

IN VITRO P-GP EXPRESSION AFTER ADMINISTRATION OF CNS ACTIVE DRUGS

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Abstract

P-glycoprotein (P-gp) is a transmembrane efflux pump, part of the ABC transporters family (ATP-binding cassette) playing an important role in the absorption (intestine), distribution (CNS and white blood cells) and elimination (liver, kidney) of xenobiotics, as well as endogenous products, present in various cell types. The clinical significance of P-gp is depicted by drug resistance of the cells, which ultimately leads to compromising therapy. Modulating the expression of P-gp transporters could be an effective therapeutic strategy in mitigating the side effects of neuronal drugs, improving pharmacokinetic and pharmacodynamic properties of the substrates whose effectiveness is limited by P-gp. In the present study we assessed the influence of several CNS active drugs (valproic acid, risperidone, thioridazine, fluoxetine, lithium), as well as combinations of these drugs associated with quinidine (a classic inhibitor of the P-gp efflux pump), on the expression of P-glycoprotein, by means of quantitative indirect immunofluorescence. We assessed the expression of P-gp *in vitro*, on the murine neuroblastoma cell line N₂a, after the administration of these drugs, as well as their associations. The obtained results revealed significant changes in the expression of P-gp in the neuroblastoma cell line, denoting the inhibition of the efflux pump. An absolute novelty, for the current research in the pharmacotherapy field, is the synergistic *in vitro* potentiation of the inhibitory effect on the expression of P-gp, revealed by two of the studied drugs: valproic acid and thioridazine. Of all five studied drugs (valproic acid, risperidone, thioridazine, fluoxetine, lithium), lithium showed the strongest effect on the expression of P-gp.

Rezumat

Glicoproteina P (P-gp) reprezintă o pompă transmembranară de eflux, parte a familiei de transportori ABC (ATP-binding cassette), cu rol important în absorbția (intestin), distribuția (CNS și celulele albe din sânge) și eliminarea (ficat, rinichi) xenobioticelor, precum și a produselor endogene, prezente în diferite tipuri de celule. Semnificația clinică a P-gp este reprezentată de rezistența la medicamente a celulelor, ceea ce duce în cele din urmă la o terapie compromițătoare. Modularea expresiei transportorilor P-gp poate fi o strategie terapeutică eficientă în reducerea efectelor secundare ale medicamentelor cu tropism neuronal, îmbunătățind proprietățile farmacocinetice și farmacodinamice ale substratelor a căror eficiență este limitată de P-gp. În studiul prezent a fost evaluată influența mai multor medicamente active la nivelul sistemului nervos central (acid valproic, risperidonă, tioridazină, fluoxetină, litiu), precum și combinații ale acestor medicamente asociate cu chinidina (un inhibitor clasic al pompei de eflux P-gp), asupra expresiei glicoproteinei P, prin tehnica imunofluorescenței indirecte cantitative. A fost evaluată expresia P-gp *in vitro*, pe o linie de celule de neuroblastom murin N₂a, după administrarea acestor medicamente, precum și asocieri ale acestora. Rezultatele obținute au evidențiat modificări semnificative ale expresiei P-gp asupra liniei de celule de neuroblastom, ceea ce denotă inhibiția pompei de eflux. O noutate absolută pentru cercetările actuale în domeniul farmacoterapiei, este sinergismul de potențare *in vitro* a efectului inhibitor asupra expresiei P-gp, revelată de două dintre medicamentele studiate: acid valproic și tioridazina. Dintre toate cele cinci medicamente studiate (acid valproic, risperidona, tioridazină, fluoxetină, litiu), litiu a arătat cel mai puternic efect asupra expresiei P-gp.

Keywords: P-gp expression, immunofluorescence, neuroblastoma cell line, CNS active drugs

Introduction

The penetration of the blood–brain barrier (BBB) is one of the most important challenges in the therapeutic development of central nervous system (CNS) acting drugs.

According to the research conducted in the past few decades, BBB is more than an essential barrier for drug delivery; it is a complex, dynamic interface tailored on the needs of the CNS which reacts to the physiological changes and is affected by and can even stimulate disease development [19, 25].

