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# A combined approach to early detect *in vitro* drug-induced hemostatic changes in preclinical safety

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ABSTRACT

Early detection of drug-induced alterations of hemostasis is challenging. Drugs can affect different components of the Virchow's triad and measurement of plasmatic coagulation times lacks sensitivity. New techniques for a more global assessment of the hemostasis are now available: the impedance platelet aggregometry, the thromboelastography and the thrombin generation measurement. The aim of this study was to evaluate three techniques (*i.e.*: Multiplate®, TEG® and CAT) for the *in vitro* detection of the effect of a drug known to induce hemostatic alterations in a preclinical safety environment. Cyclosporine A was chosen and tested at 4 concentrations after solubilization in DMSO in Wistar rats and Beagle dogs. The results obtained were comparable between both species except for the thrombin generation in platelet rich plasma. Enhanced platelet aggregability was observed after ADP stimulation and alterations of the thromboelastograms consisted in decreased maximum amplitude and increased LY30. A dual effect on thrombin generation was observed and suggested that CsA may interact with platelets in rat platelet rich plasma and speed up thrombin generation. The results of this study indicate that using a combined approach on hemostasis testing in preclinical safety it is possible to detect *in vitro* drug-induced alterations of hemostasis.



## 1. Introduction

Drugs can have pathological effects on hemostasis with thrombosis being of major concern and an important cause of market withdrawal (Kasliwal et al., 2005). In drug development, the early detection of drug-induced thrombosis is challenging because drugs can affect different components of the Virchow's triad (*i.e.* blood vessel wall, blood constituents and blood flow) (Ramot and Nyska, 2007). Different mechanisms can lead to thrombosis, such as endothelial lesions, changes in pro- and anti-coagulation mediator endothelial secretion and modification of the adhesion and aggregation responses of platelets (Ramot et al., 2013). In preclinical studies, further limitations arise from, for example, low sensitivity of the methods used when hemostasis is evaluated under non-physiological conditions or when animal models are used.

Different laboratory techniques are available for assessing various aspects of hemostasis, from platelet aggregation to whole blood viscoelastic properties and thrombin generation in clotting plasma (Hemker et al., 2002; Paniccia et al., 2015). Platelet aggregometry assesses platelet function *in vitro* after stimulation with a selected agonist at a defined concentration (*e.g.*: ADP, collagen, arachidonic acid, thrombin-derived peptides such as Par-4). The impedance Multiplate® aggregometer measures changes in the electronic resistance of whole blood that are proportional to the numbers of the platelets aggregating on the electrodes (Halimeh et al., 2010). This method has previously been optimized for use in the Beagle dog and Wistar rat (Defontis et al., 2013). Thromboelastography is a point-of-care diagnostic technique yielding a global assessment of hemostasis. It is based on continuous recording of the blood viscoelastic properties. Its advantage is a better reflection of the cell-based coagulation model, compared to the routinely used plasma coagulation times (Kol and Borjesson, 2010). Measurement of thrombin generation in clotting plasma has recently gained popularity after the development of a fluorogenic method on the Calibrated Automated Thrombogram (CAT). This methodology allows direct

*Abbreviations:* ADP, adenosine diphosphate; AUC, area under the curve; CAT, calibrated automated thrombogram; CsA, cyclosporine A; DMSO, dimethyl sulfoxide; ETP, endogenous thrombin potential; HDL, high density lipoproteins; Par-4, protease activated receptor-4; PPP, platelet poor plasma; PRP, platelet rich plasma; TEG, thromboelastography; ttPeak, time to peak.

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measurement of thrombin, the end product of the coagulation cascade, in platelet-poor and platelet- rich plasma and directly reflects bleeding or thrombotic tendencies (Kol and Borjesson, 2010). Overall, the above mentioned techniques have been mainly developed to detect bleeding conditions but could also be useful to identify thrombotic risk as well. However, there is a critical lack of validated reference compounds for hypercogulability and subclinical thrombosis testing with these techniques.

Extensive reviews on drugs associated with thrombotic complications in patients have been published and immunosuppressive agents such as cyclosporin are known to affect hemostasis (Al-Nouri et al., 2015a,b; Garwood, 2010; Ramot and Nyska, 2007; Ramot et al., 2013). In the last decades, the use of cyclosporin A in solid transplantation medicine has been linked with a remarkable increase in graft-survival rates (Dunn et al., 2001), but following its use in clinical settings, several studies have raised concerns about its adverse effects such as renal thrombotic microangiopathy and endothelial dysfunction leading to exposure of the sub-endothelium (Bombeli et al., 1996; Evans et al., 1997; Mercanoglu et al., 2004; Nolasco et al., 2009; Rezzani et al., 2001). The hypercoagulable effect of cyclosporin has since then been linked to the cyclosporin-induced alteration of lipid membranes, the externalization of phosphatidylserine (Tomasiak et al., 2007) and the increase in soluble P-selectin levels as a marker of platelet activation (Sahin et al., 2009).

