P-MO 050

Evaluation of ELISA-based VASP assay with whole blood samples from several animal species, and compatibility with various platelet antagonists

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ABSTRACT

The recent development of an ELISA-based adaptation of the VAsodilator-Stimulated Phosphoprotein (VASP) assay, one of the most specific tests to evaluate platelet P2Y12 inhibition, has opened the way for the use of this biomarker in large scale clinical studies in human.

Data presented within this poster demonstrate that this assay can also be used with whole blood samples from several animals, such as rat, rabbit and mouse.

Moreover, the assay appeared to be adaptable to evaluate antiplatelet drugs based on phosphodiesterases inhibition or soluble guanylate cyclase stimulation.

Thus, this assay should be considered as a valuable tool to evaluate new anti-platelet drugs targeting those cell signaling pathways.

INTRODUCTION, AIM OF THE STUDY

VASP is an intracellular platelet protein which can be phosphorylated by cAMP- or cGMP-dependent protein kinases (PKA & PKG) and plays a key role in platelet activation. The new ELISA-based CY-QUANT VASP/P2Y12 assay has proved to be a valuable tool to evaluate platelet P2Y12 antagonists in human. However, many anti-platelet drugs under current development target different pathways such as phosphodiesterases (PDE3 and PDE5) inhibition or soluble guanylate cyclases (sGC) stimulation. Thus, our aim was to determine whether the assay could be used to investigate the efficiency of such drugs, and moreover to examine its compatibility with non-human whole blood (WB) samples.

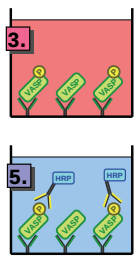
CONCLUSIONS

In addition to be a valuable tool for the evaluation of platelet P2Y12 receptor blockade by thienopyridines in human platelets, the new ELISA-VASP assay from BioCytex (CY-QUANT VASP/P2Y12) is compatible with whole blood from several animal species (rat, rabbit, mouse) and adaptable for the evaluation of several antiplatelet drugs including phopho-diesterases type 3 and 5 inhibitors, as well as nitric oxide donors. This ELISA-based format allows high throughput screening of many samples in parallel, and makes it particularly adapted to large scale studies.

PRINCIPLE OF THE TEST

CY-QUANT VASP/P2Y12 is a 96 wells microplate-based adaptation of BioCytex's gold standard flow cytometry-based PLT VASP/P2Y12 assay.

It enables the determination of platelet phophorylated VASP (VASP-P) in human whole blood under various experimental conditions.

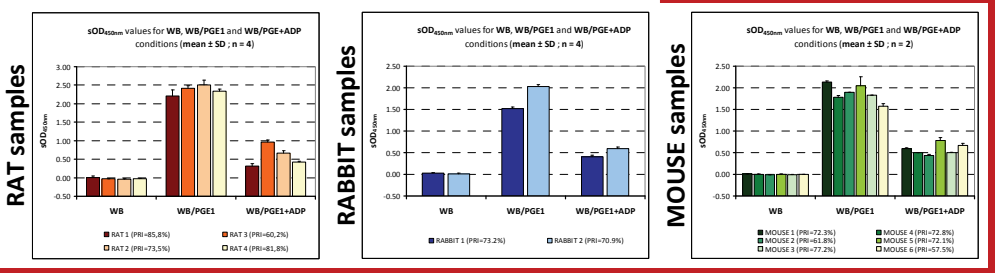


1. **WB** ACTIVATION (with **PGE1** / **PGE1+ADP**)
2. CELL **LYSIS**
3. ANTIGEN **CAPTURE** (Anti-**Total VASP** mAb)
4. *WASHING*
5. ANTIGEN **DETECTION** (Anti-**VASP-P** mAb)
6. *WASHING*
7. **REVELATION** (TMB)
8. **PRI** (Platelet Reactivity Index) **CALCULATION** from OD_{450nm}

COMPATIBILITY WITH WHOLE BLOOD FROM ANIMAL SPECIES

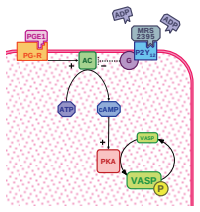
sOD_{450nm} were measured with CY-QUANT VASP/ P2Y12 kit using WB from rats, rabbits and mice, either untreated or activated with PGE1 alone or with a PGE1+ADP mixture (kit reagents), and PRI calculated from sOD_{450nm} for PGE1 and PGE1+ADP conditions.

