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Nilotinib inhibits the Src-family kinase LCK and T-cell function *in vitro*

Dear Editor:

In a recent issue of the *Journal of Cellular and Molecular Medicine*, it was reported that the tyrosine kinase inhibitor nilotinib (AMN-107, Tasigna®; Norvartis Pharmaceuticals, Basel, Switzerland), used for the treatment of chronic myeloid leukaemia, is able to inhibit the function of normal human T lymphocytes *in vitro* [1]. In addition, this group demonstrated nilotinib inhibits T-cell receptor (TCR) activation and the phosphorylation of signalling proteins involved in TCR activation. We have also investigated the effects of nilotinib on T cells and have expanded on the findings of Chen *et al.* by demonstrating that like imatinib [2], nilotinib is able to inhibit LCK, a Src-family kinase that plays a critical role in TCR activation [3].

Experiments were performed using normal human peripheral blood mononuclear cells (PBMCs) isolated by density centrifugation. Experimental use of human material was approved by the Royal Adelaide Hospital Ethics Committee and blood was collected with informed consent. 5′6 carboxyfluorescein diacetate succinimidyl ester (CFSE) staining of human PBMCs was performed as described previously [4]. In the presence of varying concentrations of nilotinib and imatinib (Novartis Pharmaceuticals), T cells were stimulated with 10 μg/ml phytohaemagglutinin (PHA) or Concanavalin A (ConA) (Sigma, St. Louis, MO, USA) or directly using 75 ng/ml of an anti-CD3 antibody (Mabtech, Stockholm, Sweden). Following a 5-day incubation, the proliferation of T cells determined by analysing CFSE dye dilution in cells stained positive by an anti-CD3 PE antibody (BD Biosciences, San Jose, CA, USA) (Fig. 1A). T-cell data were then analysed by Modfit analysis program (Verity Software, Topsham, ME, USA) and the proliferation index determined for each sample. Graphing these values allowed IC50 values for each drug to be determined. Inhibition of LCK by nilotinib and imatinib was analysed by evaluating the effects of each drug on LCK phosphorylation of a substrate peptide. Active LCK kinase (Cell Signaling, Danvers, MA, USA) was incubated with varying concentrations of both drugs in a standard kinase reaction buffer containing a mix of unlabelled and γ-32p labelled ATP and a Src-family kinase peptide substrate (Upstate Biotechnology, Lake Placid, NY, USA). The level of γ-32pATP labelled substrate, and hence LCK activity, was determined by blotting reactions on p81 filter paper (Whatman, Kent, UK) and determining radioactivity of each sample using a bench top scintillation β-counter.

Nilotinib inhibited T-cell proliferation at IC50's of 2–5 μM depending on the stimulus used (Fig. 1B). The IC50 values were roughly half that we observed with imatinib. We also observed a similar effect of nilotinib on T-cell activation marker expression and cytokine production (data not shown). LCK kinase activity was inhibited by nilotinib with an IC50 of 550 nM (Fig. 1C). Imatinib inhibited LCK at a similar concentration as reported in the literature [2] with an IC50 of 1250 nM, approximately twice the IC50 of nilotinib. In contrast to our findings, another group [5] found nilotinib to inhibit LCK weakly with an IC50 of 5200 nM. While this is somewhat less potent inhibition to that we obtained, the different IC50s could be due to technical differences in the kinase assays used. Abl has also been implicated in T-cell function [6–8] and it is possible that Abl inhibition by nilotinib may cause, or add to LCK blockade for the inhibitory effect on T-cell function. Nilotinib has 20-fold increased potency against Abl compared with imatinib [9] and we found it to have twice the potency against LCK. As nilotinib inhibited T cells approximately twice as strongly as imatinib, not 20 times as strongly, our data would suggest LCK inhibition may be the main mechanism by which the drug inhibits T-cell activation. Our findings agree well with those of Chen *et al.* [1] and expand on their results by demonstrating nilotinib inhibits the activity of the Src-family kinase LCK, and propose inhibition of LCK as the likely mechanism by which nilotinib interrupts TCR signalling and the function of T cells.

