ORIGINAL ARTICLE

Antipsychotics inhibit The ATP-binding cassette transporters of Albino rats' blood brain barrier endothelial cells

EKRAMY MAHMOUD ELMORSY[1,2], SYED SAJED HUSSAIN[1], RASHAD QASEM ALI[1]
[1]*Department of Pathology, faculty of Medicine, Northern Border University, Saudi Arabia*
[2]*Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Mansoura University, Egypt.*
Correspondence to Dr. Ekramy Elmorsy, Email: ekramyelmorsy@mans.edu.eg, Mobile: 00966501275835

ABSTRACT

The ATP-binding cassette (ABC) transporters have an important role in disposition of many drugs. Antipsychotics (Aps) are known to be substrates and potential inhibitor for these transporters with unclear underlying mechanisms. This study investigated the effects of APs: chlorpromazine (CPZ), risperidone (RIS), clozapine (CLZ), haloperidol (HAL) on the functions of P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) transporters in Wistar albino rats' isolated micro-vascular endothelial cells (RMVECs) of blood brain barrier (BBB) using wide range of therapeutic and toxic concentrations. APs was shown to inhibit the function of both transporters in a concentration dependant manner especially RIS with significant decrease in their ATP synthesis, ATP hydrolase activities and expression of the transporters proteins in a concentration dependant manner. This can be a potential source for drugs interaction at the level of these transporters.
Keywords: Antipsychotics, Cytotoxicity, P-glycoprotein transporter, Breast cancers resistant protein and blood brain barrier.

INTRODUCTION

Adenosine triphosphate (ATP)-binding cassette (ABC) transporters have a major effect the distribution and elimination of drugs from and to the brain. This large superfamily consists of membrane proteins which are able to transport a wide variety of substrates across membranes against concentration gradients with ATP hydrolysis as a driving force[1]. ABC transporters often consist of multiple subunits, one or two of which are transmembrane proteins and one or two of which are membrane-associated ATP hydrolase, which utilize the energy of ATP to provide the energy needed for the uptake or the export of the substrates[2].

P-glycoprotein (P-gp) is the first identified and best studied ABC transporter is the MDR1 gene product. P-gp is a 170-kDa phosphorylated glycoprotein, which acts as a multi-specific, ATP-driven drug efflux pump[3]. Over expression of P-gp in tumour cells causes multidrug resistance in these cells. P-gp is expressed in endothelial cells of the blood-brain barrier. Several studies, comprising isolated membranes, freshly isolated endothelial cells, isolated capillaries, and tissue slices, show a predominant distribution of P-gp at the apical membrane[4].

P-gp has a broad substrate specificity including organic cations, weak organic bases, some organic anions and some uncharged compounds, such as polypeptides and polypeptide derivatives. The first P-gp inhibitor described is the calcium channel blocker verapamil. It inhibits the efflux of drugs that are P-gp substrates and restores drug sensitivity in multi-drug resistant leukaemia cell lines[5]. Another first-generation inhibitor is the immunosuppressive drug cyclosporine A[3].

BCRP transporter was first identified in a highly doxorubicin-resistant breast cancer cell line (MCF-7/AdrVp), and was therefore named breast cancer resistance protein (BCRP)[6]. Besides the BBB, BCRP is expressed in placenta, bile canaliculi, colon, and small intestine [7]. Like P-gp, BCRP is localized at the apical surface of the micro-vessel endothelium[8]. The substrate specificity of BCRP is broad, comprising a wide variety of drugs (e.g. mitoxantrone, topotecan, and prazosine), carcinogens and dietary toxins [9]. BCRP has several substrates in common with P-gp, such as doxorubicine, daunorubicine, and rhodamine-123[7].

Antipsychotics (APs) are the primary medications used for treatment of various psychotic and bipolar disorders. They are well known to be cytotoxic to different cell lines [10]. APs also known to be substrates for p-gp and BCRP transporters, with studies proving that both transporters affect their pharmacokinetics[11]. On the other hand, APs was shown to inhibits both transporters for variable degrees[12,13]. In this study, the effect of 4 APs [CPZ, HAL, RIS) and CLZ] on P-gp and BCRP transporters in HMVECs of BBB will be studied using Wistar Albino rats' microvascular endothelial cells (RMVECs) of blood brain barrier (BBB) as a cell culture model. Then, the effect of APs on the isolated cells ATP production, ATP hydrolase and expression of the transporters proteins will be evaluated as underlying mechanisms for the inhibitory effect of APs on the studied transporters.

