Tacrolimus diffusion across the peripheral mononuclear blood cell membrane impact of drug transporters

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Tacrolimus diffusion across the peripheral mononuclear blood cell membrane: impact of drug transporters

Running title: Tacrolimus diffusion in PBMC

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ABSTRACT

Measuring tacrolimus (TAC) concentration in peripheral blood mononuclear cells (PBMC) could better reflect the drug effect on its target (calcineurin (CaN) in lymphocytes) than whole blood concentrations. Mechanisms influencing TAC diffusion into PBMC are not well characterized. This work aimed at describing, *ex-vivo,* TAC diffusion kinetics into PBMC and investigating the contribution of membrane transporters to regulate TAC intracellular concentration as well as the impact on CaN activity.

PBMC were incubated with TAC for 5 min to 4h and under several experimental conditions: 37°C (physiological conditions) 4°C (inhibition of influx and efflux active transport), 37°C + transporter inhibitors (verapamil, carvedilol, probenecid, bromosulphophtalein respectively inhibitors of P-gp, OAT, OATP). TAC concentration and CaN activity were measured in PBMC using liquid chromatography coupled with mass spectrometry.

TAC intra-PBMC concentration was maximal after 1h of incubation. Mean TAC PMBC concentrations were significantly lower in samples incubated at 4°C compared to the 37°C groups. Addition of verapamil slightly increased TAC accumulation in PBMC while other inhibitors had no effect. A significant correlation was found between TAC intra-PBMC concentration and the level of inhibition of CaN.

Using an *ex-vivo* cellular model, these results suggest that P-gp is involved in the drug efflux from PBMC while influx active transporters likely to regulate TAC intra-PBMC disposition remain to be

identify. TAC concentration in PBMC is correlated with its pharmacodynamic effect. Then, TAC intra-PBMC concentration appears to be a promising biomarker to refine TAC therapeutic drug monitoring.

Keywords: tacrolimus; calcineurin; peripheral blood mononuclear cells; membrane transporter; diffusion

ABBREVIATIONS

TAC: tacrolimus; CaN: calcineurin; PBMC: peripheral blood mononuclear cells; P-gp: P-glycoprotein; BSP: bromosulphophtalein; PBS: Phosphate buffer saline; UHPLC-MS/MS: Ultra High-Performance Liquid Chromatography tandem mass spectrometry; SEM: standard error of mean; I_{max}: maximal inhibition of the CaN activity; FKBP: FK Binding Protein

INTRODUCTION

Tacrolimus (TAC) is the main immunosuppressive drug prescribed in solid organ transplant patients. Its effect is mediated through the inhibition of intracellular calcineurin (CaN), a serine-threonine phosphatase enzyme, which results in the inhibition of interleukine-2 (IL-2) synthesis by T-cells. Because TAC exerts its pharmacological effect in the cytosol, the determination of intracellular TAC concentrations has been suggested to be of better relevance than the conventional whole blood therapeutic drug monitoring of TAC. The amount of TAC in lymphocyte cells or, for practical reasons, in peripheral blood mononuclear cells (PBMC, a leukocyte fraction enriched in lymphocytes), could represent the fraction of TAC which exerts immunosuppressive effects.

Numerous clinical studies have demonstrated a lack or a weak relationship between TAC whole blood concentrations and TAC concentrations in PBMC in various organ transplant patients [1–3]. These results could, at least partly, be explained by the action of drug transporters located in the membrane of PBMC. Actually, the role of one of these drug transporters, P-glycoprotein (P-gp), an efflux pump

encoded by *ABCB1* gene, has already been suggested to be a determinant of TAC PBMC disposition [4–6]. However, many other drug transporters, such as multidrug resistance proteins and, to a lesser extent, breast cancer resistance protein, have been reported to be present in the lymphocyte membrane and could also modulate TAC disposition into the cell [7]. TAC is also a possible substrate for influx transporters and the influence of these proteins on TAC cellular accumulation remains to be investigated. This issue is of major interest since drug disposition in PBMC could determine the level of inhibition of the target enzyme. In the case of TAC, an increase of CaN activity is suggested to occur before acute cellular rejection and could be one of the most relevant pharmacodynamic indicator for predicting this adverse event [8]. In this context, the aim of the present study was to explore TAC disposition in PBMC using an *ex-vivo* PBMC model. More specifically, our objectives were i) to highlight the role of drug transporters on drug intracellular distribution and ii) to assess intracellular pharmacokinetic/pharmacodynamic relationship between TAC concentration in PBMC and CaN activity.

