed human and murine platelets were isolated as previously described [16].

2.8. Platelet aggregation

Human donor platelet rich plasma (PRP) (100×10^9/L platelets) were treated with a range of doses (0.05–160 μM) of nilotinib, imatinib or dasatinib for 10 min at 37 °C before stimulation with 5 μM ADP, 1 μg/mL acid soluble type I collagen or GPVI-selective agonist, 0.5 μg/mL CRP. Platelet aggregation responses to agonists were recorded using a 4-channel light transmission aggregometer as previously described [17,18].

2.9. Flow cytometry

We investigated whether the prothrombotic effect of nilotinib may represent upregulated platelet glycoprotein expression or induction of conformational change and activation of the major platelet integrin αIIbβ3 measured by binding of JON/A monoclonal antibody/PE. C57BL/6 mice were treated with PBS or nilotinib 25 mg/kg and after 4 h, whole blood was collected and washed platelets were isolated. Platelets (100×10^9/L) were then labelled with respective antibodies to different platelet glycoproteins and their surface expression determined by flow cytometry as previously described [19,20]. In addition, washed human platelets were pretreated with various TKIs, imatinib, nilotinib or dasatinib prior to labelling with anti-human CD62P-FITC antibody. Flow cytometry was performed to determine the percentage of P-selectin exposure as a marker of platelet activation in response to alpha granule release [17,18]. Dense granule release was determined by flow cytometry as previously described [15].

In vitro analysis of murine platelet adhesion and thrombus formation using whole blood Whole blood from C57BL/6 mice was collected by cardiac puncture under anaesthesia and incubated with either 5–10 μM imatinib, 5–10 μM nilotinib, 0.1–1 μM dasatinib or sham control for 30 min at 37 °C. 0.5% (w/v) rhodamine labelled TKI-treated whole blood was perfused over Type I collagen matrix (500 μg/ml) for 6 min duration using a Zeiss Axiovert microscope. Thrombus formation was recorded in real time using Z-stack analysis and deconvolution of 3D reconstructions using a Zeiss Axiovert microscope and Axiovision Rel4.6 software. Thrombus growth and stability characteristics were determined in real-time by measuring fluorescently labelled platelets bound to damaged endothelium [15].

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