Evidence against a role of P-glycoprotein in the clearance of the Alzheimer's disease A β _{1–42} peptides

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Abstract It has been proposed that the amyloid- β peptides (A β) cause the neuronal degeneration in the Alzheimer's disease brain. An imbalance between peptide production at the neuronal level and their elimination across the blood–brain–barrier (BBB) results in peptide accumulation inside the brain. The identification and functional characterization of the transport systems in the BBB with the capacity to transport A β is crucial for the understanding of A β peptide traffic from the brain to the blood. In this context, it has been suggested that the P-glycoprotein (P-gp), expressed in endothelial cells of the BBB, plays a role in the elimination of A β . However, there is little, if any, experimental evidence to support this; therefore, the aim of this investigation was to determine whether P-gp is capable of transporting A β peptides. Our results show that ATPase activity measured in plasma membrane vesicles of K562 cells overexpressing P-gp is not increased by the presence of A β ₄₂, suggesting that A β ₄₂ is not a P-gp substrate. Similarly, P-gp of pirarubicin was unaffected by A β ₄₂. Moreover, the overexpression of P-gp does not protect cells against A β ₄₂ toxicity. Taken together, our results support the conclusion that A β ₄₂ is not transported by P-gp.

Keywords Alzheimer's disease · Amyloid- β peptides · Blood–brain–barrier · P-Glycoprotein

Introduction

Alzheimer's disease (AD) is the most important age-related neurodegenerative disorder characterized by a gradual loss of episodic memory (Niedowicz et al 2011). It is estimated that 60 % of dementia cases are due to AD, and more than 35

million people are currently living with the disease worldwide (Niedowicz et al 2011; Wimo et al 2013). It is expected that the number of persons that suffers AD will increase to more than 66 million by 2030 (Wimo et al 2013). The two pathological hallmarks of AD are neurofibrillary tangles and senile plaques. These plaques are proteinaceous extracellular deposits of amorphous aggregates of amyloid- β peptides (A β) (Cvetković-Dožić et al 2001; Selkoe 2001; Walsh and Selkoe 2004; Perl 2010). A β is generated in neurons by proteolytic cleavage of a transmembrane precursor protein (APP) by β -secretase and γ -secretase. As a result of this cleavage, species of 39–43 amino acids are generated and released from the membrane. The 40-residue A β (1–40) peptide represents the most abundant A β isoform inside the brain (80–90 %), whereas the 42-residue amyloid- β peptide (A β ₄₂) is less abundant. The isoform A β ₄₂ is believed to play a central role in AD pathogenesis (Selkoe 2001; Götz et al 2011; Lazarov and Demars 2012).

It has been shown that the A β peptides are neurotoxic. In the AD brain, an imbalance between the production of A β peptides by the neurons and their elimination through the blood–brain–barrier (BBB) may produce an increase in the levels of the peptides in the brain. This idea is the central core of what is known as “the amyloid cascade hypothesis,” which postulates that the deposition of the toxic amyloid- β peptides in the brain is a central event in AD pathology (Hardy and Higgins 1992; Haass and Selkoe 2007; Mucke 2009). The mechanisms responsible for extruding A β from the brain to the blood to maintain A β at normal concentrations remain unknown.

P-Glycoprotein (P-gp) is expressed on the luminal side of endothelial cells of the BBB and has been proposed as a potential A β ₄₂ transporter (Bendayan et al 2006). Lam et al. (2001) reported enhanced P-glycoprotein ATPase activity in vesicles derived from the multidrug-resistant cell line CH^RB30 in the presence of A β (1–40) and A β (1–42). In addition, Cirrito et al. (2005) studied the accumulation of

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A β in the brains of *mdr1a/b*^{-/-} double knockout (P-gp null) mice, suggesting that P-gp could prevent the risk of developing AD. Similar results observed in intact mice brain capillaries conducted by Hartz et al. (2010). Conflicting data, however, have been reported by others indicating that A β ₄₂ is not a P-gp substrate. For example, Ito et al. (2006) described in rat BBB a LRP1-mediated transport of A β ₄₂. In this study they found that the role of P-gp was insignificant and concluded that the transport of A β ₄₂ could be done by other non-identified protein. In a BBB in vitro model made of endothelial cells hCMEC/D3, Nazer et al. (2008) found that the overexpression of either LRP1 or P-gp alone had no effect in the transport of A β ₄₂.

We have been studying the P-gp and its role in the multidrug resistance (MDR) phenomenon in tumor cells (Salerno et al 2002; Darghal et al 2006; Saengkhae et al 2007). P-gp is an ubiquitous ABC family transporter with a broad range of substrates. In view of the molecular structures of the numerous known P-gp substrates, A β is an unlikely substrate of P-gp.