MATERIAL AND METHODS

Reagents and materials

Tacrolimus was purchased from LGC standards (Folsheim, France). Ascomycin was purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Methanol and acetonitrile of LC/MS grade were purchased from Carlo Erba Reagents (Val-de-Reuil, France). Ammonium acetate, ammonium sulfate, zinc sulfate heptahydrate and formic acid were obtained from Fisher Chemicals (Waltham, MA, USA). Water was purified using a Milli-Q® Ultrapure Water System (Merck Millipore, Milford, MA, USA). Heparin Choay® was purchased from Sanofi (Paris, France), RosetteSep™ Human Granulocyte Depletion Cocktail was provided by Stemcell (Vancouver, BC, Canada). Phosphate buffer saline (PBS) free from magnesium and calcium was obtained from Life technologies (Paisley, Scotland, UK). Ficoll density gradient was performed using the UNI-SEP maxi U-10 Novamed device (Courtaboeuf, France). Erythrocyte lysis buffer was obtained from Qiagen (Hiden, Germany). RPMI-1640 medium was purchased from Sigma Aldrich (Saint-Quentin-Fallavier, France). Verapamil hydrochloride, probenecid and carvedilol were obtained from Sigma Aldrich (Saint-Quentin-Fallavier, France). Bromosulphophtalein (BSP) was provided by Santa Cruz Biotechnology (Dallas, TX, USA).

Isolation and purification of the PBMC

Human whole blood was collected in patients with hemochromatosis attending the Liver Disease Department of Rennes University Hospital (France) for a phlebotomy. The study was approved by the local ethical committee (approval 13-44, July 12 2013) and patients' consents were obtained to use their blood for research purpose. Blood samples were heparinized immediately after the phlebotomy and sent timely to the laboratory for the isolation of PBMC. First, eight aliquots of approximately 15 mL of blood were incubated for 5 min with 100 µL of specific antibodies in order to remove contamination by neutrophils. Then, blood was diluted (twofold) in PBS and cells were isolated by density gradient centrifugation at 1200 g for 20 min at 18°C. The PBMC layers were gathered and washed with 40 mL of PBS and the suspension was centrifuged at 350 g for 10 min at 10°C. The pellet was mixed with 20 mL of buffer to lyse residual red blood cells. PBMC were then washed twice with 40 mL of PBS and centrifuged slowly at 120 g for 15 min at 18°C to remove residual platelets. The supernatant was discarded and a suspension enriched in PBMC was obtained by adding 8 mL of fresh PBS. The amount of PBMC in the cell suspension and the efficiency of the purification were determined by flow cytometry according to a validated procedure previously described [9]. Aliquots of 2.10^6 cells were spread in 21 glass tubes and diluted to 2 mL with a mixture of RPMI + L-glutamine (0.3 mg/L) and fœtal bovin serum (10% v/v). Final cells suspensions (10^6 cells/mL) were kept overnight at 4°C before experiment of tacrolimus diffusion. In these conditions, the viability of cells was preserved.

TAC transport in PBMC assay

The transmembrane mechanisms involved in the regulation of TAC disposition in PBMC were investigated by sequential experiments in presence or absence of fixed concentrations of transporter inhibitors (added to the cell suspension 2 h before TAC addition). First, cells were incubated with TAC in RPMI medium under agitation for 0 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h and under three experimental conditions: 37°C (control to mimic physiological conditions), 4°C (to inhibit influx and efflux active transport), 37°C + 40 µM verapamil (to inhibit P-gp). Cells were placed at their temperature of incubation 2h before TAC addition. This assay was performed by incubating 2.10^6 cells in 2 mL with 500, 1000, 2000 pg of TAC (i.e 250, 500 and 1000 pg/10^6 cells respectively) to

assess the influence of TAC extracellular concentration on intracellular accumulation. Two other sets of experiments were performed with TAC at 500 pg/10^6 PBMC (i.e. extracellular concentration generating intracellular concentration closer to those reported in patients *in vivo*) by replacing verapamil by carvedilol 10 µM (inhibition of efflux by P-gp) or a probenecid and BSP cocktail 100 µM (inhibition of OAT/MRP and OATP respectively).