According to Banks W. A. *et al.*, this entanglement hardens the simple strategies for drug delivery to the CNS, while enhancing the drug development methods. At the level of BBB we find P-glycoprotein, an ATP-dependent drug transport protein, mainly located in the apical membranes of a number of epithelial cell types, including the blood luminal membrane of the brain capillary endothelial cells that make up the blood–brain barrier [2, 30].

If the blood–brain barrier lacks optimal functional P-glycoprotein, we evidence an increased penetration of a number of important drugs in the brain, which, based on the pharmacological target of these drugs on the central nervous system (CNS), it may trigger increased neurotoxicity, or essentially modified pharmacological effects of the drug [16, 18, 19]. Taking into account the diversity of drugs affected by P-glycoprotein transport, a great step forward would be the application of these clinical discoveries to the design of drugs with either very poor or very good brain penetration, whichever is deemed more appropriate. We should also have in view, for an effective therapy, the use of P-glycoprotein blockers, aimed at enhancing the blood–brain barrier permeability of certain drugs for which the brain penetration capability is unsatisfactory [30].

The quantitative assessment of brain tissue distribution of drugs is a topic of extreme importance in neuropharmacotherapy [10, 15]. There are a number of methods designed to assess the actual drug concentrations in the brain. Clinical trials use imaging methods (computed tomography (CT), positron emission tomography (PET), magnetic resonance imaging (MRI)) whose major disadvantage is the high cost and eventual invasiveness [23, 25]. There are also used *in vivo* experiments on laboratory animals, but their relevance to medical practice is quite limited, considering that there are substantial differences among species in terms of the specific metabolizing enzymes and transporters [10, 21, 34].

Materials and Methods

Materials. Culture media: Minimum Essential Medium Eagle (MEM) with 10% fetal bovine (Sigma, Germany); Trypan blue solution (Sigma Germany); 1X Phosphate Buffered Saline (PBS Buffer, Invitrogen, Germany), 1% penicillin-streptomycin and 0.1% gentamicin (SERVA Electrophoresis GmbH, Germany), 1:20 primary antibody anti-rabbit (Acris, USA), 0.3% Triton X-100, (Sigma, Germany), 1:100 secondary antibody anti-rabbit FITC (fluorescein isothiocyanate) labelled (Invitrogen, Germany), 4 µg/mL DAPI (4',6-diamidino-2-phenylindole) (Sigma, Germany).

Drugs. Quinidine (Q), valproic acid (V), fluoxetine (F), risperidone (R), thioridazine (T) and lithium (L) were purchased from Sigma Aldrich, 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 grown on a monolayer to confluence in 80% MEM Eagle with 10% fetal bovine serum, 1% penicillin-streptomycin and 0.1% gentamicin; subsequently, the plates were multiplied and divided into the 96-well cell culture. For this study, we used 1.5×10^4 cells/mL suspension, and after 24 hours it was incubated with the CNS active drugs, and also combinations of those drugs, divided in 24 groups.

Cytotoxicity studies. Solutions of drugs: quinidine, valproic acid, fluoxetine, risperidone, thioridazine and lithium, were prepared in 50 µM culture media. 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, to 1 mL of cell suspension with 100 µL from each tested drug, we added trypan blue solution and the cells were counted separately, using a confocal microscope, as viable (opaque) and non-viable (blue-stained).

Immunofluorescence assay. Indirect quantitative immunofluorescence was implied. In immunofluorescence, antibodies are labelled with a fluorochrome (fluorescent dye) such as FITC (fluorescein isothiocyanate). These labelled antibodies (directly or indirectly) bind to the antigen and then they can be detected and quantified by fluorescence. In our

assay, the contact time between the drug and the neuroblastoma cells was 24 hours. After removing the culture medium the plates were fixed in 40% acetone, and then incubated for 60 minutes at 37°C with the 1:20 primary anti-rabbit antibody, then washed three times for 10 minutes with PBS Buffer and with 0.3% Triton X-100, incubated for 60 minutes at 37°C with the 1:100 second anti – rabbit antibody labelled with FITC, washed with PBS Buffer, three times for 10 minutes, contrasting with 4 µg/mL DAPI, 10 minutes in the dark light, washed for five minutes with PBS Buffer two times, and in the end the samples were fixed in 50% glycerol.