In this context, the aim of the present work was to study the ATPase and the transport activity of the P-gp in the presence of A β ₄₂, in order to verify whether the “interaction” between these two proteins actually modifies the functioning of this ABC transporter. For this purpose, we studied: (i) the ATPase activity of P-gp in the presence of A β ₄₂, in plasma membrane vesicles; (ii) the transport of pirarubicin, a well-known P-gp substrate, in the presence of A β ₄₂ in intact cells overexpressing P-gp; and (iii) the toxicity of A β ₄₂ in the same cell lines.

Materials and methods

Drugs and chemicals

Doxorubicin (DOX) (Teva) and pirarubicin (PIRA) were solubilized in water. Concentrations were determined by diluting stock solutions approximately 10⁻⁵ M with $\epsilon_{480}=11,500\text{ M}^{-1}\text{ cm}^{-1}$. Stock solutions were prepared just before use. Triton X-100 was dissolved in water. HEPES buffer solution contains 20 mM HEPES, 132 mM NaCl, 3.5 mM KCl, 1 mM CaCl₂, and 0.5 mM MgCl₂ at pH 7.3, with or without 5 mM glucose. Phosphate-buffered saline (PBS) buffer solution contains 4.3 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl, and 1.4 mM KH₂PO₄ at pH 7.3. Oubain, H₂SO₄, ammonium heptamolybdate, ascorbic acid, and potassium antimony (III) tartrate were solubilized in H₂O.

The sodium orthovanadate solutions (100 mM Na₃VO₄) were prepared following indications of Goodno (1982). Before use, the solutions of Na₃VO₄ were boiled until translucent (~5 min). All reagents were of the highest quality available and were purchased from Sigma. Deionized double-distilled water was used throughout the experiments.

Electronic spectroscopy

Electronic spectra were recorded on a Varian Cary 300 spectrometer at 37 °C.

Fluorescence spectroscopy

All fluorescence measurements were performed using a Perkin Elmer LS50B spectrofluorometer.

Preparation of the A β ₄₂ peptide solutions

A β ₄₂ peptide (EZBiolab Laboratories) was initially solubilized, at a final concentration of 1 mM, in 1,1,1,3,3,3 hexafluoro-2-propanol or hexafluoroisopropanol (HFIP) (Fluka). Monomers were prepared as described by Ryan et al. (2010) with some modifications. Briefly, stock solution in HFIP was aliquoted in Eppendorf tubes (20 μ L/tube) and dried under N₂ gas atmosphere. The A β ₄₂ dried films were stored at -20 °C until processing. Aliquots were resuspended at a final concentration of 5 mM in DMSO (Sigma), sonicated using a bath sonicator for 10 min, and diluted to 100 μ M with a PBS buffer+0.05 % sodium dodecyl sulfate (SDS) (Sigma). To study the aggregation kinetics of A β ₄₂, the stock solutions were diluted to 20 μ M with PBS buffer (pH 7.3) and aggregation process started with incubation at 37 °C in a water bath for 72 h.

Aggregation kinetics

In order to follow the kinetics of aggregation of A β ₄₂, aliquots of the solution of A β ₄₂ (30 μ L) were taken at different times of incubation, and the process was followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12 % Tris-glycine gels. Silver staining method (Rabilloud et al 1988; Chevallet et al 2006) was used to detect the peptide in gels.

Cell lines and cultures

The K562 cell line is a highly undifferentiated erythroleukemia originally derived from a patient with chronic myelogenous leukemia (Lozzio and Lozzio 1975). K562 leukemia cells and the P-glycoprotein overexpressing K562/ADR cells were cultured in RPMI 1640 medium with GlutaMAXTMI (GIBCO) medium supplemented with 10 % fetal calf serum (GIBCO) at 37 °C in a humidified incubator with 5 % CO₂. The overexpressing P-gp cell line, K562/ADR, was cultured with 400 nM doxorubicin, until 4 weeks before experiments. K562 cultures were initiated at a density of 10⁵ cells/mL grew exponentially to about 10⁶ cells/mL in 3 days. In order to have enough cells in the exponential phase for assay, cultures were initiated at 5 $\times$ 10⁵ cells/mL and allowed

to grow for 24 h until use. All cultured cells were counted with a Coulter counter before use.

