



Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc



Bioenergetic disruption of human micro-vascular endothelial cells by antipsychotics



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ARTICLE INFO

Article history:

Received 14 March 2015

Available online 28 March 2015

Keywords:

Antipsychotics

Mitochondria

Blood brain barrier

Endothelia

Heterogeneity

Toxicology

ABSTRACT

Antipsychotics (APs) are widely used medications, however these are not without side effects such as disruption of blood brain barrier function (BBB). To investigate this further we have studied the chronic effects of the typical APs, chlorpromazine (CPZ) and haloperidol (HAL) and the atypical APs, risperidone (RIS) and clozapine (CLZ), on the bioenergetics of human micro-vascular endothelial cells (HBVECs) of the BBB. Alamar blue (AB) and ATP assays showed that these APs impair bioenergenesis in HBVECs in a concentration and time dependent manner. However since these effects were incomplete they suggest a population of cell bioenergetically heterogeneous, an idea supported by the bistable nature by which APs affected the mitochondrial transmembrane potential. CPZ, HAL and CLZ inhibited the activity of mitochondrial complexes I and III. Our data demonstrates that at therapeutic concentrations, APs can impair the bioenergetic status of HBVECs, an action that help explains the adverse side effects of these drugs when used clinically.

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1. Introduction

The BBB is a specialized system of capillary endothelial cells that protects the central nervous system (CNS) from harmful and pathogenic blood constituents such as toxic solutes and bacteria. Moreover the BBB is paramount to the homeostatic maintenance of CNS where it regulates the supply of ions, nutrients and metabolic substrates, as well as the removal of metabolic waste products [1]. In humans the BBB is formed by the brain vascular endothelium where endothelial cells are joined by multiple bands of zona adherens and tight junctions (zona occludens) [2]. The tight junctions effectively close off transepithelial transport via the paracellular pathway. As a result, the majority of solutes cross the BBB of the endothelial cell by facilitated diffusion and primary/secondary active transport. Primary active transport is driven by the work done by their inherent enzymic ATPase activity. In addition to the cationic ATPases, members of the ABC transporter family such as P-glycoprotein (P-GP) and Multidrug resistance related protein (MRP) also have ATPase activity and act to regulate the flow of lipophilic substances across the BBB [1]. Secondary active transport processes

convert the potential energy of trans-plasma membrane solute gradients into the movement of sugars, amino acids as well as various cations and anions up their electrochemical gradients. Consequently, the transport role of the BBB is highly energy dependent; as evidenced by the greater number and volume of mitochondria compared to that of endothelia in peripheral vasculature [3]. Subsequently, the bioenergetic fitness of mitochondria of endothelial cells of the BBB is paramount to a healthy CNS. Many drugs that are toxic to mitochondria are highly lipophilic. Their ability to cross the BBB from the blood plasma to the CSF is prevented by the BBB [1] and compromised by the counter-transport activities of PGP and MRP, consequently a tipping point is envisaged where if a drug reaches a sufficiently high enough intracellular concentration to impair the mitochondrial function of BBB endothelial cells then a combined action of a decrease in intracellular ATP and ATPase-dependent pump activities will culminate in a catastrophic failure of the endothelial cell to act as an effective barrier for that drug; a process manifest as a drug permeable conduit from the plasma into the CNS with associated neuropathologies [4]. Indeed, we have recently shown that a variety of APs can impair BBB functions at concentrations seen at the higher end of their therapeutic windows and with over-dosage [4]. The fact that this effect was associated with decreased cellular redox potential and increased ROS production, implicates that the adverse

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action of APs on endothelial cells of the BBB was most likely mediated by mitochondrial dysfunction. We have now investigated this possibility by studying the direct chronic effects of antipsychotics on mitochondrial function in cultured endothelial cells derived from the vasculature of the human BBB (HBVECs).

2. Materials and methods

2.1. Chemicals and reagents

The selection of neuroleptic drugs was based upon those routinely prescribed for patients being treated for schizophrenia or bipolar disorder: chlorpromazine (CPZ), haloperidol (HAL), risperidone (RIS) and clozapine (CLZ) (Abcam). Stock solutions of drugs were all made in ethanol (vehicle). Drugs were tested with the cells in culture. Mitochondria were isolated with the Qproteome Mitochondria Isolation Kit (Cat. no. 37612). Unless stated all other chemicals were purchased from Sigma–Aldrich.