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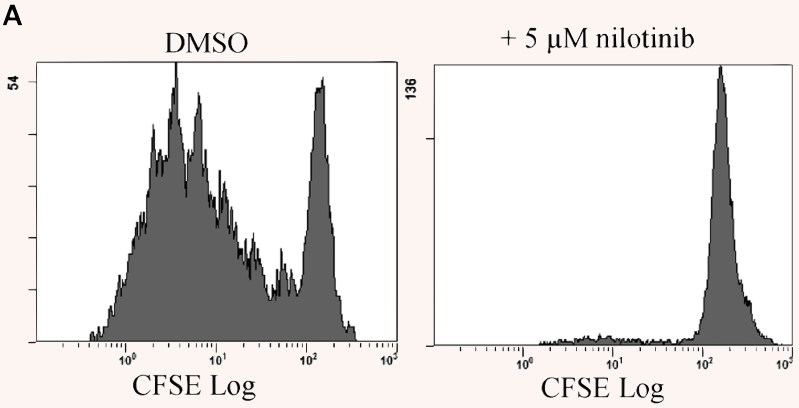
A



B

		PHA	Con A	anti-CD3
Nilotinib (μM)	IC50	2.4±2.1	4.5±3	2.15±1.9
Imatinib (μM)	IC50	4.2±1.1	5.5±2.9	5.8±3.2

C

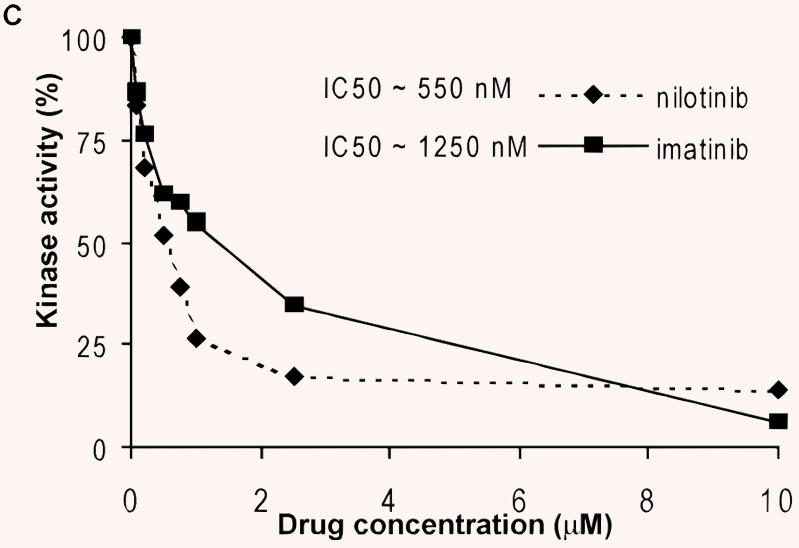


Fig. 1 Nilotinib inhibits T-cell proliferation and LCK activity. Following PHA stimulation, nilotinib was able to strongly inhibit the proliferation of T cells as determined by CFSE tracking (**A**). Using this CFSE data, the proliferation index at various drug concentrations was determined and used to calculate IC50 values for the inhibition of proliferation (**B**). Values represent the mean from five donors each analysed in different experiments and significant donor variability was seen as represented by large standard deviations. The effects of varying concentrations of nilotinib and imatinib on LCK kinase activity was determined and normalized to a percentage of maximum kinase activity when no drug was present allowing IC50 values to be determined (**C**). Data presented represent the mean of three independent experiments.

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No influence of the VAMP8 rs1010 single nucleotide polymorphism on platelet functions *in vitro*

Dear Editor:

Platelet activation plays an important role in the pathogenesis of arterial thrombosis [1], as illustrated by the preventive effect of antiplatelet agents on cardiovascular events such as acute coronary syndrome and myocardial infarction (MI). Platelet granule secretion is a key step in sustained platelet aggregation and clot formation. The main secretory organelles in platelets are dense granules and α-granules. Dense granules contain small molecules such as ATP, serotonin, calcium and ADP which, once secreted, contribute to the recruitment of other platelets. The α-granules contain large proteins such as fibrinogen, von Willebrand factor and growth factors, and their membranes express a specific receptor, P-selectin, that is used as a marker of degranulation. During platelet secretion, heteromeric complexes are formed between membrane proteins called soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) present on vesicles or granules (v-SNAREs) or on the target membrane (t-SNAREs) [2]. VAMP3 and VAMP8 (v-SNAREs) bind to syntaxin 4 (t-SNAREs) in platelets [3]. Recently, three independent studies comprising about 1820 cases and 1100 controls linked a *VAMP8* single nucleotide polymorphism (SNP: rs1010 A/G) to the risk of MI [4]: the rs1010 G *VAMP8* allele was associated with early-onset MI ($P = 0.025$, [OR] 1.75; CI 1.17–2.62).

Here we sought a functional effect of the rs1010 SNP on platelet functions *ex vivo*, independently of known cardiovascular risk factors and hormone status, in a homogeneous population of 100 healthy male Caucasian volunteers aged 18–35 years. They were recruited as described elsewhere [5] and were extensively phenotyped in terms of platelet function.

Venous blood was collected from each volunteer on two occasions one week apart, between 8:00 and 10:00 a.m. after an overnight fast, in 0.105 mol/l sodium citrate (BD Vacutainer, Becton Dickinson, Le Pont de Claix, France) with a 19-gauge needle. Platelet-rich plasma (PRP) was obtained by centrifugation at 150 × *g* for 10 min. at room temperature. Autologous platelet-poor plasma was used to adjust the platelet count of PRP to 250 × 10^9/l. Aggregation studies were performed within 2 hrs after blood collection, with the following agonists: arachidonic acid 1 mmol/l (Helena Biosciences Europe, Saint-Leu la Forêt, France), ADP 2 μmol/l (Sigma Aldrich, Saint Quentin Fallavier, France), the thromboxane receptor agonist U46619 1 μmol/l (Calbiochem, Merck Eurolab, Fontenay-sous-Bois, France), and Horm collagen 1 μg/ml (Nycomed Pharma, Paris, France). Maximal aggregation was recorded during a 5-min. period, and the results for the paired samples were averaged. We also determined the concentration of the thrombin receptor activating peptide (SFLLRN, Diagnostica Stago, Asnières, France), causing double-wave aggregation in the presence of 100 μmol/l amastatin. This concentration reflects the minimal SFLLRN concentration necessary to provoke granule secretion and sustained platelet aggregation. To evaluate maximal platelet secretion capacity, collagen (100 μg/ml), U46619 (10 μmol/l) or SFLLRN (100 μmol/l) was added to PRP in the presence of eptifibatide (Integrilin®, 4 μg/ml) to prevent aggregation. The expression of P-selectin (CD62P or GMP140) on the platelet surface was then quantified by flow cytometry as described elsewhere [5]. Genomic DNA was isolated from peripheral blood mononuclear cells by using the Qiamp Maxi Kit® (Qiagen, Courtaboeuf, France)

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Table 1 *Ex vivo* platelet functions according to the rs1010 genotype

	Platelet maximal aggregation (%)					Number of P-selectin copies on platelets			
Genotype	**Arachidonic acid, 1 mmol/l**	**ADP 2 μmol/l**	**U 46619 1 μmol/l**	**Collagen 1 μg/ml**	**Collagen lag time (sec)**	**Basal level**	**Collagen 100 μg/ml**	**U46619 10 μmol/l**	**SFLLRN 100 μmol/l**
AA (n = 39)	75.9 (69.0–79.1)	40.4 (26.9–67.4)	70.1 (48.8–75.9)	72.4 (67.6–76.7)	1.0 (0.8–1.2)	376 (287–552)	5607 (4534–7213)	10396 (7887–11229)	11356 (10211–12551)
AG (n = 49)	77.4 (72.9–79.8)	37.0 (27.7–67.9)	73.3 (67.0–79.4)	73.7 (68.9–80–7)	1.0 (0.8–1.2)	506 (288–621)	5443 (3209–7540)	9858 (8212–11708)	11309 (9579–12776)
GG (n = 12)	75.4 (71.9–78.0)	56.9 (24.5–73.1)	71.4 (65.9–77.9)	72.3 (59.4–75.2)	1.0 (0.9–1.2)	510 (300–577)	6579 (3913–7217)	9921 (5803–11969)	10268 (9259–12765)
P	0.38	0.83	0.30	0.45	0.90	0.34	0.74	0.9	0.62