Quantification of tacrolimus in PBMC and CaN activity determination

After each incubation time, aliquots of cells suspension were splitted in two samples, one for the determination of intracellular concentration of TAC and another one for the measurement of CaN activity (each one of 1 mL containing 1 million of cells). Samples were centrifuged 4 min at 5000 rpm, (the supernatants were discarded and the cell pellets were lysed with 1 mL of methanol or 20 µL of lysis buffer for the intracellular concentration and the enzyme activity measurement, respectively). Samples were stored at -80°C prior to analysis. TAC concentration in PBMC was determined using a validated Ultra High Performance Liquid Chromatography tandem mass spectrometry (UHPLC-MS/MS) method adapted from a previously published method [2]. The activity of CaN was measured by HPLC-Ultraviolet according to Blanchet et al. [10]. The study experimental design is illustrated in figure 1.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Comparisons between independent experimental conditions were performed with Kruskal-Wallis test. Comparisons between control and test conditions samples were performed with a Wilcoxon paired-test. Correlations were assessed using Spearman test. The R software (version 1.1.3) was used for the statistical analysis. A p-value < 0.05 was considered statistically significant.

RESULTS

Influence of TAC extracellular concentration on TAC intracellular accumulation

The accumulation of TAC in PBMC increased with TAC extracellular concentrations and appeared maximal (Cmax) after 1 hour (figure 2). The relationship between TAC accumulation in PBMC and TAC extracellular concentrations seemed roughly linear over the studied range (Cmax at T1h: 50.6, 82.5, 208.9 pg/millions of cells at 250, 500, 1000 pg/mL respectively). A strong correlation was observed between intracellular concentration at Cmax and the intracellular area under the curve ($AUC_{0\text{-}4h}$) ($r=0.98$, $p<0.001$). The subsequent experiments were conducted using a TAC extracellular concentration of 500 pg/mL i) to avoid the saturation of transporters likely to occur with an excess of drug in the medium (despite saturation did not seem to have been reached at 1000 pg/mL) and ii) to allow to obtain intra-PBMC concentrations closer to that obtained in transplant recipients treated with TAC [11,12].

Effect of transporters inhibition on the accumulation of TAC in PBMC

The mechanism behind TAC distribution across the cell membrane was investigated by comparing the effects of several inhibitors on the intracellular concentration of the drug (figure 3). First, we observed that TAC accumulation in PBMC was significantly decreased at 4°C compared to the control condition. Indeed, from 30 min of incubation, TAC concentration in cells at 4°C was about 60% lower than in control cells. A slight but significant increase of TAC accumulation in cells was observed with verapamil from 30 min to 1 h of incubation (+14% and +11% respectively). During the last period of incubation (from 2 h), the same trend was observed but did not remain statistically significant. In contrast, neither carvedilol nor BSP or probenecid had any effect on TAC concentrations in PBMC.

Relationship between TAC intracellular accumulation and CaN activity in PBMC

As shown in Figure 4, the effect of verapamil on CaN activity appeared noticeable only when a high TAC extracellular concentration was applied (1.4 fold increase of the CaN maximal inhibition (I_{max}) at 1000 pg/mL). A trend to a lower CaN inhibition was observed at 4°C. The enzyme inhibition increased

with TAC extracellular concentrations when influx or efflux transporters were inhibited (verapamil and 4°C) but not in control condition. Overall, Imax was reached after 2h of incubation with tacrolimus whereas intracellular Cmax occurred after 1h of incubation (data not shown).

A significant correlation was found between TAC intra-PBMC concentration and corresponding CaN I_{max} (figure 5). Even at high TAC intra-PBMC concentration, a total inhibition of CaN activity was not achieved. An IC_{50} could be graphically determined at 160 pg of TAC per million of cells (or 0.160 ng/mL).

DISCUSSION

Membrane transporters play an essential role in the pharmacokinetics of drugs since they mediate exchanges between biological compartments. Their functionality can be modulated by extrinsic factors such as drug-drug interactions or intrinsic factors like genetic polymorphisms or physio-pathological conditions [13,14]. Thus studies aiming at deciphering the role of transporters on drug concentrations might be particularly relevant to explain pharmacodynamic variability. TAC has a narrow therapeutic index and intra- and inter-patient variabilities of its pharmacokinetics and pharmacodynamics have been extensively described [15]. Hence, new insights on the involvement of influx and efflux transporters on TAC distribution in its target cell and on the interplay with its intracellular pharmacological target (CaN) are of value to improve our understanding of drug response variability (figure 6).