2.2. Cells

HBVECs were grown and maintained in RPMI-1640 medium which also contained heat inactivated 20% Foetal Bovine Serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% minimum essential media (MEM) vitamins, 1% MEM non-essential amino acids, 100 units of penicillin-G ml⁻¹, and 100 µg of streptomycin sulphate ml⁻¹ as previously described [4]. When confluent, our HBVECs demonstrated morphological and transport properties consistent with a functional endothelium identity [4].

2.3. Alamar blue and ATP assay

The Alamar blue (AB) assay was used to measure redox state (Invitrogen). Cells were seeded at 10⁴ per well in 96 well plates (Nunc), pre-incubated for 24hr in RPMI followed by a further incubation for 4hr, 24hr, 48hr or 72hr in the presence of vehicle or test drug. The ATP assay (Abcam) followed the same incubation steps as above, but with the experiment terminated by the addition of cell lysis buffer. Data is expressed as a percentage of control. Each experiment was performed in triplicate, with the mean result counting as a single experimental value.

2.4. Mitochondrial membrane potential

The cationic dye: DePsipher (Trevigen) was used with flow cytometry to monitor the distribution of mitochondrial membrane potential ($\Delta\Psi_m$) in cells pre-incubated for 24 h in RPMI in the presence of vehicle or test drug. Data was analysed with Weasel software, V2.7.4.

2.5. Cell respiration

The O₂ consumption rate (OCR) of endothelial cell suspensions of known cell density was measured polarographically using Clark oxygen electrodes (Rank Brothers) following the protocol of Daunt et al. [5]. Experiments were performed at 37 °C in a solution that contained (in mM): 5.6 KCl, 138 NaCl, 4.2 NaHCO₃, 1.2 NaH₂PO₄, 2.6 CaCl₂, 1.2 MgCl₂, 10 HEPES (pH 7.4 with NaOH), and 0.1% (wt/vol) BSA. Prior to the measurement of OCR, cells were pre-incubated for 24 h in RPMI in the presence of vehicle or test drug. Background, non-respiratory, oxygen consumption was determined by the addition of 6 mM NaN₃ to block oxidative respiration at cytochrome aa₃. OCR was measured for each condition as the rate of change in [O₂]_{aq} over a 5-min time period.

2.6. Mitochondrial complex assay

Confluent HBVECs were pre-incubated for 24 h with either vehicle or vehicle and test drug. For the complex assays, a mitochondrial enriched fraction and cell lysate was prepared as described by Ref. [6]. Complex I was assayed following the protocol of [7]. Specific Complex I activity was calculated as the difference between that measured in the absence and presence of 2 µM rotenone. Complex III activity was measured by the ability of the cell lysate to reduce cytochrome c monitored by the change in absorbance at 550 nm. Background was corrected by subtraction of the absorbance of samples without lysate. Specific complex III activity was calculated as the difference between that measured in the absence and presence of 2 µM antimycin A. The activities of the complexes were normalised to that measured in vehicle control with the assumption that these possessed 100% activity.

2.7. Statistical analysis

Concentration-response relationships were constructed from single dose concentration experiments. The concentration-response relationships were quantified by fitting the data with the following equation:

$$Y = \frac{100}{(1 + ([D]/IC_{50}))}$$

Where Y is the magnitude of drug effect as a percentage of the control, [D] is the concentration of drug; IC₅₀ is the concentration of drug that produces a half-maximal effect. IC₅₀ values are quoted with 95% confidence intervals. Data were tested for Normal distribution with the D'Agostino & Pearson omnibus normality test and were compared using the appropriate statistical test as indicated in the text. These procedures were all performed using PRISM 6 (GraphPad Software Inc., San Diego, CA). The fitting of equations to data used a least squares algorithm as supplied with PRISM. Statistical significance is defined as P < 0.05 and is flagged as * in graphics, ** when P < 0.01, *** when P < 0.001 or **** when P < 0.0001.