MATERIALS AND METHODS

Ethical issues: All experiments were approved by the local bioethics committee of Northern Border University, Saudi Arabia (A/40/28). Rats were sacrificed under sodium pentobarbital anaesthesia.

Chemicals and reagents: Antipsychotics CPZ, HAL and RIS were purchased from Sigma-Aldrich (St. Louis, MO, USA), while CLZ was purchased from Abcam (Cambridge, MA, USA). Stock solutions of all drugs were made in ethanol (vehicle). Drugs were dissolved in DMSO and PRMI-1640 media. The selected APs are commonly prescribed for patients being treated for schizophrenia or bipolar disorder.

Verapamil, Rh123 and Ko143 were brought from Sigma-Aldrich (St. Louis, MO, USA). For the solubilizing solution, 125ml was made of 12.5 ml 10% triton (100X) and

0.1N HCL. Then the volume is adjusted to 125 ml with isopropanol.

Regarding western blotting, P-gp was probed using the C219 antibody (Thermo Fisher Scientific, Waltham, MA, USA) in dilution 1:200 and anti-mouse IgG-HRP antibody (Thermo Fisher Scientific, Waltham, MA, USA) in dilution 1:1000. While for probing of BCRP transporter, the primary antibody Bxp-53 (Enzo Life Sciences, Farmingdale, NY) in dilution 1:5000 and Peroxidase Conjugated AffiniPure rabbit anti-rat IgG antibody at 1/10000 dilution (Protein Tech group, Chicago, USA) were used. Western blotting was visualized via enhanced chemi-luminescence detection (ECL kit, Amersham, Buckinghamshire, UK).

Cell line isolation: Isolation of rCMECs was conducted following the previously published methods of Nakagawa et al. (2009) [14]. After isolation, cells were purified using a 33 % continuous Percoll gradient, then the Cells were washed and plated on 35 mm collagen IV/fibronectin-coated plates (0.1 mg/ml). For culture, Endothelial Cell Medium supplemented with 4 mg/ml puromycin and 100 mg/ml heparin was used. Then the cells were incubated in 5 % CO2 at 37 ºC. After 3 days, puromycin was removed from the media.

Isolated cells characterisation and confirmation of the presence of ABC-transporters in RMVECs of BBB: Presence of ABC transporters was confirmed in our cell line functionally by **Rhodamine 123 assay.** Rhodamine123 (Rh-123) was shown to be a substrate for both P-gp and ABCG2 transporters [15]. Functionally, the transporters were evaluated by studying the ability of the cells to retain more amount of Rh-123 by using the well-known inhibitors verapamil for P-gp transporter and Ko143 for ABCG2 transporters. The endothelial cells of the BBB were seeded (20x 10^3 /well) in 24 wells quartz plates (Porvair Sciences) and left till 90% confluence. Wells with media without cells were used as a blank. Cells were incubated with Rh-123 (10µg/ml Hanks) for 15 minutes. Rh-123 was removed and wells were washed twice. Verapamil (1 µM, 10 µM, 100 µM and 1000 µM) and Ko143 (1nM, 10nM and 100nM) were incubated with the cells in Hanks solution for 30 minutes. Then, cells were washed twice again with PBS and destroyed by the solubilizing solution. The retained Rh-123 was assessed by fluorescence plate reader (Dyne technologies, Chantilly, VA, USA) using wave lengths (480nm and 530nm) for excitation and emission respectively. Blanks were subtracted from all wells' readings. Retained Rh123 due to different concentrations of the inhibitors was expressed as a percentage from the corresponding control (cells in Hanks without transporters' inhibitors) readings.