The examination was performed using a fluorescence microscope Leica DMIL, EBQ 100 Isolated, UV, B. Images were acquired by a Nikon D40 device [24]. Results were assessed using ImageJ software which transforms the image in quantitative expression. In our case, Image J application allows the quantification of the P-gp expression.

For the statistical analysis, we also used Student t test. p values < 0.05 were considered statistically significant.

Results and Discussion

Cytotoxicity Assay. In terms of cell cytotoxicity, the studied drugs (valproic acid, fluoxetine, risperidone, thioridazine and lithium) in 50 µM concentration showed no cytotoxic effects on N2a cells.

Immunofluorescence assay. Figure 1 shows some representative images of cells used after staining with specific antibodies to P-gp.

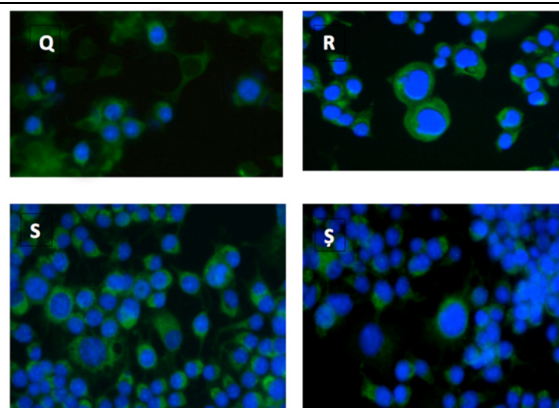
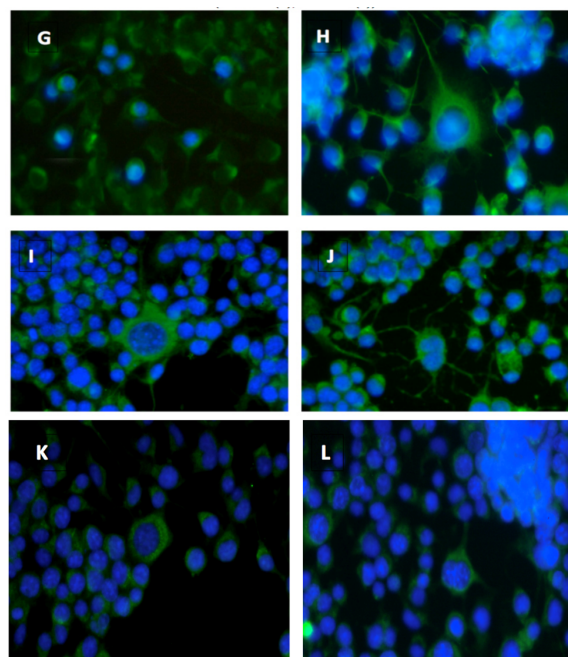


Figure 1.

P-glycoprotein expression in murine neuroblastoma cells.

G - Group 7 (Li), H - Group 8 (V + R) I- Group 9 (V + T), J - Group 10 (V + F), K - Group 11 (V + Li), L - Group 12 (R + T)

Q - Group 17 (Q + F), R - Group 18 (Q + Li),

S - Group 19 (Q + V + R), Ş - Group 20 (Q + V + T)

The images obtained were processed quantitatively and results are expressed as a percentage of immunofluorescence intensity (%). Table I shows the immunofluorescence intensities (%) recorded for the murine neuroblastoma N2a cells.

Table I

Immunofluorescence intensities (%) recorded for the neuroblastoma N2a cells

Group no.	Murine neuroblastoma groups (50 µM)	Immunofluorescence intensity (%)
1.	M	100 ± 14.21
2.	Q	35.5 ± 6.31
3.	V	70.35 ± 11.7
4.	Q + V	51.68 ± 8.59
5.	R	76.47 ± 12.01
6.	Q + R	71.23 ± 11.45
7.	T	82.37 ± 12.32
8.	Q + T	67.97 ± 10.8
9.	F	77.89 ± 13.6
10.	Q + F	55.09 ± 8.81
11.	Li	62.05 ± 11.23
12.	Q + Li	58.31 ± 9.45
13.	V + R	75.24 ± 12.52
14.	Q + V + R	60.78 ± 9.2
15.	V + T	89.1 ± 13.57
16.	Q + V + T	75.1 ± 12.24
17.	V + F	59.13 ± 10.56
18.	Q + V + F	39.49 ± 7.35
19.	V + Li	56.23 ± 7.45
20.	Q + V + Li	49.32 ± 7.24
21.	R + T	69.67 ± 11.58
22.	Q + R + T	59.87 ± 10.99
23.	V + R + T	53.28 ± 8.66
24.	Q + V + R + T	49.19 ± 7.4

A low fluorescence intensity is correlated with low P-gp expression, depicting the inhibition of the efflux pump by the studied drugs.

