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Full Length Article

In vitro anti-platelet potency of ticagrelor in blood samples from infants and children☆

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ABSTRACT

Introduction: Ticagrelor, a novel platelet inhibitor acting on the ADP-dependent $P2Y_{12}$ receptor, is currently approved for treating adults with acute coronary syndrome. The effect of ticagrelor in children has not been explored. As a first step, we here evaluate if the *in vitro* anti-platelet potency of ticagrelor in blood samples from children of different age is different as compared with in blood samples from adults.
Materials and Methods: Blood samples from 36 healthy children grouped by age (0–2 months, n = 6; 2–6 months, n = 6; 6 months-2 years, n = 6; 2–6 years, n = 10; 6–12 years, n = 8) and 13 adults were collected for *in vitro* analysis using vasodilator stimulated phosphoprotein phosphorylation (VASP) assay in whole blood and ADP-induced light transmission aggregometry (LTA) in platelet rich plasma. Ticagrelor (0.01 – 10 μmol/L) was added *in vitro* and its potency was assessed by calculating the concentration that provided 50% inhibition of the maximum response (IC_{50}).
Results: The in vitro potency of ticagrelor in blood from adults and in blood from children of any age group were comparable, both when analyzed with LTA and with VASP.
Conclusions: These *in vitro* results are consistent with the hypothesis that ticagrelor would achieve a comparable anti-platelet effect in children of different ages as in adults at equal plasma exposure.

1. Introduction

Normal platelet function is essential for haemostasis but may increase the risk for thrombotic events in patients with cardiac or extra-cardiac vascular disease. Accordingly, treatment with agents that inhibit platelet function is indicated in selected groups of patients with vascular diseases, including patients with acute and chronic coronary artery diseases, cerebral vascular diseases and peripheral arterial diseases. In paediatric patients platelet inhibition is indicated e.g. after implantation of systemic-to-pulmonary shunts [1], prosthetic heart valves [2] and ventricular assist devices [3]. The most widely used anti-platelet drug for paediatric patients is acetylsalicylic acid (ASA) [4], sometimes in combination with clopidogrel, a prodrug and irreversibly binding $P2Y_{12}$ antagonist [5].

Ticagrelor is a direct acting reversibly binding $P2Y_{12}$ antagonist that provides a higher and more consistent inhibition of ADP-induced platelet aggregation than clopidogrel [6,7], has a faster on-set and off-set of the pharmacodynamic effects [8], and is superior to clopidogrel in preventing further cardiovascular events in adult patients with acute coronary syndrome [9]. However, ticagrelor has not been evaluated in children.

The primary aim of this study was to compare the *in vitro* anti-platelet potency of ticagrelor in blood from groups of different aged children with blood from adults. A secondary aim was to compare baseline ADP-induced platelet aggregation between children and adults.

2. Methods

2.1. Patients

Thirty-six healthy children 0–12 years old and 13 healthy adults were enrolled in a prospective observational study from April 2012 to May 2013. The study was approved by the Regional Medical Research Ethics Committee and conducted in accordance with the Declaration of Helsinki. Informed written consent was given by all parents. Inclusion criteria were healthy children scheduled for minor surgical procedures. Children with known coagulation defects, medication or other conditions which

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might affect platelet function were excluded. All children were anesthetized by the same two anesthesiologists (FS and BSR). Enrolled children were divided according to age into six groups (0–2 months, n = 6; 2–6 months, n = 6; 6 months-2 years, n = 6; 2–6 years, n = 10; 6–12 years, n = 8). Children characteristics are presented in Table 1. The age range for the adults was 20–65 years. Weight data of the adults are not available.

2.2. Anesthesia

An intravenous Venflon cannula (BD Medical, North Ryde, Australia) was inserted, and the patient received atropine (10 μg/kg), alfentanil (10 μg/kg) and propofol (3–8 mg/kg). Some patients were anesthetized using mask inhalation induction with sevoflurane prior to being deeper anesthetized intravenously as described above. If the patient was to be intubated, atracurium (0.5 mg/kg) was added. The anaesthesia procedure was unchanged during the study period. None of the patients received any premedication except for paracetamol.

2.3. Study Protocol

Blood samples from the children were collected immediately after induction of anesthesia, from a peripheral vein using a Venflon cannula used for sampling only. The collected blood volume did not exceed what is recommended in current EU guidelines [10]. In addition, venous blood samples were collected from healthy adult volunteers. Blood samples of adults and children >10 kg were collected in sodium citrate tubes (Sarstedt AG, Nümbrecht, Germany), and samples of small children and infants (<10 kg) in Minicollect tubes (Becton Dickinson & Co, Franklin Lakes, USA), all with a final concentration of sodium citrate of 10.9 mmol/L.

Blood samples were centrifuged at 240 x g for 15 min to obtain platelet rich plasma (PRP). Ticagrelor was added *in vitro* to PRP and whole blood samples at 8 concentrations (0–10 μmol/L) and pre-incubated for 1 h at room temperature prior to Light Transmission Aggregometry (LTA) and VASP analysis, respectively.

Platelet count and VASP analysis were performed in all patients. Since minimum blood volume required to carry out the LTA analysis was 10 mL, this method, due to regulations [9], could only be performed in blood from children with a body weight of more than 15 kg (n = 18).

