

IN VITRO P-GLYCOPROTEIN INHIBITION ASSAY ON N2a MURINE CELL LINE

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Abstract

P-glycoprotein (Pgp), also called MDR1, the product of the multidrug resistance (MDR) gene, is an ATP-dependent efflux transporter that affects the absorption, distribution, and excretion of a great number of clinically important drugs.

Modulation of P-glycoprotein (Pgp) through inhibition or induction can lead to interactions at the level of the central nervous system (CNS) or intestinal, renal, or biliary efflux, having consequences upon treatment optimization.

In order to reveal the Pgp effect of some CNS important drugs, we investigated five structurally diverse active substances: valproic acid (V), fluoxetine hydrochloride (F), risperidone (R), thioridazine hydrochloride (T) and lithium carbonate (L) for their ability to inhibit Pgp, compared to quinidine (Q), a classic Pgp inhibitor. Pgp inhibition was studied in temporal dynamics (different cell-drug contact times), by a fluorimetric assay using calcein-acetoxymethylester as fluorophore, on the murine neuroblastoma cell line N2a.

Modulation of P-glycoprotein was accomplished using a physiological concentration of glutathione (GSH), on the cell line treated with the studied drugs.

Rezumat

Glicoproteina P (Pgp), recent identificată pentru rezistența multimedicațională (MDR), este un transportor de eflux ATP-dependent, ce afectează absorbția, distribuția și excreția unui număr mare de medicamente, importante din punct de vedere farmacoterapeutic.

S-a demonstrat că modularea activității glicoproteinei P (Pgp), prin inhibiție sau inducție poate conduce la interacțiuni moleculare la nivelul sistemului nervos central, intestinal, renal sau biliar, având consecințe majore asupra eficienței farmacoterapiei.

Am investigat cinci compuși diferiți structural, activi la nivelul sistemului nervos central: acid valproic (V), clorhidrat de fluoxetină (F), risperidonă (R), clorhidrat de tioridazină (T) și carbonat de litiu (L) referitor la capacitatea lor de a inhiba activitatea Pgp, comparativ cu chinidina (Q), un inhibitor clasic al acesteia. Activitatea Pgp a fost studiată în dinamică temporală (diferiți timpi de contact celule-medicamente), pe linia celulară murină de neuroblastom N2a, printr-o metodă fluorimetrică, folosind esterul acetoximetilat al calceinei, ca sondă fluoroforă.

Modularea expresiei glicoproteinei P a fost realizată prin adăugarea în sistem a unei concentrații fiziologice de glutatation redus (GSH).

Keywords: calcein-AM(calcein-acetoxymethyl ester), P- glycoprotein inhibition assay, MDR (multi drug resistance), cell culture, quinidine, valproic acid, fluoxetine, risperidone, thioridazine, lithium, GSH (glutathione), N2a cell line.

Introduction

P-glycoprotein (Pgp) is a member of the ATP-binding cassette (ABC) superfamily and represents a major component of the blood-brain barrier and the intestinal barrier, and it contributes to renal and biliary elimination of drugs. At the blood-brain barrier level Pgp is localized in the apical membrane of brain capillary endothelial cells and transports substrates toward the blood compartment. Therefore, Pgp can limit the penetration into and retention within the brain and thus modulate effectiveness and central nervous system (CNS) toxicity of numerous compounds [3].

P-glycoprotein is a transmembrane efflux pump involved physiologically in the elimination of degradation products of cell metabolism (cellular clearance function). In addition to the specific interaction with Pgp, the CNS penetration of compounds depends on its permeability.

The experimental data led to the hypothesis that, in addition, Pgp is overexpressed in the neuroblastoma cells (like N2a cell line), where it is involved in the cellular efflux of CNS drugs. Thus, after administration of CNS drugs and their intracellular transport, Pgp pump takes over the compound immediately and removes it in the extracellular space, preventing therefore the cytotoxic effect on cells. For these reasons, highlighting molecules with inhibitory properties on Pgp pump has become a topic of great interest in CNS pharmacotherapy.

In addition, there is increasing evidence that ABC transporter-mediated drug efflux at the blood-brain barrier (BBB) may limit brain drug delivery of several CNS drugs, thereby leading to treatment failure in various brain disorders.[20] In the field of psychiatry, much attention had been given to the role of efflux pumps on the pharmacokinetic profile of antidepressant drugs. Emerging evidence suggest that P-gp, in particular, may limit the ability of several antidepressants to cross the BBB, thus resulting in inadequate brain concentrations and therefore contributing to the poor success rate of current antidepressant therapies [21].