In animals, numerous studies evaluating the effect of cyclosporin on renal function have been published and the rat is used as an animal model of nephorotoxicity (Jackson et al., 1987; Klawitter et al., 2009; Ouyang et al., 2014). In veterinary medicine, cyclosporin is used in dogs for treatment of inflammatory and auto-immune diseases and beside the most common gastrointestinal adverse effects, increases in creatinine and blood urea concentrations following treatment when compared to the baseline values have been reported which may suggest an effect of CsA on renal function (Archer et al., 2014; Steffan et al., 2005).

### 1.1. Objective

To evaluate three techniques (*i.e.*: Multiplate®, TEG® and CAT) for the *in vitro* detection of the effect of a drug known to induce hemostatic alterations in a preclinical safety environment.

## 2. Materials and methods

### 2.1. Animals

All procedures were approved by the Novartis Institutes for BioMedical Research and were performed at the Klybeck site of the Novartis Pharma AG in Basel, Switzerland. The animals did not receive any medication for 14 days prior to sampling.

The Beagle dogs (Marshall Europe, Green Hill, Montichiari, Italy) were between 12 and 22 months of age. The Wistar rats were supplied by Harlan laboratories B.V, Netherlands and the mean body weight was 400 g.

### 2.2. Blood sampling and analysis

#### 2.2.1. Blood sampling

*2.2.1.1. Beagle dogs.* Jugular venipuncture was performed using a 20G needle for S-Monovette (Sarstedt), after minimal manual compression of the vein. Blood was collected into either 2.6 ml S-Monovette hirudin tubes (Sarstedt) or in 10 ml S-Monovette trisodium citrate tubes (Sarstedt).

*2.2.1.2. Wistar rats.* After $CO_2$ euthanasia, blood was taken by cardiac puncture and punction of the caudal *vena cava* using a 20G needle connected to a multi-adapter (Sarstedt). The anticoagulant used was either hirudin (S-Monovette, 2.6 ml, Sarstedt) or trisodium citrate (S-Monovette, 2.9 ml, Sarstedt).

#### 2.2.2. Whole blood aggregometry (Multiplate®)

Whole blood aggregometry was performed as described in a previous study on hirudinized blood (Defontis et al., 2013) and the different optimal concentrations ($EC_{50}$) of each platelet agonist, as determined in that study, was use. Samples from 15 Beagle dogs (9 males, 6 females) were included for which the agonist concentrations were: 0.95 μM ADP and 0.23 μg/ml collagen. Samples from 10 male Wistar rats were analyzed and the platelet agonist concentrations used were: 2.70 μM ADP, 0.85 μg/ml collagen and 165.7 μM Par-4 agonist. The Par-4 agonist was not used in the Beagle dog since it had previously been shown to fail to induce platelet aggregation in this animal species (Defontis et al., 2013). Whole blood platelet aggregation was performed according to the manufacturer's instructions on two Multiplate® analyzers with the Multiplate® software version V2-03. Electronic controls were run daily before any measurement. Multiplate Mini Test cells were used for rat samples since they allow the use of small blood volumes (175 μl) (Lee et al., 2012). Briefly, samples were diluted 1:2 in an isotonic NaCl solution. After a 3-min incubation time and stirring at 37 °C, platelet aggregation was triggered by adding the agonist solution. Progressive platelet aggregation on the electrode pair of the Multiplate® test cells causes a resistance change (named "impedance") proportional to the number of platelets adhering to the electrodes. After a 15-min recording time, the final results are displayed as the area under the curve (AUC, expressed as U).

#### 2.2.3. Thromboelastography (Haemoscope TEG® 5000)

The viscoelastic properties of blood were assessed on samples from 15 Beagle dogs (9 males, 6 females) and 10 male Wistar rats. Single measurements were performed on citrated blood using three Haemoscope TEG 5000 (TEG® Hemostasis System) analyzers. Coagulation was initiated by mixing 340 μl citrated blood with 20 μl of a calcium-containing solution (0.2 M CaCl2). The recorded parameters are presented in Table 1.

**Table 1**
List of the recorded parameters on the Haemoscope TEG® 5000.

| Recorded parameter (unit) | Definition |
|---|---|
| R (min) | Period of latency from the time when blood was placed in the TEG analyzer until initial fibrin formation |
| K (min) | Time to reach a certain level of clot strength |
| Angle (degree) | Measure of the rapidity of fibrin build-up and cross linking |
| Maximum amplitude (MA, mm) | Direct function of the maximum dynamic properties of fibrin and platelet binding *via* glycoprotein (Gp) IIb/IIIa. |
| G/dyn/cm$^2$ | Measure of clot firmness in shear elasticity units |
| A (mm) | Measure of the trace width at the latest time point |
| LY30 (%) | Measure of the rate of amplitude reduction 30 min after MA has been reached; represents clot stability |