Plasma membrane vesicles preparation

Vesicle membranes were prepared as described by Garrigos et al. (1993): K562/ADR cells were centrifuged (1000×g, 15 min, 4 °C). The cells were then resuspended in an hypotonic buffer (10 mM Tris–HCl, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 1 mM EGTA, and 5 mM NaN₃ pH 7.8) at a final concentration of 2×10⁷ cells/mL and sonicated during 10 s (three times in ice) (VIBRACELL, Sonics and Materials Inc., Dantbury Connecticut, USA) at 20 % of maximal power. Lysed cells were centrifugated (1400×g, 15 min, 4 °C), and supernatant was placed in a tube with a layer of 46 % *p/v* sucrose in hypotonic buffer, recentrifugated (7000×g, 20 min, 4 °C). Layer at the sucrose interphase was removed, twice diluted with hypotonic buffer and sedimented (135,000×g, 15 min, 4 °C). The pellets containing membrane vesicles were resuspended in hypotonic buffer containing 100 mM NaCl. Membranes were frozen at –20 °C until use.

Protein determination

The protein content of K562/ADR plasma membrane was determined with the Bio-Rad Quick Start™ Bradford kit, using bovine serum albumin (BSA) as standard.

Measurement of ATPase activity

Membrane suspensions of K562/ADR cells (30 µg proteins) were incubated in a buffer solution containing 50 mM Tris, 2 mM EGTA, 2 mM DTT, 50 mM KCl, 0.5 mM Ouabaine, and 5 mM NaN₃ (pH=6.8). ATPase reaction was started by the addition of 1 mM ATP, and reaction mixtures were incubated for 1 h at 37 °C in a water bath under stirring. Aliquots of 0.1 mL reaction mixtures were mixed with 0.1 mL of 5 % SDS in order to stop ATP hydrolysis. Inorganic phosphate levels in samples were measured by a modification of method from Bartolommei et al. (2013). Briefly, the SDS containing samples were supplemented with 0.8 mL of a buffer containing 125 mM H₂SO₄, 0.5 mM ammonium heptamolybdate, 10 mM ascorbic acid, and 40 µM potassium antimony(III) tartrate, and incubated for 1 h at room temperature under stirring. Absorbance was recorded at 880 nm. Controls of reactions were included in all assays, specifically verapamil (a P-gp substrate) and sodium orthovanadate (a classic inhibitor of ATPase activity).

Determination of the P-gp-mediated efflux of pirarubicin in the presence or absence of A β ₄₂

K562/ADR cells (10⁶ cells/mL, 2 mL per cuvette) were incubated for 30 min in HEPES buffer with sodium azide, but without

glucose (energy-deprived cells). The cells remained viable throughout the experiment, as checked with Trypan blue (not shown). After addition of pirarubicin (PIRA), the decrease in the signal was monitored until steady state was reached. Since the pH of the medium was chosen to equal the intracellular pH, at steady state, the extracellular-free drug concentration (C_e) was equal to the cytosolic-free drug concentration (C_i). Before the addition of glucose, the cells were treated or not with different concentrations of A β ₄₂ (concentrations, 0.5, 1, and 2 µM) during 5 min. Then, glucose was added, which led to the restoration of control ATP levels within 2 min and to an increase in the fluorescence signal due to the efflux of anthracycline. ATP-dependent PIRA efflux was determined from the slope of the tangent of the curve $F=f(t)$, where F is the fluorescence intensity at the time of addition of glucose. Since under these conditions $C_i=C_e$, the passive influx and efflux were equal at the moment of addition of glucose, the net initial efflux represents the P-gp-mediated active efflux only (Mankhetkorn et al 1996; Marbeuf-Gueye et al 1999).

Toxicity assays

The different cell lines were incubated with different concentrations of A β ₄₂ peptide (1, 2, and 4 µM). The assay was carried out in 96-well plates. Briefly, 10⁵ cells/well were incubated for 72 h at 37 °C and 5 % CO₂ in presence or absence of A β ₄₂ peptide. Toxicity was evaluated using Trypan blue dye. In all experiments, the cells were grown in serum free culture medium in order to prevent the binding of A β to albumin (Biere et al 1996).

Results

A β ₄₂ aggregation kinetics

The goal of this study was to test the hypothesis that P-gp is a transporter for A β ₄₂. A major impediment to the study of A β ₄₂ is its low aqueous solubility and rapid aggregation. In order to ensure consistent responses induced by A β ₄₂, we followed the dissolving protocol proposed by Ryan et al. (2010). Using this protocol, we were able to obtain suspension of A β ₄₂ consistently made of monomeric forms. The first step of the A β aggregation process is the formation of low molecular weight species. Conformational changes occur with time, giving rise to the formation of higher molecular weight species responsible of the peptide toxicity. As shown in Fig. 1, the A β ₄₂ aggregation process was visualized by SDS-PAGE electrophoresis of a solution of A β ₄₂ (20 µM), incubated during 72 h in PBS buffer at 37 °C. Under these conditions, the aggregation from monomeric to oligomeric aggregates occurred within 72 h of incubation. The electrophoresis pattern