In the present study, TAC diffusion in PBMC was assessed using an *ex-vivo* model of freshly collected cells. This model was chosen because it closer represents clinical conditions than recombinant cell lines overexpressing a selected protein isoform. Nevertheless, this approach has some drawbacks. Indeed, the magnitude of the effect due to a particular protein can be weakened and its contribution regarding drug transport can be more difficult to bring into light. This kind of model could also be more sensitive to inter-experiments variability, though it reflects the actual variability observed *in vivo*.

This study confirmed the key role of active transporters in TAC disposition in PBMC.

Our results highlight the involvement of P-gp in the efflux of TAC out of PBMC since intra-PBMC TAC concentrations were increased with verapamil (P-gp inhibitor). They are consistent with previous studies showing that recombinant cells overexpressing P-gp had reduced TAC intracellular concentration compared to control or demonstrating the association between a single nucleotide polymorphism in ABCB1 gene (1199G>A) and a significant increase of TAC accumulation in PBMC [4–6]. Nonetheless, in our study, the effect of verapamil was less marked than expected, maybe because of the low specificity of this inhibitor. Similarly, in a study focused on the elucidation of transport mechanisms of HIV drug, Janneh *et al.* [16] observed a lack of effect of verapamil on drug accumulation in PBMC whereas incubation with more specific inhibitors led to significant changes in intra-PBMC concentrations. Because specific and potent inhibitors are usually synthetic compounds unable to be administrated in patients for therapeutic reasons, we decided to use verapamil since it could more likely cause a drug-drug interaction with TAC in clinical practice. Nonetheless, carvedilol has also been reported to inhibit P-gp [17] and its influence on TAC accumulation was also evaluated. No effect on TAC accumulation was observed with carvedilol compared to control suggesting that P-gp would not be the exclusive transporter involved in TAC efflux. However, a noticeable variability of carvedilol effect was observed between samples leading to a non-significant average effect. In transporter inhibition experiments, the concentration of inhibitors is a critical parameter which can strongly impact the results. In our study, we chose the concentrations of inhibitors according to the literature and in particular according to Ki reported for P-gp [18,19]. We used concentrations above the Ki of the inhibitors for P-gp. Besides, a limit of our experimental design is the lack of determination of the level of actual protein expression in the cells membrane of each volunteer, or the research of genetic polymorphisms usually known to alter P-gp activity [20]. Such investigations could not be performed in the current study but it could have provided additional information to analyze the data given that high differences in the relative expression of transporters in PBMC membranes have been previously described [16].

A major finding of the present work is the reduced TAC intracellular accumulation observed when both influx and efflux active transports were inhibited (4°C). These results might suggest that active influx transporters could play a role in TAC uptake in PBMC. We chose to investigate the contribution

of two families of influx transporters (OAT and OATP) likely to be expressed in PBMC membranes [21,22] and whom influence on drug pharmacokinetics have been highlighted in several works [13,23–26]. Neither probenecid (OAT inhibitor according to [26]) nor BSP (OATP inhibitor according to [19]) had any impact on TAC accumulation in our experiments suggesting that these transporters are not involved in TAC uptake in PBMC. Again, the choice of these inhibitors could be discussed since probenecid can also inhibit MRP efflux protein [27,28]. The identification of influx solute carrier transporters (SLC) expressed in PBMC, whom TAC might also be a substrate, remains to be established. Pharmacogenetics studies underlined the potential interest, in TAC pharmacokinetics, of other SLC transporter isoforms which could be candidates for future studies [29,30]. Besides, based on our data, we cannot fully rule out the hypothesis that the decrease of TAC accumulation in cells at 4°C would be due to a lower passive diffusion across the lipid membrane. Indeed, since TAC is a lipophilic drug (Log P≈3.3), it is expected that its concentration measured in PBMC results in the combined effect of passive and active diffusion in both directions across the cell membrane [31]. We postulated that at low temperature, only the passive diffusion phenomenon was observed. However, for some substrates, temperature can alter passive diffusion as well [31] by changing the shape of the cell and the membrane stiffness.