3. Results

3.1. Alamar blue and intracellular ATP assays

All four APs decreased the ability of HBVECs to reduce AB, an action that increased with time and concentration (Fig. 1), however, the block was never complete. For HAL and CLZ the maximal block was independent of incubation time, whereas it increased with incubation time for RIS (r = -0.98, p < 0.01, Pearson) and CLZ (r = -0.92, p < 0.05, Pearson). The IC₅₀ for HAL, CPZ and RIS all significantly decreased with incubation time (r = -1, p < 0.05, Spearman; Fig. 1E) whereas that for CLZ it did not (r = 0.6, p = 0.2, Spearman; Fig. 1E). After 72 h incubation, CPZ produced the most potent inhibition with a pIC₅₀ ~ 5.1 M (8 µM).

After 24 h incubation in APs, intracellular ATP was also decreased in relation to the concentration of drug used (Fig. 1F). Comparison of the IC₅₀ values for the effect of APs on AB reduction and intracellular ATP levels, shows that the latter appears more sensitive, although only CPZ and RIS were significantly so (p < 0.05, Student's t-test).

3.2. Oxygen consumption

In the absence of glucose, HBVECs respired with a linear rate. This was significantly decreased relative to the vehicle control

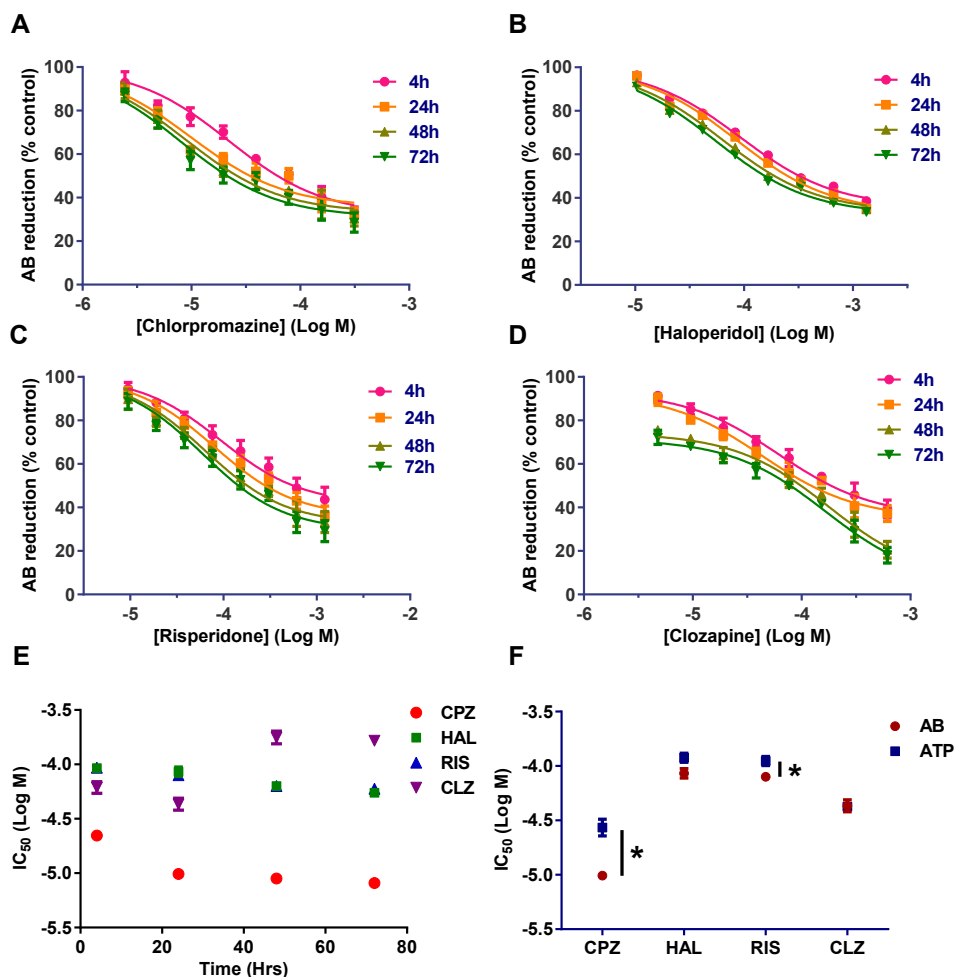


Fig. 1. Effect of APs on the bioenergetic status of HBVECs from the BBB. A–D) Effect of AP concentration on the reduction of AB measured after 4, 24, 48 and 72 h post drug treatment for the drugs indicated. Data are given as percentages of the vehicle controls. Solid lines are drawn to the equation given in the methods with IC_{50} values given in the text and panel E). E) IC_{50} as a function of incubation time for various APs as indicated. F) Comparison of IC_{50} for the decrease in AB reduction and cytosolic ATP levels after 24 h incubation in the APs as indicated. Data are shown as means \pm S.E.M (n = 6–9).