In this paper we aimed to investigate the Pgp activity impact of some structurally diverse central nervous system-active drugs: valproic acid sodium salt (V), fluoxetine hydrochloride (F), risperidone (R), thioridazine hydrochloride (T) and lithium carbonate (L), which were selected based on literature data suggesting controversial interactions with Pgp [6,9,18].

We assessed the cytotoxicity of each studied compound, using the trypan blue cells viability assay, and we characterized the inhibitory potencies of the compounds by using calcein AM (calcein-acetoxymethylester) Pgp inhibition assay on the activity of the efflux pump MDR1 (also called P-glycoprotein), for different cell-drug contact times, compared to quinidine (Q), a classic Pgp inhibitor.

In vitro calcein-AM inhibition assay can be used to detect compounds that inhibit Pgp mediated efflux of the fluorescent P-gp substrate, calcein. This assay can differentiate Pgp inhibitors from noninhibitors by measuring the fluorescence of calcein.

Modulation of P-glycoprotein activity was assessed using a physiological concentration of glutathione, on the cell line treated with the studied drugs.

Glutathione (GSH) is a ubiquitous thiol-containing tripeptide (L- γ -glutamyl-L-cysteinyl-glycine) that is synthesized in the cytosol from the precursor amino acids glutamate, cysteine and glycine. The cell contains millimolar concentrations of GSH (up to 10 mM) that are maintained in this reduced form by a cytosolic NADPH-dependent reaction catalysed by glutathione reductase [17]. It plays a crucial role in antioxidant defense mechanisms, modulates the cell response to redox changes, detoxifies the metabolites of drugs, regulates gene expression and apoptosis, and is involved in the transmembrane transport of organic solutes. Owing to its reactivity and high intracellular concentrations, GSH has been implicated in the resistance to several chemotherapeutic agents [22]. Recent studies showed that GSH inhibited multi-drug resistance P-glycoprotein activity in excised small intestine and moreover it was revealed the up-regulation of P-glycoprotein expression by glutathione depletion [16]. The modulation of Pgp expression by GSH is therefore of key interest in therapy, especially at the blood–brain barrier (BBB) level.

Materials and Methods

Materials. Culture media: Minimum Essential Medium Eagle (MEM), with Earle's salts, L-glutamine and sodium bicarbonate, supplied by Sigma Aldrich (Taufkirchen, Germany); Trypan blue solution from Sigma Aldrich (Taufkirchen, Germany); PBS X 1 (phosphate buffered saline), purchased from Invitrogen (Karlsruhe, Germany), calcein-AM (calcein-acetoxymethyl ester) purchased from Fluka (Taufkirchen, Germany); DMSO (dimethyl sulfoxide) was purchased from Sigma Aldrich (Taufkirchen, Germany).

Drugs. Quinidine (Q), valproic acid sodium salt (V), fluoxetine hydrochloride (F), risperidone (R), thioridazine hydrochloride (T) and lithium carbonate (L) were purchased from Sigma Aldrich (Taufkirchen, Germany); GSH (reduced glutathione) was purchased from Merck (KGaA, Germany). Other routine reagents were of analytical purity.

Cell line. The mouse neuroblastoma cell line N2a, was a kind gift from the Institute for Diagnosis and Animal Health, Bucharest, Romania. Cells were not grown in the presence of any selection agent to maintain the Pgp expression. For both studies, the cytotoxicity and calcein inhibition assays, we used 1.5×10^4 cells/ mL suspension.