Effect of Aps on the P-gp and BCRP transporters: Rh-123 assay was repeated as formerly described. However, instead of the well-known transporters inhibitors, the cells were incubated with the studied APs in concentrations 0.1, 1, 10, 100, and 1000 µM), and the fluorescence were measure by fluorescence plate reader (Dyne technologies, Chantilly, VA, USA) using wave lengths (480nm and 530nm). Rh-123 was normalised to the total cell number of each well. All experiments were conducted for at least 4 times.

Effect of APs on ATP synthesis: The Cells (10^3 cells/well) were cultured in 96 wells plates till 90% confluences and treated with APs in 0.1, 1, 10 and 100 µM concentrations for 3, 6, 12, 24, and 48hours. Intracellular ATP was measured based on lumescence using a commercial kit following the manufacturer's protocol (Abcam, Cambridge, UK). ATP levels were normalised to the number of cells after subtraction of the blanks (wells without cells), then they were quantified as a percentages relative to controls ATP contents. All assay points were performed in triplicate in every experiments and every experiment was repeated for at least 3 times.

Effect of APs on ATP hydrolysis assay: cells were seeded in on 35 mm collagen IV/fibronectin-coated 6 well plates at a density of 5×10^6 cells/well and incubated till confluence and treated with Aps in concentrations 10 and 100 µM for 24 hours. Then ATPase hydrolysis reaction with the purified proteins was conducted following Rule et al. (2016) [16]. Briefly, the diluted protein was added to the reaction buffer and incubated at 37°C for an hour during which 5µl of the buffer was collected in aliquots after 60 minutes to be diluted 1:50 in HNG buffer (5x HEPES/NaCl/glycerol) and frozen at -80 for 10 minutes. Then aliquots were thawed at room temperatures and transferred to 96 well plate (50 µl/well in triplicates from each sample). Wells for standard inorganic phosphate were prepared in serial dilution from0-40µM. Then, 100µl of malachite green/molybdate Pi detection reagent was added to each well and the plate was incubated at the room temperature for 25 minutes. The absorbance was read at 650 nm using the microplate reader. The experiment was repeated for at least three times.

Immunoblot analysis: cells were seeded in on 35 mm collagen IV/fibronectin-coated 6 well plates at a density of 5 × 106 cells/well and incubated till confluence and treated with Aps in concentrations 10 and 100 µM for 24 hours. Western blotting was conducted following ***Papa et al. (2008)[17].*** Briefly, the samples were loaded onto a 4% acrylamide/bisacrylamide gel. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed and proteins were transferred to the nitrocellulose membrane. The membrane was blocked for two hours at 37°C with 5% powdered skimmed milk in PBS containing 0.05% tween 20 (PBS-T). Washed membranes were incubated overnight at 4°C with the primary antibodies. Washed membranes were then incubated for two hours at room temperature with the secondary antibodies in PBS-T containing 1% milk powder. Membranes were washed in PBS-T and P-gp or BCRP were visualized using enhanced chemi-luminescence detection using enhanced chemiluminescence (ECL) detection kit (Amersham, Buckinghamshire, UK) and the bands' density was evaluated by the FluorChem system (Alpha Innotech, San Leandro, CA).

Statistical analysis: Data were given as mean ± standard error of the mean (SEM). Multiple groups comparisons were conducted by One-way ANOVA test with Turkey post-hoc test. *P*-values were considered to be statistically significant at a value of $P<0.05$. All statistical calculations were done using PRISM 3 (Graph Pad Software Inc., San Diego, CA).

RESULTS

The function of P-gp transporters and BCRP was assessed using well known transporters inhibitors and substrate. Both Verapamil and Ko134 were found to significantly block pumping of Rh-123 as shown in figure with more retained Intracellular Rh-123 (Figure 1A and 1B). There was no

significant difference between the inhibitory effect of verapamil in both concentrations 100 and 1000µM on P-gp transporters. On the other hand, no significant difference was shown between the effect of K0134 in concentrations 10 and 100nm on BCRP transporter of the studied the isolated cell line.

Antipsychotics were found to block pumping of Rh123 and increasing the retained Rh123 and inside the treated cells, as shown in figure (1C). CPZ was found to be the inhibit pumping in 10 µM concentration, while the other 3 APs showed significant effect in 100 µM concentration. While the effect of CPZ was reversed at 1mM concentration with significant lower levels of retained Rh123 in comparison to the controls.