(ANOVA) after 24 h chronic exposure to the APs tested at their IC_{50} for inhibition of AB reduction (Fig. 2A). The greatest decrease on OCR was observed with CPZ and HAL and the least with CLZ and RIS (Fig. 2A). Within 2 min of adding 10 mM glucose the OCR decreased (Crabtree effect), although the relative abilities of the APs to decrease OCR was conserved (Fig. 2B).

3.3. Mitochondrial complexes

After 24 h chronic exposure to the APs, applied at their IC_{50} for inhibition of AB reduction, all four drugs significant decreased the activity of complex I and complex III in relation to vehicle (Fig. 2C and D; One sample t-test). CPZ was the most efficacious AP at both complex I ($p < 0.001$, ANOVA) and complex III ($p < 0.05$, ANOVA).

3.4. Mitochondrial membrane potential

Flow-cytometry revealed that in vehicle control, HBVECs possessed a spread of $\Delta\psi_m$ values; although the majority of cells had energised values of $\Delta\psi_m$ (predominantly orange), a few were de-energised (lower end of orange to green) and a very few had a completely collapsed $\Delta\psi_m$ (green only fluorescence; Fig. 3A). Of the 4 APs incubated for 24 h at their IC_{50} for AB reduction, CPZ had the great impact on mitochondrial membrane potential (Fig. 3), causing

the greatest proportion of cells with a collapsed $\Delta\psi_m$, and eradication of cells with partially energised $\Delta\psi_m$. The percentages of the cell population with exclusively green fluorescence (lower right region Fig. 3) for CLZ and RIS were insignificantly different to vehicle control, unlike CPZ and HAL, for which this was a major component of the dye redistribution (Fig. 3B). CPZ, HAL and CLZ, but not RIS, were all associated with a significant decrease in the high end of orange–green fluorescence (upper left region Fig. 3).

4. Discussion

The aim of the present study was to investigate to what extent four widely used APs had on the mitochondrial function in HBVECs of the BBB.

4.1. Alamar blue and intracellular ATP assay

For assessment of the steady state redox potential we performed AB (resazurin) assays. On cell penetration, resazurin is irreversibly reduced to resorufin as the result of different redox enzymes [8]. Like other colorimetric assays of redox state, e.g. MTS and MTT, AB output is a product of the population average redox potential and cell number. As we have previously shown [4], the former effect dominates since that the number of viable HBVECs, determined via