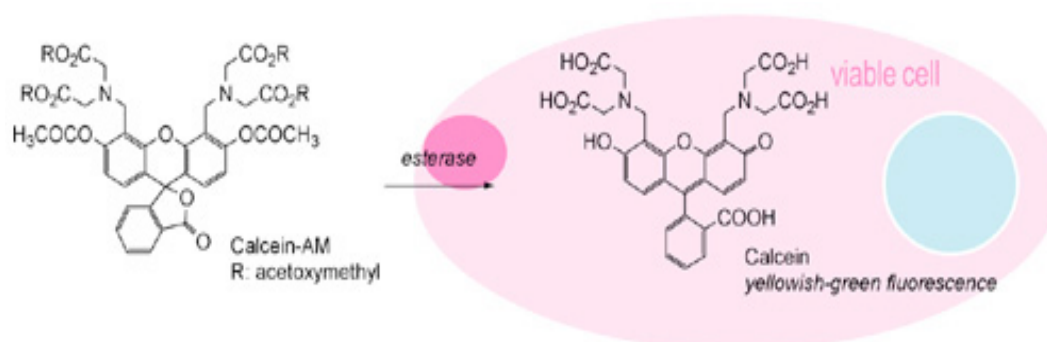
Cytotoxicity studies. Solutions of drugs: quinidine, valproic acid sodium salt, fluoxetine hydrochloride, risperidone, thioridazine hydrochloride and lithium carbonate, were prepared in culture media MEM in different concentrations: 200 μ M, 100 μ M, 50 μ M, 25 μ M and 12.5 μ M.

We established the cytotoxicity of each compound, using the trypan blue viability assay. This assay is used to determine the number of viable cells present in a cell suspension. Live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not. In this test, a cell suspension is simply mixed with the dye and then visually examined to determine whether cells take up or exclude the dye. A viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm.

Briefly 1 mL of cell suspension with 100 μ L from each tested drug, we added trypan blue solution and the cells were counted separately, as viable (opaque) or non-viable (blue-stained).

Calcein inhibition assay. Calcein-AM is a derivative of calcein acetoxymethylester, and is transported inside the cell *via* P-glycoprotein (Pgp transmembrane pump). The cytoplasmatic esterases act on acetoxymethyl group, removing it by enzymatic hydrolysis (Figure 1). Thus, at the cytoplasmatic level (intracellular) is found calcein, a fluorescent compound, spectrofluorimetrically estimable.

Whereas calcein-AM is a substrate of Pgp, calcein is not [8]. Cells expressing high levels of Pgp rapidly extrude nonfluorescent calcein-AM from the plasma membrane, thus preventing accumulation of fluorescent calcein in the cytosol. Because the transport capacity of Pgp is inversely proportional to the accumulation of intracellular calcein fluorescence, inhibition of Pgp will lead to intracellular calcein accumulation (augmentation of fluorescence signal) [18]. Therefore, an increase in relative calcein accumulation represents inhibition of P-gp function.

**Figure 1**

Cytoplasmatic chemical reaction: the conversion of calcein-AM to calcein

The experiments were conducted within the Biochemistry Department, Faculty of Pharmacy from Bucharest, using a Perkin Elmer LS 50B spectrofluorimeter equipped with an external thermostat sample system, magnetic stirring in sample medium and polarized fluorescence analysis system, with 485-nm excitation and 535-nm emission filters.

The basal fluorescence of cell suspensions was registered. We used a 2×10^{-5} mM calcein-AM solution. From this solution we added 100 μL and the fluorescence intensity was recorded. 100 μL of drugs solutions of different concentrations (200 μM , 100 μM , 50 μM , 25 μM and 12.5 μM) were added, and the dynamic temporal fluorescence intensity ($t = 0$, $t = 15$ min, $t = 30$ min, $t = 60$ min) was registered.

In order to assess the *in vitro* role of GSH in the modulation of Pgp activity, in the next step we added a GSH solution in a dose of 10 mM, to all the samples, and we repeated the experiment in temporal dynamics, registering the fluorescence intensities. The concentration used through the study was previously reported as the mean intracellular physiological concentration of GSH [16,17].

Results were assessed using ANOVA analysis. We also quantified the Pgp inhibitory effect using the following equation:

Pgp inhibitory effect (%)=

$$\frac{[(\text{RFU}_{\text{sample}} - \text{RFU}_{\text{control}}) / \text{RFU}_{\text{control}}] \times 100}{}$$

where RFUsample (in relative fluorescence units) is the fluorescence intensity in the presence of tested compound RFUcontrol is the fluorescence intensity in the presence of quinidine.

Furthermore, the dose-effect relationships were described as linear or logarithmic mathematical models (if applicable).

Results and Discussion

Cytotoxicity Assay. In terms of cell cytotoxicity, the studied compounds (quinidine (Q), valproic acid sodium salt (V), fluoxetine hydrochloride (F), risperidone (R), thioridazine hydrochloride (T) and lithium carbonate (L)) showed no cytotoxic effects on N2a cells, even for the highest dose.

Calcein inhibition Assay

We registered the fluorescence signals for each studied substance (200 μ M, 100 μ M, 50 μ M, 25 μ M and 12,5 μ M), in temporal dynamics (0,15,30, 60 minutes cells-drug contact).