The present work also explored the relationship between TAC concentration in PBMC and its pharmacodynamic effect on CaN and the influence of drug transport on this relation. Inhibition of active transporters (4°C) seemed to have reduced the pharmacodynamic effect of TAC. This could be related to the lower TAC concentrations available to interact with its target in the cytosolic compartment. Verapamil increased TAC effect on CaN only at the highest concentrations of the drug (extracellular concentration of 1000 pg/mL). In these conditions, however, intra-PBMC TAC concentrations were higher than the median range observed *in vivo* in transplanted patients [11,12]. Similarly, Vafadari *et al.* investigated the effect of P-gp activity modulation by verapamil and genetic polymorphisms on IL-2 synthesis in T lymphocytes [32]. They reported an enhanced TAC-mediated inhibition of IL-2 synthesis in T cells by verapamil in 3435CC (i.e. wild-type) patients. However, the intracellular concentration of TAC was not determined in their study.

Furthermore, our data show a significant correlation between the increase of TAC intra-PBMC concentration and the ability of TAC to inhibit CaN activity. It emphasizes the reality of the intracellular

TAC pharmacokinetic/pharmacodynamic relationship and strengthens the relevance of the monitoring of TAC intra-PBMC concentration to refine therapeutic drug monitoring as suggested elsewhere [33,34]. Indeed, monitoring TAC treatment efficacy using pharmacodynamic biomarkers would be of particular interest [35] but it remains technically cumbersome. Therefore, using TAC intra-PBMC concentration would be a surrogate marker easier to implement in clinical practice. Among pharmacodynamic parameters reflecting TAC effect, the clinical utility of CaN activity measurement was reported by several authors [8,36,37] and an important inter-patient variability was described in their works as in our study. Such a variability as well as the imperfect correlation observed with the intracellular concentration ($r = -0.5$) could be explained by wild differences in the relative level of FK Binding Protein (FKBP) isoforms in PBMC from different patients [38]. We also observed uncomplete inhibition of CaN in PBMC despite high intracellular concentrations of TAC. Similarly, Kung et *al.* reported a partial inhibition of CaN at saturating TAC concentrations in PBMC and showed that the amount of active FKBP in cells was the limiting factor [39,40]. In our study, we estimated that CaN IC_{50} was reached for an intra-PBMC TAC concentration around 0.16 ng/mL, a threshold hardly achieved with usual TAC exposure. Indeed, our team already reported a limited inhibition of CaN in liver transplant recipients with mean TAC whole blood trough concentration of 5.4 ± 3.1 ng/mL [11]. Moreover, CaN IC_{50} has been shown to be reached for TAC whole blood trough concentration up to 26.4 ng/mL which is over the usual upper recommended therapeutic threshold (i.e. 15 ng/mL) [41].

Finally, our results have some limits. First, our data should be confirmed in a larger study with more experimental replicates to overcome the hurdle of lack of statistical power. Secondly, our observations were performed in PBMC. Despite the fact that this subcellular fraction is mainly composed of lymphocyte T (around 60%), we cannot certify that TAC diffusion would be the same in a cell suspension of lymphocyte T only.

CONCLUSION

Our results suggest that TAC disposition in PBMC is likely to be determined by influx and efflux active transporters using an *ex-vivo* cellular model. P-gp seems involved in the drug efflux whereas OAT and OATP do not appear to influence the drug uptake in PBMC. Importantly, TAC concentration in PBMC is correlated with its pharmacodynamic effect through CaN activity inhibition. Consequently, an accurate identification of the PBMC membrane transporters network involved in TAC intracellular accumulation should be further investigated to manage drug response variability and to make a step forward in precision medicine in solid organ transplant recipients treated with TAC.

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Disclosures

The authors declare no conflict of interest regarding this study.

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FIGURES LEGENDS

Figure 1: Experimental workflow of the TAC intra-PBMC accumulation assay

PBMC: peripheral blood mononuclear cells, TAC: Tacrolimus, P-gp: P-glycoprotein, UHPLC-MS/MS: ultra-high performance mass spectrometry

Figure 2: Effect of TAC extracellular concentrations on the accumulation of TAC in 1 million PBMCs incubated for 4h at 37°C. TAC concentration in cells over time is expressed by mean +/- SEM (n=3). Trapezoidal area under the curve (AUC0-4h) at 250, 500 and 1000 pg/mL were significantly different (kruskal-wallis test, $p<0.05$).