Antipsychotics were found to significantly inhibit ATP synthesis inside the treated cells for variable degrees in concentrations and exposure durations dependant patterns, as shown in figure (2A-D). CPZ was the most potent as it was found to decrease ATP significantly in 10 µM concentration. In parallel, APs was shown to have inhibitory effect on the ATP hydrolase activities in the treated cell (figure 2E)

Western blotting assay had shown that all the tested APs significantly decreased the expression of both P-gp and BCRP transporters' protein to different degrees, 24 hours' post-treatment (Figure 3 A and 3B)

Fig. 1: The inhibitory effect of verapamil (1A) and Ko134 (1B) and antipsychotics (Aps) (1C) [chlorpromazine (CPZ), Haloperidol (HAL), Risperidone (RIS) and clozapine (CLZ)] on pumping of Rh-123 by Albino rats' isolated microvascular endothelial cells of the blood brain barrier via P-gp and BCRP transporters. The inhibitory effects of Verapamil, Ko134, and APs were concentration dependant. Data were shown as means ±SD. * means p-vale <0.05 while, ** means p-value <0.01, while *** means p-value <0.001

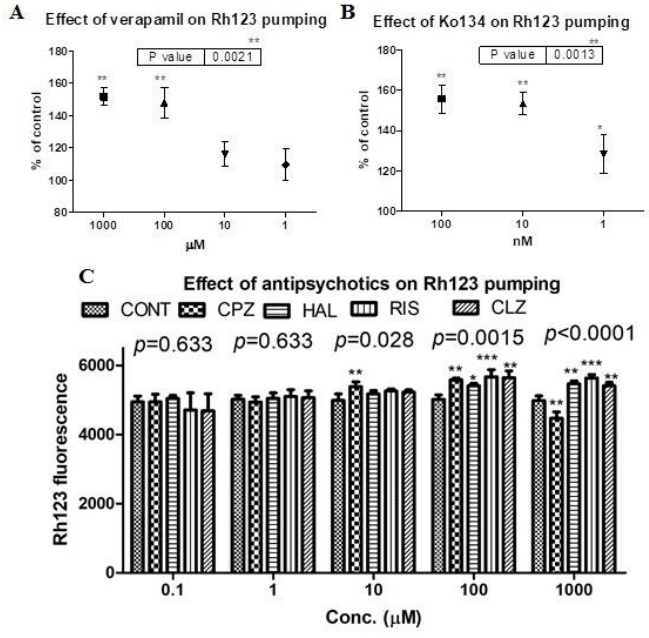


Fig. 2: The effect of antipsychotics (APs); chlorpromazine (CPZ), Haloperidol (HAL), Risperidone (RIS) and clozapine (CLZ) on intracellular ATP concentration (2A-2D) and ATP hydrolase activities (2E) in the Albino rats' isolated microvascular endothelial cells of the blood brain barrier. All APs significantly reduced ATP production in the treated cells and reduce the activities of the ATP hydrolase. Data were shown as means ±SD. * means p-vale <0.05 while, ** means p-value <0.01, while *** means p-value <0.001