Each sample was performed in triplicate.

Certain drugs (namely risperidone, thioridazine, fluoxetine) exhibited significant inhibitory effects (corellated with an increase of the samples fluorescence) compared to quinidine, the classical inhibitor of Pgp ($p < 0.05$) (Figure 2). During the study, valproic acid did not reveal any inhibition compared to quinidine, regardless the dose and time.

Evaluation of risperidone, thioridazine, fluoxetine and lithium effects, revealed differences regarding their inhibition profiles. These suggest that drugs may have different interactions with Pgp transport.

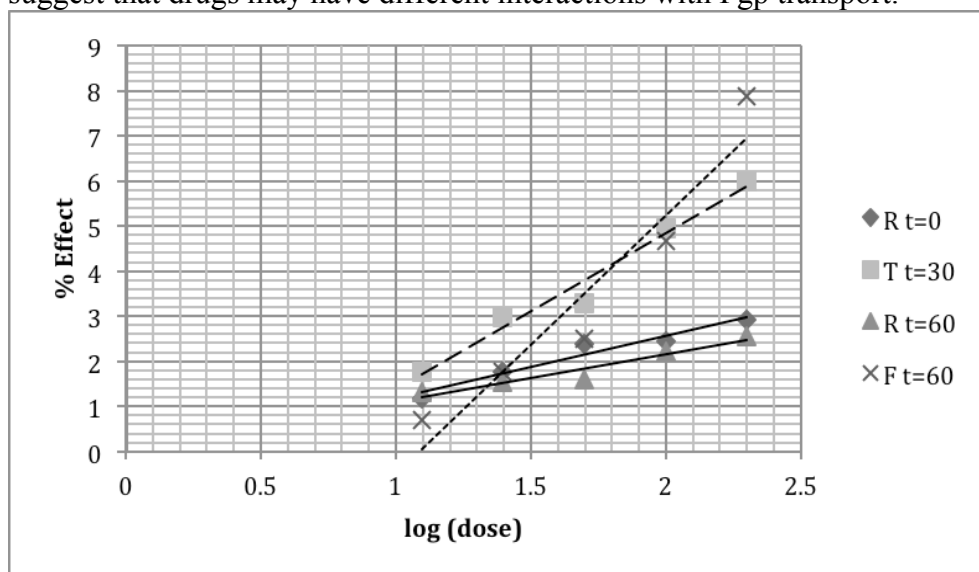


Figure 2

The inhibitory dose-dependent effect for risperidone, thioridazine and fluoxetine at different cell-drug contact times

The inhibitory effect for fluoxetine, risperidone and thioridazine showed a dose dependent relationship. Thus, by increasing the drug concentration the inhibitory effect on Pgp is increasing, following a linear model (Figure 2).

For risperidone $t=0$ min ($y = 0.6028\ln(x) - 0.2134$) and thioridazine $t=30$ min ($y = 1.5033\ln(x) - 2.0832$), we registered a logarithmic evolution of the inhibitory effect, while risperidone $t=60$ min ($y = 0.0065x + 1.337$) and fluoxetine $t=60$ min ($y = 0.0371x + 0.6264$), followed a linear description of Pgp inhibition (Figure 2).

In the case of lithium, a particular behavior should be noted. Thus, low doses exhibited highest Pgp inhibitory effects. Our findings raise new questions regarding the possible molecular mechanisms of this controversial CNS active drug (Figure 3).