TAC: tacrolimus, PBMC: peripheral blood mononuclear cells, SEM: standard error of the mean

Figure 3: Kinetic of accumulation of TAC (500 pg/mL) in PBMC and effect of transporters inhibitors. Results are expressed as mean PBMC concentrations ratio +/- SEM (n=6). *P<0.05 (Wilcoxon paired test) compared to control (incubation at 37°C without inhibitor).

TAC: tacrolimus, PBMC: peripheral blood mononuclear cells, SEM: standard error of the mean, BSP: bromosulfophthalein

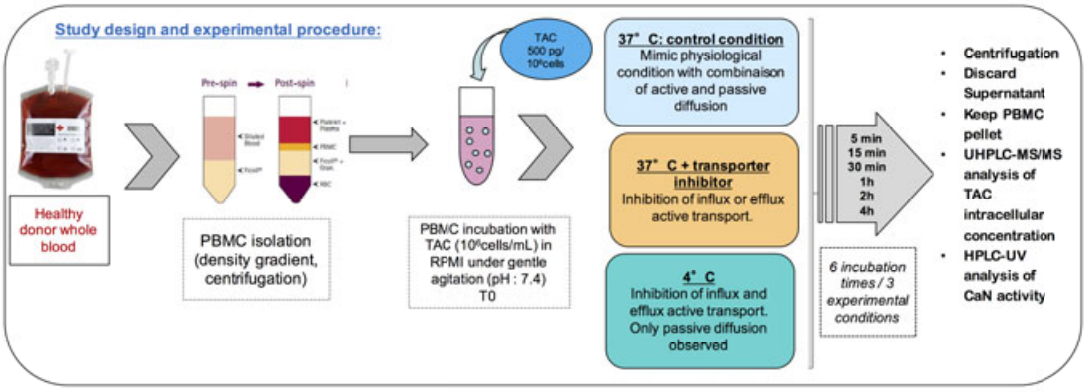
Figure 4: Effect of transporters inhibition on CaN activity depending on TAC extracellular concentration. CaN activity inhibition (Imax) was calculated as the maximal percentage of decrease of the CaN activity compared to T0 (before the addition of TAC in the medium) on the incubation period 0-4h. Results are expressed as mean +/- SEM (n=3).

Figure 5: Correlation between maximal TAC concentration in PBMC and maximal inhibition of the CaN activity (Imax). (n=24, pooled data from experiments at 37°C, at 37°+verapamil, or 4°C with TAC concentration of 250 or 500 or 1000 pg/mL (Spearman correlation coefficient = -0.52, p=0.009)

CaN: calcineurin, TAC: tacrolimus, PBMC: peripheral blood mononuclear cells

Figure 6: Mechanistic model of the influence of drug transport on TAC intra-PBMC accumulation

TAC: tacrolimus, PBMC: peripheral blood mononuclear cells, ABC-transporter: ATP-binding cassette transporteur, SCL transporter: solute carrier transporter



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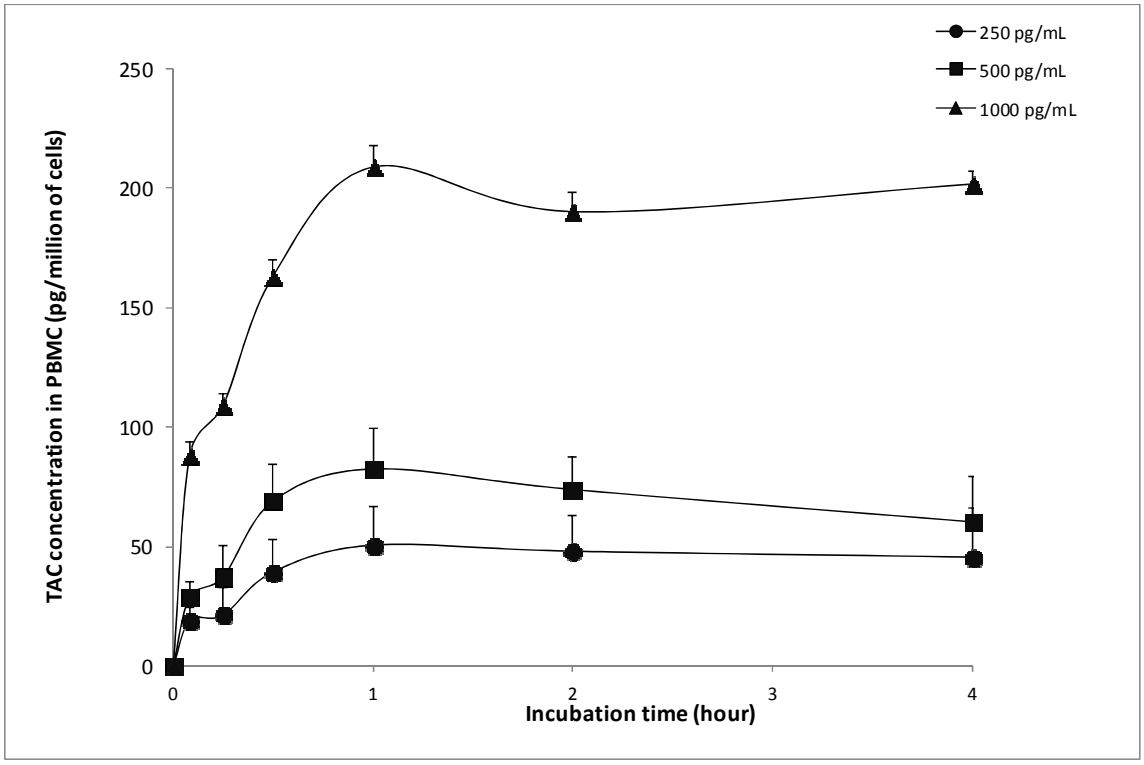