Figure 2 indicates the graphic interpretation of the obtained experimental data.

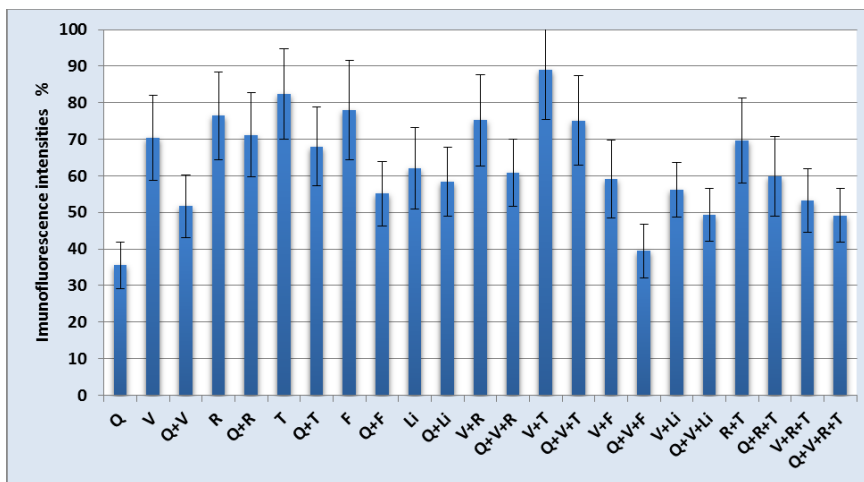


Figure 2.

Graphical representation of the immunofluorescence intensities obtained for the neuroblastoma groups

From the five studied drugs (valproic acid, risperidone, thioridazine, fluoxetine, lithium), lithium showed the strongest effect on the expression of P-gp ($62.05 \pm 11.23\%$ inhibition) compared to the control. A similar effect was obtained for the association of V + F ($59.13 \pm 10.56\%$) and V + Li ($56.23 \pm 7.45\%$).

These experimental data represent a direct quantification of P-gp expression on the neuroblastoma N₂a cells. The immunofluorescent lower signal values developed by these cells, after incubation with the tested drugs directly reflect the inhibitory potential of these drugs on the P-gp pump expression.

Following the experimental results, the highest immunofluorescence intensity statistically significant

was recorded for the following groups: V + T ($89.10 \pm 13.57\%$) > T ($82.37 \pm 12.32\%$) > R + T ($69.67 \pm 11.58\%$) compared to control cells N₂a ($p < 0.05$). This fact accounts for a weak inhibitory effect on the P-gp expression.

All these results indicate a significant change in P-gp expression induced by the studied drugs. Moreover, without exception, all drugs with quinidine co-administration lead to an enhancement of the inhibition of the efflux pump.

In order to assess this interesting phenomenon, the inhibitory effect of drugs vs. same drugs + quinidine was mathematically interpreted.

Table II shows the results obtained for the 24 murine neuroblastoma groups.

Table II

The percentage of the immunofluorescence intensities, for all neuroblastoma groups

Studied combinations of drugs	Immunofluorescence intensities percentage (%)
V vs. Q + V	18.67
R vs. Q + R	5.24
T vs. Q + T	14.4
F vs. Q + F	22.8
Li vs. Q + Li	3.74
V + R vs. Q + V + R	14.46
V + T vs. Q + V + T	14
V + F vs. Q + V + F	19.69
V + Li vs. Q + V + Li	6.91
R + T vs. Q + R + T	9.8
V + R + T vs. Q + V + R + T	4.09

As such, the highest amplification effect of inhibition on the P-gp expression was registered in the following order: Q + F > Q + V + F > Q + V. The P-gp expression is significantly altered in the neuroblastoma cell line by the studied CNS active

drugs. From this point of view, lithium has been proven to have the most potent inhibitory effect on the P-gp expression in comparison with valproic acid, fluoxetine and risperidone. A similar effect was recorded for fluoxetine in combination with

valproic acid (V + F) and valproic acid with lithium (V + Li).