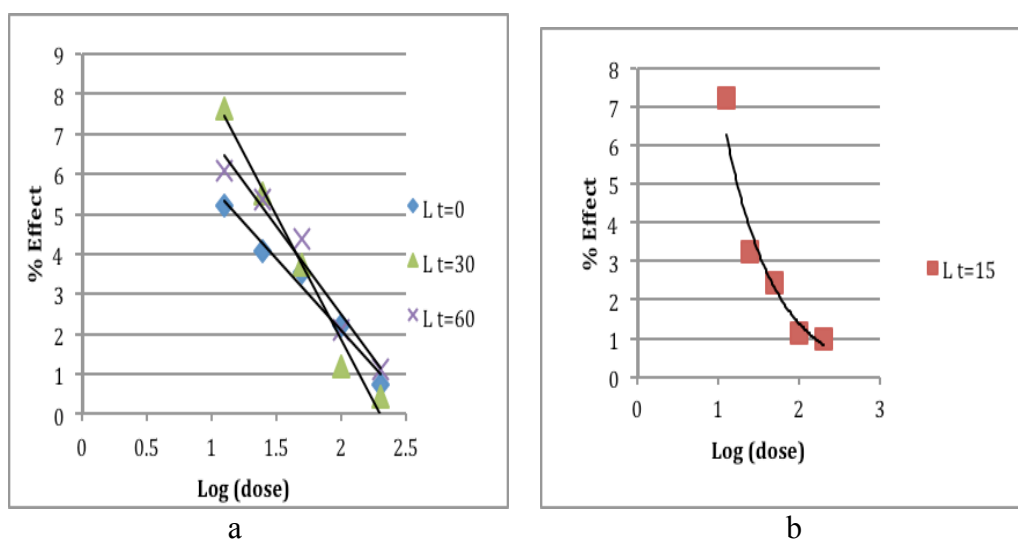


Figure 3

The inhibitory dose-dependent effect for lithium
a-linear equations, ($t=0,30,60$ min), b-exponential equation ($t=15$ min)

GSH modulated inhibition assay

Considering its Pgp modulating effect, we added 10 mM GSH to all samples and registered the consequent fluorescence intensities.

Table I summarizes our results expressed as Pgp inhibitory effects(%). In the context of GSH incubation, all samples developed Pgp activity alteration (inhibition) with respect to dose and time. Once more, GSH proves its Pgp activity- relationship. Our results bring new evidence for the potential use of this biomolecule in the clinical approach of

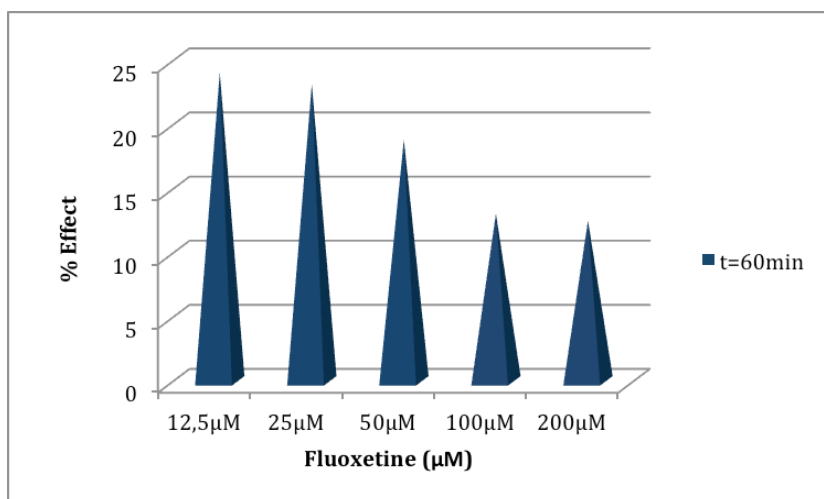
multidrugresistance. The most concrete GSH adding effect was registered for fluoxetine at t=60 min, risperidone at t=0 min, t=30 min and t=60 min, and lithium at t=30 min.

Table I

Pgp inhibitory effect of drugs in the presence of GSH physiological concentrations (10 mM) at specific doses of drug for all studied cell-drug contact times