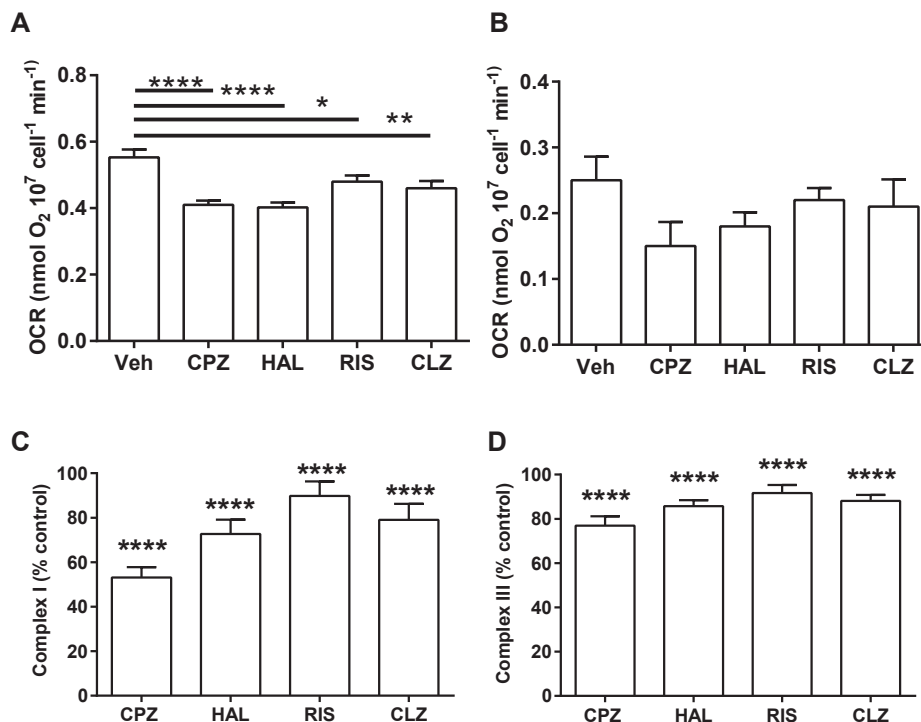


Fig. 2. Effect of APs on the oxygen consumption rate (OCR) and mitochondrial complex activity of HMVECs of BBB after 24 h pre-exposure to the APs indicated at their IC_{50} values for AB reduction. A) OCR in the absence of exogenous substrate B) OCR after the addition of 10 mM glucose. Note the decreased OCR in response to the sugar. Data shown as means \pm S.E.M ($n = 5-6$). C & D) Effect of APs on the reduction of mitochondrial complexes I (C) and III (D). Data is expressed as a percentage of vehicle control and is shown as means \pm S.E.M ($n = 9$).

trypan blue exclusion or lactate dehydrogenase leakage, lags in time and also occurs at much higher concentrations than the effect of the APs on AB reduction, furthermore the decrease in respiration seen in the OCR is for data normalized to cell density. The fact that the block of AB reduction was incomplete at the highest AP concentrations and incubation times employed indicates cell heterogeneity [9], an idea also supported by the range of $\Delta\psi_m$ values observed: data clearly indicative of cellular heterogeneity in the mitochondrial energetic status of HBVECs. Furthermore, since CPZ completely collapsed $\Delta\psi_m$ for some cells leaving other cells apparently unscathed, is also evidence for bioenergetic heterogeneity in our HBVECs, where it appears that, at least for this particular AP, a tipping point is reached that leads to a switch between an energised and deenergized mitochondrial state: bistability.

CPZ was 3–10 x more potent an inhibitor of AB reduction than HAL, RIS and CLZ, whom all possessed IC_{50} values within a 1 \log_{10} unit range. CPZ also had the greatest increase in potency with incubation time. The IC_{50} of 22 μ M for CPZ in HBVECs after 4 h incubation is similar to that seen in endothelial cells from human umbilical vasculature (25 μ M) whereas the IC_{50} value for CPZ in dividing and non-dividing human lymphocytes is far higher at 88 μ M and >400 μ M respectively [10]. Together this data suggests that endothelial cells appear to have an increased sensitivity to CPZ. The ability of APs to inhibit the redox potential as revealed with the AB assay confirm our earlier findings which utilized the MTT assay [4]: the inhibition of AB reduction was incomplete at the highest concentrations and longest incubations of AP tested.

The bioenergetic heterogeneity we observe arises from the variability in mitochondrial energetic status due to the cells being at different stages of the cell cycle [11]: where cells in the S and G2 phases are predominantly dependent on glycolytic energy catabolism, and will possess low $\Delta\psi_m$ values, whereas cells in the G1 phase are predominantly aerobic in bioenergenesis and will

possess high values of $\Delta\psi_m$ [12]. Consequently, there will be cells in G1 phase that are the most vulnerable to mitochondrial inhibitors, an idea supported by close inspection of the DePsiSpher data, where, with the exception of RIS, cells with the highest $\Delta\psi_m$ values (mostly orange fluorescence) are amongst the ones primarily lost after incubation with APs. Indeed flow cytometry with propidium iodide [4] has revealed that 30–40% of the HBVECs are in S or G2 phase, a value similar to the proportion of the cell population shown here to be resistant to the actions CPZ as revealed by both the AB and DePsiSpher assays (proportion of cells orange green).