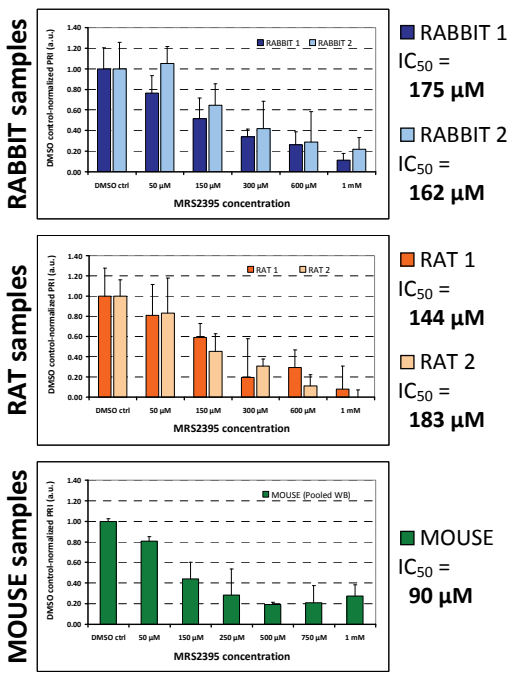
The data confirms that ELISA-VASP is compatible with WB from these 3 species and that the principle (PGE1/ PGE1+ADP activation) is transferable.



P2Y12 ANTAGONISTS

MRS2395, an antagonist for the P2Y12 purinoceptor, inhibits the ADP-mediated downregulation of adenylate cyclase (AC) in the presence of PGE1, leading to enhanced VASP phosphorylation by PKA due to increased cAMP.





RABBIT 1 IC_{50} = **175 µM**

RABBIT 2 IC_{50} = **162 µM**

RAT 1 IC_{50} = **144 µM**

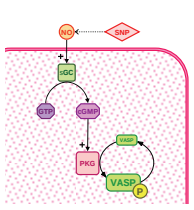
RAT 2 IC_{50} = **183 µM**

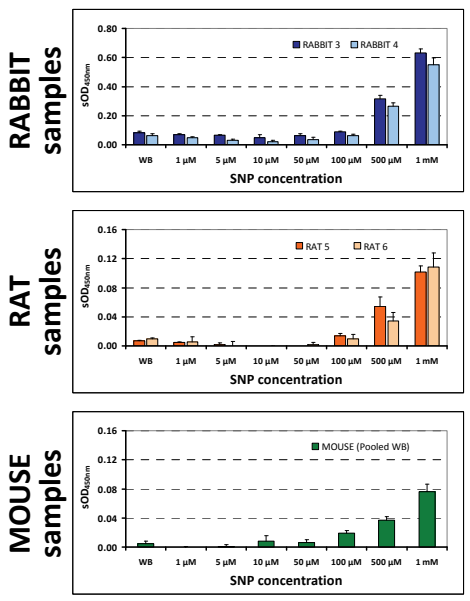
MOUSE IC_{50} = **90 µM**

The ELISA-based version of the assay is able to measure PRI changes subsequent to P2Y12 inhibition by MRS2395 in WB samples from rats, rabbits and mice. IC_{50} were comparable to human values (176 - 196 µM).

NO DONORS

Sodium nitroprusside (SNP) breaks down in circulation to release nitric oxide (NO) which stimulates cGMP production by soluble guanylate cyclase (sGC), resulting in increased VASP phosphorylation mediated by PKG.



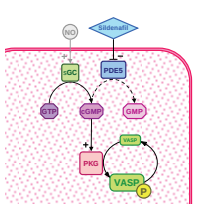


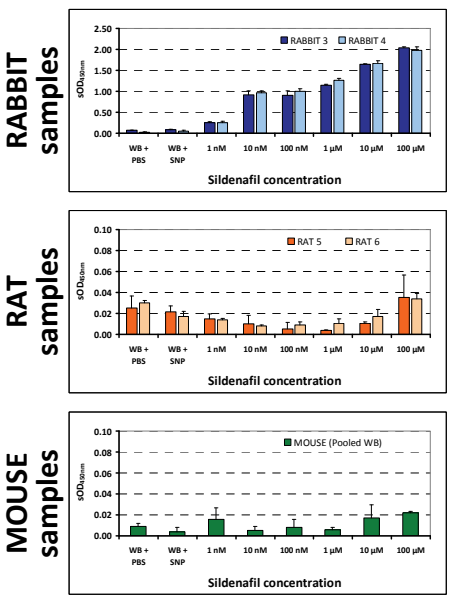
A significant increase of VASP phosphorylation is noticeable following treatment of rabbit WB samples with SNP (> 100 µM).

VASP phosphorylation is also observable in rat and mouse WB samples, but to a much lower extent under the same experimental conditions.