Left: maximal platelet aggregation (%) and collagen aggregation lag time.
Right: P-selectin (CD62P) expression level on platelets at baseline and after activation, as determined by quantitative flow cytometry.
Owing to the skewed distribution of the continuous variables, trends across genotype groups were tested with the non parametric Kruskal–Wallis test. Data are expressed as medians and interquartile ranges.

according to the manufacturer's instructions. Rs1010 SNP status was determined by amplifying the 3′UTR region of the *VAMP8* gene, using 5′-CCGGGGGACCAAGGTACCTTCTGGGGCATACAcC-3′ and 5′-CTGGGTCACTCACTCTGCC-3′ as upstream and downstream primers, respectively. The upstream primer is a mutagenic primer that bears a sequence change at the penultimate position (lower case), introducing an *Nco I* restriction site into the amplicons when the A allele is amplified. Twenty microlitres of amplification mixture was subjected to *Nco I* (20 units) digestion at 37°C overnight, and the restriction products were resolved on 2% agarose gel. Digestion of the A allele yielded two products, of 318 and 32 bp, while the amplicon of the G allele remained undigested (350 bp).

The respective frequencies of the A allele (wild-type) and the G allele (mutated risk allele) were 0.63 and 0.37, in line with published data [4]. The genotype was in Hardy–Weinberg equilibrium. As shown in Table 1, the rs1010 variant was not associated with maximal platelet aggregation, whatever the agonist, even with a low ADP concentration (used to observe interindividual variations), nor with the lag time for collagen aggregation. We also analysed the aggregation surface, that is likely to better represent the reversibility of aggregation, but no difference was found according to the genotype (data not shown). Finally, we determined the minimal SFLLRN concentration necessary to provoke platelet secretion (and thus a biphasic aggregation profile) and found it to be similar in the two genotype groups (median values 9, 10 and 9 μmol/l for AA, AG and GG subjects, respectively).

P-selectin expression levels at the platelet surface upon stimulation with various agents are shown in the table. As expected, maximal P-selectin expression was achieved with the thrombin-receptor-activating peptide SFLLRN. We preliminary controlled that, in our experimental conditions, *i.e.* in the absence of calcium (citrated platelet-rich-plasma), such activation did not induce microparticule generation, that could have major influence on the analysis (microparticules were found below 1%, data not shown). As in the aggregation tests, no difference in P-selectin expression was observed between the genotype groups, whatever the agonist.

Thus, despite the use of weak (ADP) and strong (SFLLRN) platelet agonists and an accurate method to quantify α-granule secretion, the *VAMP8* rs1010 SNP had no observable impact on platelet functions *ex vivo*. Based on the allelic frequency of the *VAMP8* SNP and on the mean and standard deviation of the CD62P level after SFLLRN stimulation, this study had 80% power to detect a difference of 10% or more between carriers and non carriers of the mutated allele. A smaller effect of this SNP cannot therefore be ruled out.

This study has two limitations: first, we focused on α-granule secretion: we did not directly measure dense granule secretion, and the platelets were not subjected to shear stress. Second, we only tested platelets from healthy men with normal platelet function. It is conceivable that the rs1010 SNP might affect functions of circulating activated platelets in patients with cardiovascular

disease. Interaction with atherosclerotic plaque activates platelets and induces P-selectin expression *in vivo*, creating a reactive surface for leucocyte recruitment. It would be particular interest to measure the number of platelet-leucocyte aggregates [6, 7] in a population of genotyped patients with cardiovascular disease.

Despite these limitations, this study has the merit of being the first to test a putative association between a *VAMP8* gene variant and platelet functions *ex vivo*. Our negative results are in line with those of a recent study that failed to confirm the link between the VAMP8 SNP and coronary heart disease in a population of 2145 patients with familial hypercholesterolaemia [8].

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