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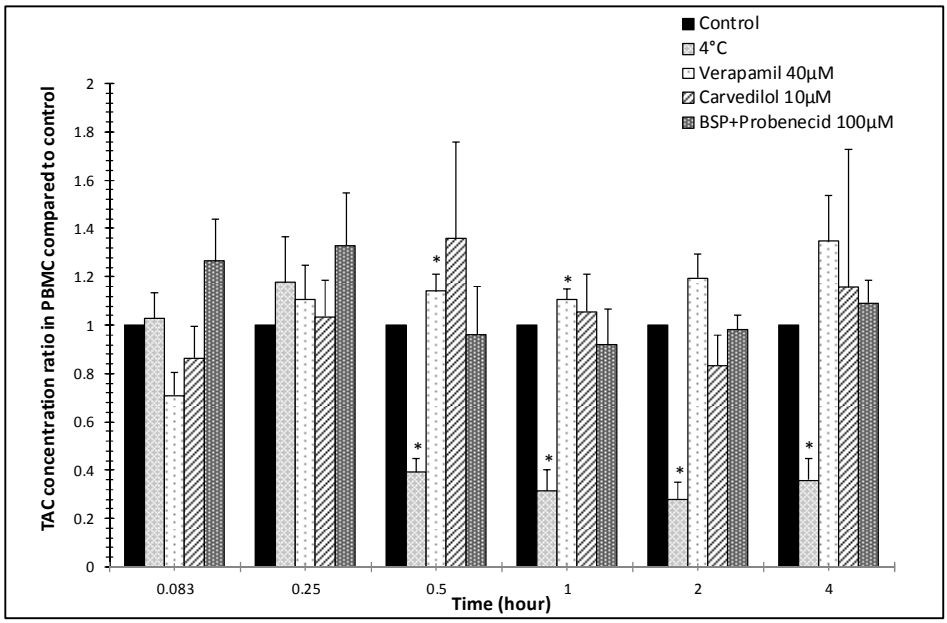

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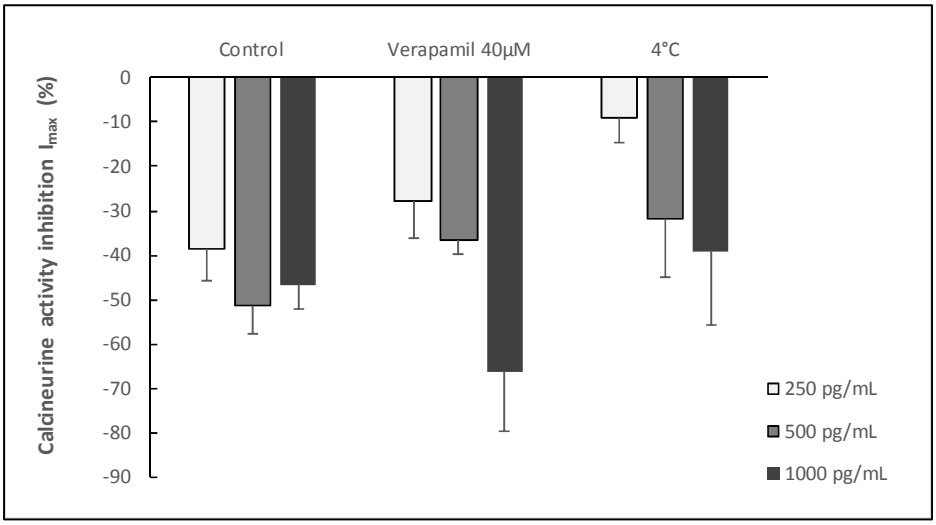