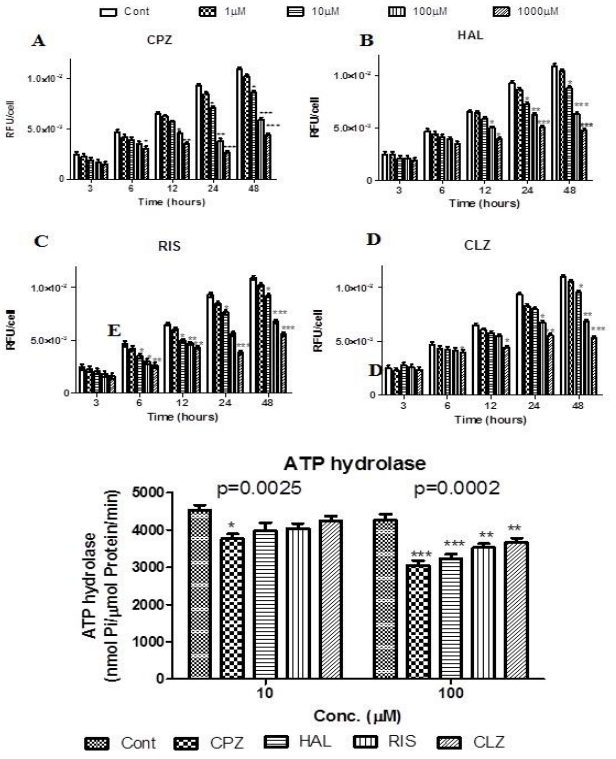
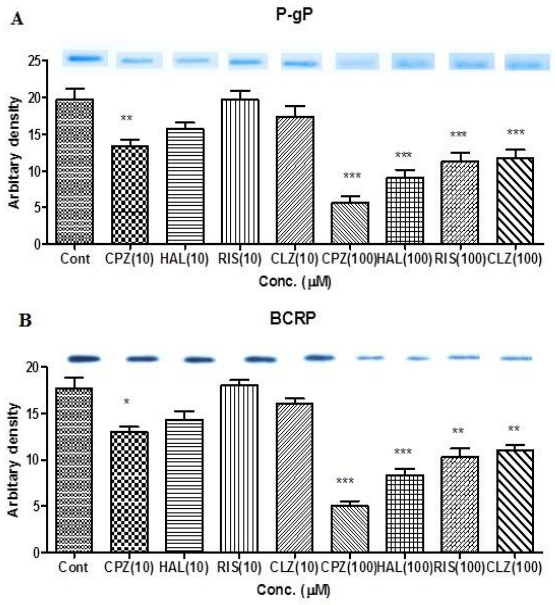


Fig.3: The effect of antipsychotics (APs); chlorpromazine (CPZ), Haloperidol (HAL), Risperidone (RIS) and clozapine (CLZ), in concentrations 10 and 100 µM, on immunoblotting of P-gp and BCRP transporters in the Albino rats' isolated microvascular endothelial cells of the blood brain barrier. Data were shown as means ±SD. * means p-vale <0.05 while, ** means p-value <0.01, while *** means p-value <0.001



DISCUSSION

This study was done to evaluate the effect of ABC transporters in the microvascular endothelial cells of BBB on the APs-induced cytotoxicity. Isolated RMVECs were used as a model to test the hypothesis. The data showed that ABC transporters inhibitors can increase the cytotoxic effect of Aps. On the other hand, the tested Aps were shown to inhibit ABC transporters. Aps were tested in a wide range of concentrations from 0.1-1000µM which cover the therapeutic, supra-therapeutic and toxic concentrations. Therapeutic serum levels were previously reported as 1.5µM for CPZ, 2 µM for CLZ, 0.64 µM for HAL and 0.28 µM for RIS [18]. While the reported toxic serum levels of CPZ, CLZ, HAL and RIS were 3 mM for CPZ[19], 5 mM for CLZ [20], 0.7 mM for HAL [21] and for RIS 0.8 mM[22]. Higher concentrations were used to get the effect of the chronic exposure to APs within the limited time frames of the experiments.

Micro-vascular endothelial cells were isolated for BBB cultures mostly from animal sources, especially from rats [23]. Isolated cells were characterised by the Presence of P-gp and BCRP which was confirmed by western blot and Rh123 assay using the previously reported verapamil and Ko143 inhibitors transporters inhibitors [24].

In the present study we used Ko134 as an inhibitor for BCRP transporter. 10nM was found to be an ideal inhibitory concentration for the subsequent experiments as there was no significant difference between K0134 10nM and 100 nM. So Ko134 (10nm) can give the best inhibitory effect with less cytotoxicity on the cells. Results of the present study showed that verapamil (100µM) would give the best inhibitory effect on P-gp transporter of RMVECs of BBB and at the same time will be safer than 1000 µM, so 100 µM was chosen as an ideal inhibitory concentration in the subsequent experiments.