The decrease in ATP levels observed with APs is the canonical functional consequence of the associated decreased redox state. In the neuronal and lymphocyte cell lines, SH-SY5Y and U937, Heiser et al. [13] reported significantly lower ATP levels after incubation with HAL. In our study, CPZ had an IC_{50} on ATP production lower than HAL, a finding possibly explained by the failure of endothelial cells to generate the more potent neurotoxic metabolite HPTP (1,2,3,6-tetrahydropyridine) from HAL [14].

4.2. Effect of antipsychotics on mitochondrial electron transport

The basal rate of oxygen consumption for HBVECs was ~ 5.5 nmole O₂ 10⁷ cell⁻¹ min⁻¹, a value similar to that seen for other cell lines [5]. Consistent with the other bioenergetic assays, CPZ had the greatest potency for the inhibition of OCR. CPZ, HAL, RIS and CLZ are well established inhibitors of Complex I of the electron transport chain in isolated mitochondrial preparations [15, 16, 17], and when tested acutely have estimated IC_{50} values of $\sim 35-120$ μ M for CPZ, ~ 35 μ M for HAL, ~ 65 μ M for RIS and >200 μ M for CLZ. CPZ can also impair, albeit less potently the activities of both complex II ($IC_{50} \sim 260$ μ M [17]) and III ($IC_{50} \sim 150$ μ M [16]) as well as acting as a partial mitochondrial uncoupler ($IC_{50} \sim 50$ μ M [16]), these other effects may synergise to bring about the drastic change observed in $\Delta\psi_m$