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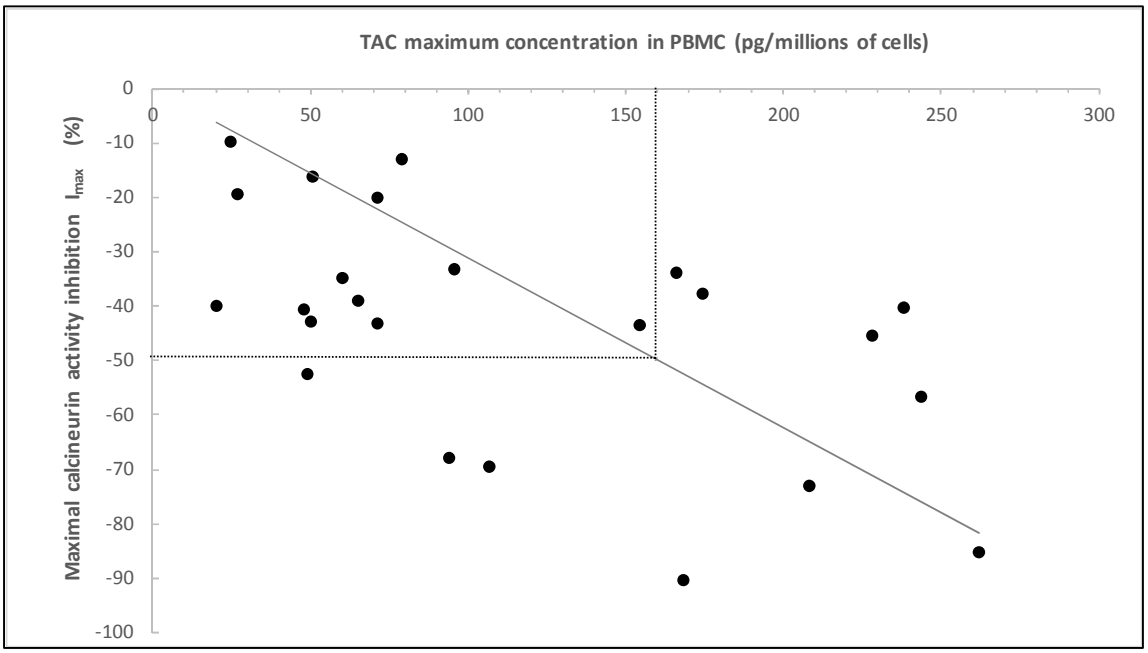

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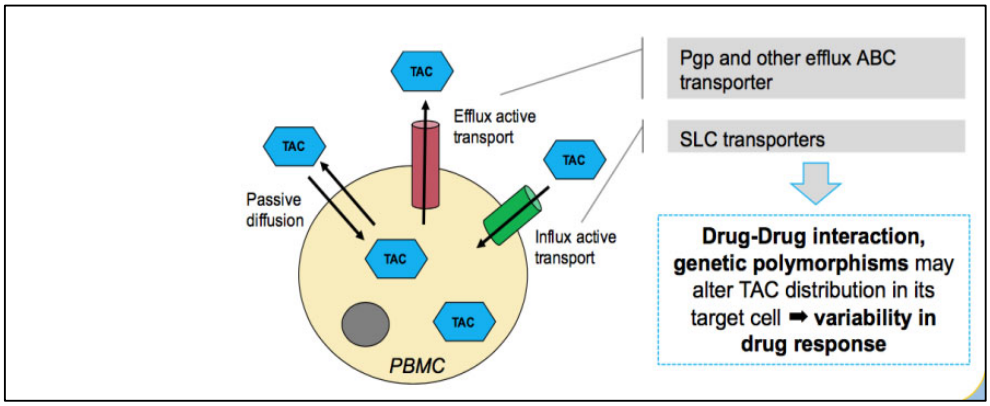

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