2.4. Analyses

2.4.1. Platelet Count

Platelet count in whole blood and PRP was analyzed with the Sysmex Instrument KX-21 N (Milton Keynes, United Kingdom).

2.4.2. VASP

The effect of ticagrelor on $P2Y_{12}$ receptor signaling was analyzed with the VASP ELISA (Biocytex, Marseille, France). The method detects the phosphorylation of VASP, which is one step in the signaling sequence which is initiated when the $P2Y_{12}$ receptor is activated by ADP.

Table 1
Patient characteristics.

Age	n	Girls/boys	Weight* ± SD (kg)	Platelet count ± SD ($x10^9$/L)	
				Blood	PRP**
0-2 months	6	1/5	4.7 ± 1.6	337 ± 156	-
2-6 months	6	0/6	5.9 ± 4.2	329 ± 86	-
6 months - 2 years	6	0/6	12.1 ± 3.8	205 ± 82	-
2 - 6 years	10	2/8	18.2 ± 11.8	243 ± 182	277 ± 118
6 - 12 years	8	7/1	30.3 ± 25.0	251 ± 91	305 ± 212
Adults	13	9/4	N/A	231 ± 84	318 ± 107

Key: PRP = Platelet rich plasma, n = number, SD = standard deviation. *Data not available for adults. **Not available in children <15 kg.

Whole blood was pre-incubated for 1 h at room temperature with 1 μL ticagrelor (final concentration range 0.01-10 μmol/L) or vehicle (5% mannitol). Fifty μL of blood was mixed with 50 μL VASP assay reagent containing prostaglandin E1 (PGE1) and in a separate test tube another 50 μL blood was mixed with 50 μL VASP assay reagent containing both PGE1 and ADP. After addition of 125 μL VASP assay lysis buffer to both test tubes samples were stored at −20 °C until analysis according to the manufacturer's instructions: An aliquot of each sample was added to a plate coated with anti-human VASP antibodies that bind both phosphorylated VASP (VASP-P) and non-phosphorylated VASP. Peroxidase-coupled antibodies specific for VASP-P were then added followed by tetra-methyl-benzidine (TMB), a chromogenic substrate to reveal peroxidase activity as an indirect measure of VASP-P levels. The optical density at 450nM was recorded using a plate reader (SpectraMax, Molecular Devices, Sunnyvale, USA). A platelet reactivity index (PRI) was calculated using the recorded optical density at 450 nm in the presence of PGE1 alone or PGE1 and ADP:

$$PRI\ (\%) = ((OD450nm\ [PGE1] - OD450nm\ [PGE1 + ADP])/(OD450nm\ [PGE1] - OD450nm\ [Blank])) * 100$$

A PRI of 100% represents the maximum platelet $P2Y_{12}$ receptor activation achieved by ADP.

2.4.3. Light Transmission Aggregometry

Blood was centrifuged at 240 x g for 15 min at room temperature. PRP was carefully removed and transferred to a clean vial. Platelet poor plasma (PPP) was prepared by centrifugation of the PRP at 2000 x g for 15 min at room temperature. Ticagrelor at increasing concentrations (0.04-10 μmol/L), or vehicle (5% mannitol) was pre-incubated with PRP at RT for 1 hour prior to initiation of aggregation by 20 μmol/L ADP. Platelet aggregation was continuously recorded for 6 min using the Platelet Aggregation Profiler (PAP-8E, Bio/Data Corporation, Horsham, USA) using PRP and PPP as 0 and 100% aggregation reference, respectively. Data for final aggregation extent, (FA), (%), at 6 min was analyzed.

The concentration ticagrelor that gave half- maximum inhibition (IC_{50}) were calculated for PRI (%) or FA (%) data using equation 201 (4-parameter Logistic Model) in XLfit version 5.2.0.0 (ID Business Solutions Ltd, Guildford, UK):

$$y = (A + (B\text{-}A/(1 + (x/IC50)s)))$$

Where y = PRI (%) or FA (%); A = minimum PRI (%) or FA (%); B = maximum PRI (%) or FA (%); s = slope of concentration response curve and x = ticagrelor concentration.

2.5. Statistical Analysis

Based on pilot experiments it was calculated that a minimum of six patients in each group was necessary to detect a 1.9-fold and 1.5-fold difference in ticagrelor geometric mean IC_{50} as measured with VASP and LTA respectively, when using a two-sided t-test on log-transformed data (with a power of 0.80, a significance level of 0.05 and assuming a standard deviation for log transformed VASP of 0.16 and standard deviation for log transformed LTA of 0.093). The chosen level for fold change indicates that for VASP, differences within the interval 90% less than adults to 90% greater than adults are considered to be comparable. The corresponding interval for LTA is 50% less than adults to 50% greater than adults.

The IC_{50} data had a skewed tendency and the statistical analysis was carried out using log transformed IC_{50} values with an estimate of standard deviation pooled from all IC_{50} values. Groups were compared using two-sided t-tests. VASP measurements of baseline ADP-induced platelet aggregation, reported as PRI, were not symmetric even after log transform, and hence groups were compared using the non-parametric Wilcoxon rank sum test. For *in vitro* potency as measured