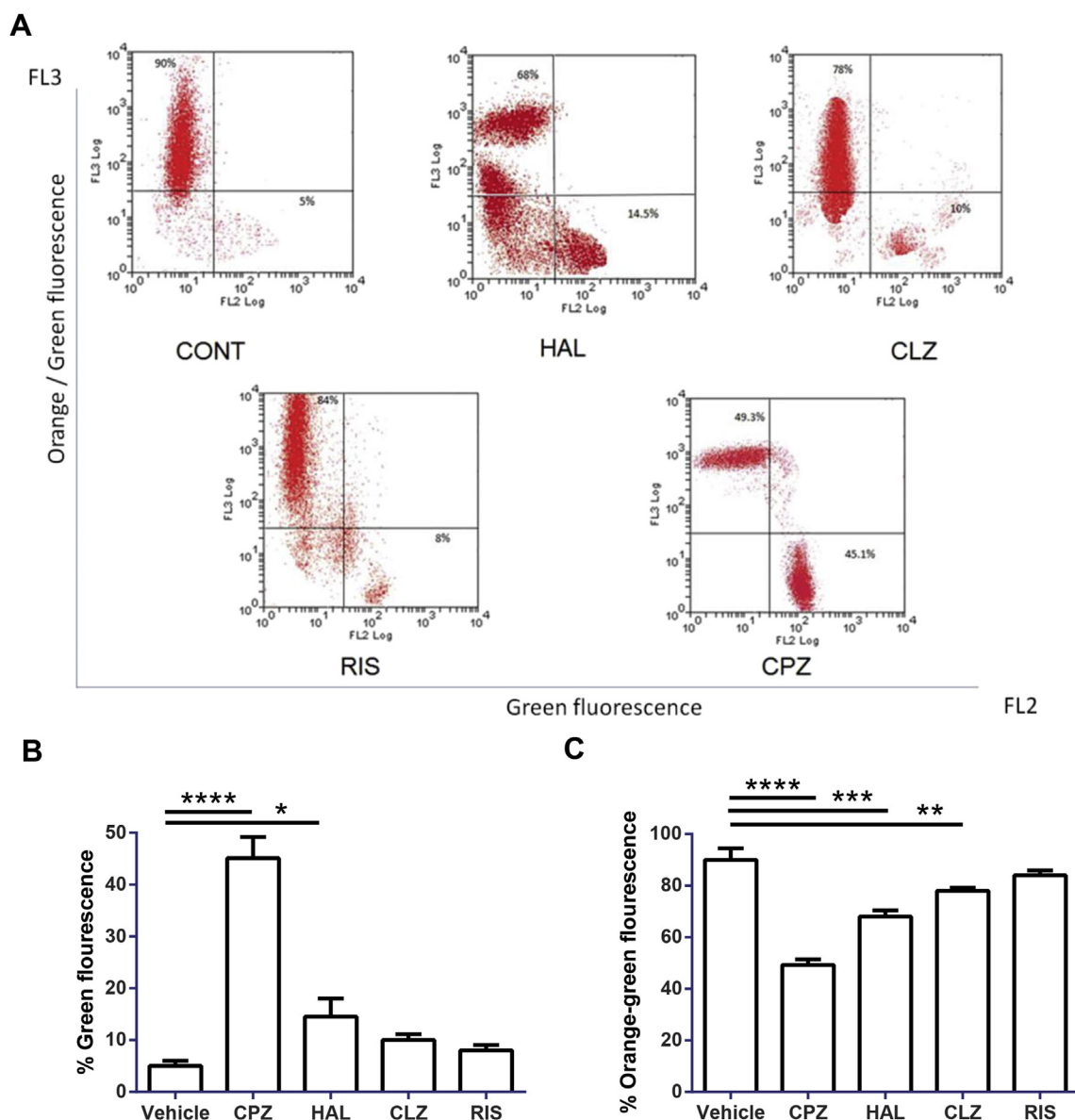


Fig. 3. Representative quantification of DePsiSpher stain by flow-cytometry after incubation with CPZ, HAL, RIS and CLZ for 24 h at their IC_{50} for AB reduction. The FL3 axis represents cells with orange–green fluorescence, while the FL2 axis represents cells with only green fluorescence and a collapsed mitochondrial membrane potential. The number shown in the quadrants are the average values from 3 experiments for that quadrant. B) Effect of APs on the proportion of cells expressing green only fluorescence. C) Effect of APs on the proportion of cells expressing orange–green. Data is expressed as a percentage of the total cell population and is shown as means \pm S.E.M (n = 12).

with this drug. Indeed, inhibition of Complex I within neurons of the cortex by APs has previously been proposed to partly explain the extrapyramidal symptoms seen with this class of drugs [18]. After 4 h incubation in intact HBVECs, the IC_{50} values for the inhibition of bioenergenesis as assayed via AB reduction were 22 μ M for CPZ, ~92 μ M for HAL, ~93 μ M for RIS and ~61 μ M for CLZ, values similar to those reported for the inhibition of complex I in isolated mitochondria [15,16], data indicative of Complex 1 as the major target for these drugs in HBVECs. This has the consequential effects of reactive oxygen species generation, impaired BBB transport function, as well as induction of apoptosis, effects we have previously reported for HBVECs in response to these APs [4]. Why the IC_{50} for the inhibition of AB by CPZ in intact cells decreased with incubation time and ultimately surpasses its IC_{50} for its direct effect on Complex I in isolated mitochondria is unclear. This is unlikely to relate to its behaviour as a blocker of P-GP mediated efflux since

both HAL and RIS also block P-GP with potencies nearly identical to that of CPZ [19].

4.3. Clinical significance

Of the many drugs that target and impair mitochondrial function [20], psychotropic medications are well renowned for this phenomenon. Many antipsychotics, in particular CPZ, HAL, RIS and CLZ, have been demonstrated to directly damage mitochondria [18,21]. Indeed, the toxicological effects of these drugs on mitochondria are thought to underlie some of their central nervous system (CNS) associated neurological side effects [4,22] that are associated with antipsychotic overuse and misuse, such as tardive dyskinesia [23], neuroleptic associated cerebral oedema [24] and neuroleptic malignant syndrome [25]. Indeed the relative potencies of these neuroleptic drugs as inhibitors of mitochondrial

bioenergetic function is similar to their relative potencies as risk factors in the reported incidence of extrapyramidal symptoms, including tardive dyskinesia (TD). Our data suggests that an impaired bioenergetic function of HBVECs may be involved in the cellular pathology underlying TD, whereby antipsychotics energetically compromise HBVECs to allow their passage across the BBB and bring about both therapeutic as well as adverse neurological actions. This means that a preclinical in-vitro assay of the ability of the newly discovered APs on mitochondrial bioenergetics will help to anticipate their efficacy as well as occurrence of extrapyramidal manifestations.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

Financial support to EE was provided by the Ministry of Higher Education, Egypt. PAS thanks the University of Nottingham for research facilities.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.bbrc.2015.03.122>.

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