In addition, the co-administration of the studied drugs with a classic P-gp inhibitor, quinidine, led to a significant decrease of the efflux pump expression. The most significant changes in the expression of P-gp have been obtained for the cells treated with Q + F, Q + V + F, Q + V ($p < 0.05$).

Quinidine and fluoxetine are included in the first generation of P-gp inhibitors according to their potency, selectivity and drug-drug interaction [26]. P-gp inhibitors are nowadays considered as a perspective solution in co-therapy for improving the pharmacological response in case of resistant patients. The co-therapy has been applied successfully in some antimicrobial and cancer treatments by increasing the drugs bioavailability [1]. According to some recent clinical studies, 20 - 50% of the high-risk patients diagnosed with neuroblastoma are not responding to the chemotherapy. The mechanism is not completely elucidated, but several studies demonstrated that both tumour suppressor protein p53 and multidrug transporters like P-gp, play important roles in chemoresistance [4, 12].

Valproic acid is an anti-neoplastic agent previously tested *in vivo* and *in vitro* on solid tumour, leukaemia and neuroblastoma [20]. Our study demonstrated a similar potency of valproic acid with fluoxetine. The inhibitory effect on P-gp expression was significant in association with the studied drugs except for thioridazine co-treatment. The results are optimistic and may represent a future solution for the bioavailability regulation of the chemotherapeutic agents in neuroblastoma treatment.

Treatment-resistant patients suffering of schizophrenia, bipolar disorders and anxiety represent an important pharmacological challenge. Therapeutic resistance is often correlated with an over-expression of multidrug transporter proteins. Our study demonstrated that the association of drugs can lead to dramatical changes in P-gp expression level (e.g. Q + V + F). The treatment with lithium alone or in association with valproic acid may influence significantly the pharmacological response of other drugs used for the treatment in case of patients with associated pathologies.

The decreasing of the cellular P-gp expression may represent a solution for increasing the drugs bioavailability in the resistant cases but the clinical use of the P-gp inhibitors is still limited due to a potential increase of the drugs toxicity. The approach of the future individual therapies should consider not only the adjustment of the drug doses, but also the cellular level of the multidrug transporter proteins for an optimal therapeutic response and fewer secondary effects.

Conclusions

According to our results, the highest immunofluorescence intensity ($p < 0.05$) was achieved for the groups of cells treated with: V + T > T > R + T compared to the control group N₂a. This demonstrates a weak inhibitory effect on P-gp expression. A low fluorescence intensity level is correlated with a low P-gp expression, with the inhibition of the efflux pump by the studied drugs.

All these results point a significant change in the P-gp expression, induced by the studied drugs. According to our *in vitro* results, the P-gp expression is significantly altered in the neuroblastoma cell line by neuronal drugs administration. From all the five studied drugs (valproic acid, risperidone, thioridazine, fluoxetine, lithium), lithium showed the strongest effect on the expression of P-gp, compared to the control. In addition, it was noted that, without exception, all drug-quinidine co-administrations led to an enhancement of the inhibition of the efflux pump. The most significant changes in the expression of P-gp in this case have been obtained for cells treated with Q + F, Q + V + F, Q + V ($p < 0.05$).

The P-glycoprotein (P-gp) plays an important role in the function of the blood-brain barrier by selectively extruding certain endogenous and exogenous molecules, thus limiting the ability of its substrates to target the brain. Therefore, co-administration of P-gp inhibitors with antidepressants to patients who are refractory to antidepressant therapy may represent a novel therapeutic approach in the management of treatment-resistant depression (TRD). Furthermore, certain antidepressants inhibit P-gp *in vitro*, and it has been hypothesized that inhibition of P-gp by such antidepressant drugs may play a role in their therapeutic action [7].

A number of studies have demonstrated a negative correlation between Pgp expression levels and chemosensitivity or survival in a range of human malignancies. In principle, Pgp mediated drug resistance can be circumvented by treatment regimens that either exclude Pgp substrate drugs or include Pgp inhibitory agents [14].

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