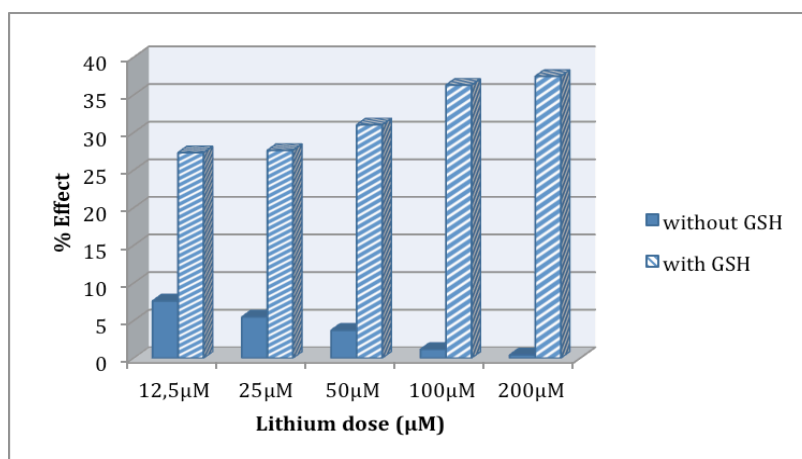
Drug/contact time	% Effect at a specific dose of drug				
	200 μ M	100 μ M	50 μ M	25 μ M	12.5 μ M
Q (quinidine)+GSH 10mM					
t=0 min	29.33	27.26	29.49	25.80	27.26
t=15 min	25.19	23.07	27.36	17.96	23.16
t=30 min	24.16	23.23	22.41	17.87	16.83
t=60min	26.27	20.85	18.26	15.37	13.68
L (lithium) +GSH 10mM					
t=0 min	32.21	26.52	39.11	34.82	35.17
t=15 min	27.62	23.43	36.59	31.24	32.95
t=30 min	37.48	36.31	31.03	27.64	27.34
t=60min	28.01	27.31	26.23	25.16	20.73
F (fluoxetine) +GSH 10mM					
t=0 min	34.57	29.15	22.74	29.25	21.13
t=15 min	32.22	24.66	20.04	27.01	18.51
t=30 min	26.12	21.42	20.50	22.28	7.68
t=60min	12.50	13.05	18.82	23.18	24.06
R (risperidone) +GSH 10mM					
t=0 min	28.23	28.16	25.36	25.01	23.63
t=15 min	31.05	35.03	28.34	29.77	19.54
t=30 min	30.63	28.09	26.32	23.31	17.92
t=60min	27.83	25.94	24.11	22.66	16.56
T (thioridazine) +GSH 10mM					
t=0 min	31.74	28.72	33.35	28.15	26.72
t=15 min	27.85	31.81	33.81	26.95	28.85
t=30 min	11.34	13.04	21.93	23.23	24.48
t=60 min	18.90	21.97	22.08	17.02	24.31
V (valproic acid) +GSH 10mM					
t=0 min	32.09	20.40	30.30	32.05	20.64
t=15 min	27.85	21.63	24.35	33.45	20.71
t=30 min	28.41	21.12	24.55	26.48	22.54
t=60 min	25.35	16.09	21.58	25.79	15.37

Fluoxetine (t=60 min) developed a particular fluctuation of Pgp activity. Thus, figure 2 described its linear dose-response relationship, while the addition of 10 mM GSH reversed the pump transport.

**Figure 4**

The dose-dependent effect for fluoxetine.

Fluoxetine at $t=60$ min, without GSH, showed a dose-effect relationship described by a linear equation (figure 2) In the presence of GSH, the inhibitory effect of fluoxetine varied inversely proportional to the dose used.

**Figure 5**

The dose-dependent effect for lithium.

There should also be noted the specific molecular response after lithium and GSH addition. As presented in figure 4, the potency of Pgp inhibition decreased with increasing doses, while GSH 10 mM altered this particular pattern. Our results evoke that Li^+ -GSH Pgp activity relationship

does not follow any mathematical model, which suggests particular interactions that need further investigations (Figure 5).

Conclusions

The study considered the possible Pgp inhibitory effect of some CNS active drugs: lithium, fluoxetine, risperidone, thioridazine, valproic acid, in different concentrations (12.5 μ M, 25 μ M, 50 μ M, 100 μ M, 200 μ M) and different cell-drug contact times ($t=0$ min, $t=15$ min, $t=30$ min, $t=60$ min) on N2a murine cell line.

Risperidone, thioridazine and fluoxetine exhibited significant inhibitory effects compared to the classical inhibitor of Pgp-quinidine. During the study, valproic acid did not reveal any inhibition compared to quinidine, regardless the dose and time.

In the case of lithium, a particular behavior was noted. Thus, low doses exhibited highest Pgp inhibitory effects. Our findings raise new questions regarding the possible molecular mechanisms of this controversial CNS active drug.

In the context of GSH addition, all samples developed Pgp activity alteration (inhibition) with respect to dose and time. Once more, GSH proved its Pgp activity- relationship. Our results bring new evidence for the potential use of this biomolecule in the clinical approach of multidrugresistance.

We also have to consider the limitations of these *in vitro* studies, because different studies have used the same assay for the specific compounds and reported contrasting results.

It can be difficult to draw definitive conclusions from *in vitro* P-gp studies due to the discrepancies in results obtained depending on the used assay and numerous other confounding factors. There remains a demand for the development of highly reliable predictive screening models for the accurate *in vitro* evaluation of P-gp activity [19].

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