PDE5 INHIBITORS

Sildenafil (Viagra™) selectively inhibits phosphodiesterase type 5 (PDE5), leading to enhanced VASP phosphorylation in platelets by PKG, as a result to the increase of intracellular cGMP.



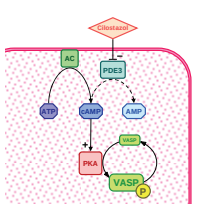


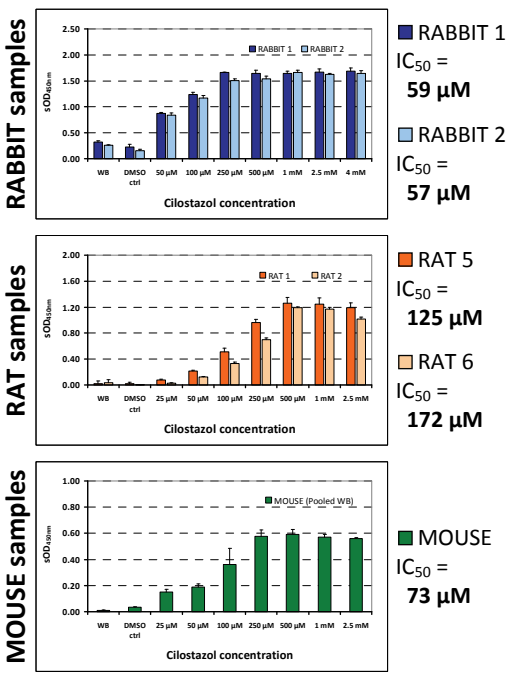
Strong concentration-dependent increase of VASP phosphorylation is noticeable following treatment of rabbit WB samples with Sildenafil in the presence of SNP (100 µM).

There is no significant VASP phosphorylation in mouse sample, and only a barely noticeable in rat sample with 100 µM Sildenafil, most likely due to a negligible effect of SNP at the concentration used.

PDE3 INHIBITORS

Cilostazol (Pletal™) is an antiplatelet drug which selectively inhibits phosphodiesterase type 3 (PDE3), enhancing VASP phosphorylation by PKA through an increase of intracellular cAMP.





RABBIT 1 IC_{50} = **59 µM**

RABBIT 2 IC_{50} = **57 µM**

RAT 5 IC_{50} = **125 µM**

RAT 6 IC_{50} = **172 µM**

MOUSE IC_{50} = **73 µM**

It is possible to measure an increase in VASP phosphorylation resulting from PDE3 inhibition by Cilostazol in WB samples from rats, rabbits and mice. Data was reproducible and IC_{50} were consistent between species.

MATERIAL AND METHODS

Chemicals : MRS2395, Cilostazol and SNP were obtained from Sigma-Aldrich France (Lyon, FRANCE), Sildenafil from Pzifer (New York, USA), CY-QUANT VASP/P2Y12 kits were produced by BioCytex (Marseille, FRANCE).

WB samples : rat samples were obtained from Syncrosome (Marseille, FRANCE), rabbit samples from Agrobio (La Ferté St Aubin, FRANCE) and mouse samples were collected at BioCytex (anticoagulant = sodium citrate). Samples were treated within 24 hours from collection. When necessary, WB samples from several mice were pooled.

Presented data : triplicate measurements were performed for each condition, mean ± SD are displayed. sOD_{450nm} = blank-corrected OD_{450nm}. IC_{50} were determined using Sigma plot software "4-parameters regression".

Animal blood compatibility assays : WB was processed and PRI were calculated according to kit procedure. One additional "WB" condition without activation was performed (PBS-citrate instead of PGE1/PGE1+ADP).

MRS2395 assays : WB was mixed (v/v) with MRS2395 solution (C_{Final} = 50 µM - 1 mM) and incubated 20 min at RT. Complete protocol was applied (including activation) and PRI were calculated according to kit procedure.

SNP assays : WB was mixed (v/v) with SNP solution (C_{final} = 1 µM - 1 mM), and incubated 5 min at 37°C. Then, samples were treated according to kit protocol omitting the activation step, and OD_{450nm} were measured.

Cilostazol assays : WB was mixed (v/v) with Cilostazol solution (C_{Final} = 0.025/0.05 - 2.5/4 mM) and incubated 1h at 37°C. Samples were treated according to kit protocol omitting the activation step, and OD_{450nm} measured.

Sidenafil assays : WB mixed with ½ vol. Sildenafil solution (C_{final} = 1 nM - 100 µM) and ½ vol. SNP (C_{final} = 100 µM) was incubated 5 min at 37°C. Samples were processed omitting the activation step, and OD_{450nm} measured.

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