The present study findings also showed evidence that commonly used APs have had various degrees of inhibitory effects on P-gp and BCRP transporters functions These findings are in agreement with the previously published work by Wang et al. (2006) [12] and Wang et al. (2008)[13], who reported that several APs, including CPZ, RIS, quetiapine, paliperidone and CLZ, are dual inhibitors of both P-glycoprotein and BCRP.

For mechanistic studies, the effect of APs on ATP production in the isolated cells was evaluated as ATP play a major role in the functional integrity of the investigated transporters. The data revealed that APs can significantly inhibit ATP production in a concentration dependant pattern. These findings are in a accordance of different studies using in vivo and in vitro models which revealed that APs can inversely affect the cellular energetic and ATP production [25].

Secondly, the effect of the tested APs on the ATP hydrolase activities was evaluated. ATP hydrolase proper function is mandatory for the function of the ABC-family transporters (Khunweeraphong et al., 2017). APS were shown to significantly decrease ATP hydrolase activities. CPZ showed the most significant effect even in 10 µM concentration. This effect of APs on the hydrolase are expected to inhibit the function of the studied transporters. Finally, APs were shown to decrease the expression of the P-gp and BCRP transporters. P-gp transporter was affected to a greater extent than BCRP transporter expression.

The main limitation of the current study was the difficulty to evaluate the effect of APs on each transporter separately as the cells have both types and may be other types of ABC transporters. However, the present findings may have potential application for safety treatment of the APs. ABC transporters have long been recognized to confer multidrug resistance in cancer and inflammation chemotherapies[26]. Accordingly, in combination with the present findings suggested that toxic doses of APs may alter pharmakco-kinetics and pharmaco-dynamics of a lot of therapeutics which may be given to these patients for therapeutic purposes and they are substrates for ABC transporters. On the other hand, therapeutics which act as inhibitors for these transporters will worsen the outcome of therapy as it will expose the neuronal cells to higher concentrations by blocking the outward pumping action of these transporters in the BBB endothelial cells.

CONCLUSIONS

Antipsychotics have inhibitory effect of p-gp and BCRP transporters of the rats' endothelial cells of the blood brain barrier to variable degrees in a concentration and exposures' durations dependant manner. The tested APs was shown to inhibit ATP synthesis, ATP hydrolase and expression of the transporters proteins in the treated cells in concentrations-based patterns. This inhibitory effect open new channels with the other drugs or chemicals which are known as transporters or inhibitors for these transporters.

Conflict of interest: The authors state no conflict of interest with the bupublished data or conclusions

Acknowledgement: Special thanks for the Deanship of research in Northern Border University, Saudi Arabia for funding this project

Funding: This work was funded by Northern Border university (grant number: 7883-MED-2018-3-9-F)

REFERENCES

1. Abbott NJ, Hughes CC, Revest PA, Greenwood J. Development and characterisation of a rat brain capillary endothelial culture: towards an in vitro blood–brain barrier. J Cell Sci. 1992;103(1): 23– 37.
2. Locher KP. Structure and mechanism of ATP-binding cassette transporters. Philos Trans R Soc Lond B Biol Sci. 2008;364(1514):239-45.
3. Linnet K, Ejsing T. B. A review on the impact of P-glycoprotein on the penetration of drugs into the brain. Focus on psychotropic drugs. Eur Neuropsychopharmacol. 2008;18: 157–169
4. Abraham J, Salama N.N, Azab A.K. The role of P-glycoprotein in drug resistance in multiple myeloma. Leuk lymphoma. 2015;56(1):26-33.
5. Fricker G, Miller D. S. Modulation of drug transporters at the bloodbrain barri. Pharmacology 200470169-176.
6. Meng H, Liong M, Xia T, Li Z, Ji Z, Zink J.I, Nel AE. Engineered design of mesoporous silica nanoparticles to deliver doxorubicin and P-glycoprotein siRNA to overcome drug resistance in a cancer cell line. ACS nano. 2010;4(8):4539-50.
7. Doyle LA, Yang W, Abruzzo LV, Krogmann T, GaoY, Rishi AK, Ross DD. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci. 1998;95(26):15665-70.

8. Doyle LA, Ross DD. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene. 2003; 22:7340-7358.
9. Cooray HC, Blackmore CG, Maskell L, Barrand M.A. Localisation of breast cancer resistance protein in microvessel endothelium of human brain. Neuro. report 2002; 13:2059-2063.
10. Eisenblatter T, Huwel S, Galla H. J. Characterisation of the brain multidrug resistance protein (BMDP/ABCG2/BCRP) expressed at the bloodbrain barrier. Brain Res. 2003;971: 221-231.
11. Donard SD, Lu XH, Ronald J. Cytotoxicity of conventional and atypical antipsychotic drugs in relation to glucose metabolism. Brain Res. 2003; 971:31–39.
12. Wang JS, Zhu HJ, Markowitz JS, Donovan JL, Devane CL Evaluation of antipsychotic drugs as inhibitors of multidrug resistance transporter P-glycoprotein. Psychopharmacol. 2006;187: 415–423.
13. Wang JS, Zhu HJ, Markowitz JS, Donovan JL, Yuan HJ, Devane CL. Antipsychotic Drugs Inhibit the Function of Breast Cancer Resistance Protein. Basic Clin Pharmacol Toxicol 2008;103(4): 336–341.
14. Nakagawa S, Deli MA, Kawaguchi H, Shimizudani T, Shimono T, Kittel A, Tanaka K, Niwa M. A new blood–brain barrier model using primary rat brain endothelial cells, pericytes and astrocytes. Neurochemistry international. 2009;54(3-4):253-63.
15. Sharom FJ. ABC multidrug transporters: Structure function and role in chemoresistance. Pharmacogenomics J. 2008;9(1):105-127.
16. Rule CS, Patrick M, Sandkvist M. Measuring in vitro ATPase activity for enzymatic characterization. JoVE (Journal of Visualized Experiments). 2016;(114):e54305.
17. Papa V, Tazzari PL, Chiarini F, Cappellini A, Ricci F, Billi AM. Proapoptotic activity and chemosensitizing effect of the novel Akt inhibitor perifosine in acute myelogenous leukemia cells. Leukemia. 2008; 22:147–160.
18. Winek CL, Wahba WW, Winek Jr CL, Balzer TW. Drug and chemical blood-level data 2001. Forensic Sci Int. 2001;122(2-3): 107-23.
19. Van Putten T. Marder SR. Wirshing WC. Aravagiri M. Chabert N. Neuroleptic plasma levels. Schizophr bull. 1991;17(2): 197-216.
20. Chang WH, Lin SK, Lane HY, Hu WH, Jann MW, Lin HN. Clozapine dosages and plasma drug concentrations. J Formos Med Assoc. 1997;96(8): 599-605.
21. Chang WH, Shieh YS, Liu HC, Jann MW, Chien CP. Plasma reduced haloperidol/haloperidol ratios in schizophrenic patients treated with high dosages of haloperidol. Eur Neuropsychopharmacol. 1994;4(2):119-126.
22. Titier K, Bouchet S, Pehourcq F, Moore N, Molimard M. High-performance liquid chromatographic method with diode array detection to identify and quantify atypical antipsychotics and haloperidol in plasma after overdose. J Chromatogr B. 2003;788(1):179-185.
23. Rist RJ, Romero IA, Chan MW, Couraud PO, Roux F, Abbott NJ. F-actin cytoskeleton and sucrose permeability of immortalised rat brain microvascular endothelial cell monolayers: effects of cyclic AMP and astrocytic factors. Brain Res. 1997;768: 10-18.
24. Virginento D, Robertson D, Errede M, Benagiano V, Girolamo F, Maiorano E, Roncali L. and Bertossi M. Expression of Pglycoprotein in human cerebral cortex microvessels. J. Histochem Cytochem. 2002;50: 1671-1676.
25. Elmorsy E, Al-Ghafari A, Aggour AM, Mosad SM, Khan R, Amer S. Effect of antipsychotics on mitochondrial bioenergetics of rat ovarian theca cells. Toxicol lett. 2017;272: 94-100.
26. Breedveld P, Beijnen JH, Schellens JH. Use of P-glycoprotein and BCRP inhibitors to improve oral bioavailability and CNS penetration of anticancer drugs. Trends Pharmacol. Sci. 2006;27: 17–24.