showed bands localized around 4.5 kDa and around 16 kDa that we defined as monomers and tetramers of A β ₄₂, respectively. These forms are seen from the start of the process. The highest aggregates larger than 66 kDa that could be observed in the figure, which we defined as the oligomeric forms, are seen after 48 h of incubation. In order to ensure mostly monomeric species of A β in the experiments that follow, we always used freshly prepared PBS buffer solutions of A β . Higher insoluble species resided in the stacker segment of the SDS-PAGE gel, not migrating into the resolving gel.

Effect of A β ₄₂ peptide on the P-gp ATPase activity

The P-gp-mediated transport of molecules is an energy-dependent process wherein the energy is provided by hydrolysis of ATP. In order to study possible interactions between A β ₄₂ and P-gp in native plasma membranes, we measured the P-gp ATPase activity from membrane vesicles of K562/ADR cells in the presence of A β ₄₂. For this purpose, the inorganic phosphate (Pi) released during ATP hydrolysis was measured. In order to isolate P-gp ATPase activity from other activities in native plasma membranes, the solutions used in this investigation contained specific ATPase blockers (see solutions in “Materials and methods”). A necessary criterion to consider a molecule as a substrate for the P-gp is that the ATPase activity of P-gp has to be stimulated by the molecule to levels higher than the basal activity level (Sharom 2008).

We measured the effect of A β ₄₂ on the ATPase activity of P-gp at A β ₄₂ concentrations from 0 to 10 μ M, and the results are shown in Fig. 2a. The various concentrations of A β ₄₂ were obtained by diluting stock solutions of A β ₄₂ with PBS. The data obtained with the mixture containing A β ₄₂ peptide were corrected by subtracting the corresponding data obtained with the solvent mixture alone. As can be noted, data points

basically laid over the dotted line that indicates the level of the ATPase basal activity of P-gp showing clearly, that A β ₄₂ did not modify the ATPase activity of P-gp.

We also studied the P-gp ATPase activity from membrane vesicles of K562/ADR cells in the presence of several concentrations of verapamil. Verapamil is a known substrate that alters P-gp ATPase activity (Spoelstra et al 1994; Sharom et al 1995b). Verapamil was dissolved in small volumes of ethanol solutions to concentrations up to 10 μ M. The same volumes of ethanol solution were tested on the membrane vesicles and showed no effect on the basal ATPase activity of the P-gp (not shown). As it is shown in Fig. 2b, low concentrations of verapamil produced moderate increases above the basal level of the P-gp ATPase activity. A maximum increase was observed at 5 μ M. As expected, verapamil concentrations beyond this level were inhibitory. This particular behavior of verapamil on the P-gp ATPase activity has been previously reported (Sharom et al 1995b).

Likewise, we performed another set of measurement of the P-gp ATPase activity from membrane vesicles of K562/ADR cells, in the presence of sodium orthovanadate (0–100 μ M). Sodium orthovanadate has been shown to be an inhibitor of P-gp ATPase activity (Sharom et al 1995a). Figure 2c shows the result of this experiment. As described before, all concentrations of sodium orthovanadate tested reduced the P-gp ATPase activity to levels below the basal level. These results are expected from a compound that is known to inhibit the P-gp ATPase activity and confirm the adequacy of the methodology that we followed to evaluate the effect of A β ₄₂ peptide on the P-gp ATPase activity.

P-gp-mediated efflux of pirarubicin in K562/ADR cells

To further study the possible participation of the P-gp in the cellular transport of A β ₄₂ we chose a method that has previously

Fig. 1 A β ₄₂ aggregation by SDS-PAGE electrophoresis. A β ₄₂ solutions, at a concentration of 20 μ M and at 37 °C, were incubated for up to 72 h in PBS buffer. The electrophoresis pattern showed monomers and tetramers localized around 4.5 and 16 kDa, during the first 4 h of incubation. Highest oligomeric aggregates, larger than 66 kDa, are seen after 48 h of incubation. The figure shows that A β ₄₂ aggregation from monomeric to oligomeric aggregates occurred within 72 h of incubation

Fig. 2 P-gp ATPase activity measured in plasma membrane vesicles of K562/ADR cells. Membrane vesicles were exposed to various concentrations of A β_{42} (**a**), verapamil (**b**), and vanadate (**c**). The activity is plotted as the percentage of the ATPase basal activity. Values represent the means $\pm$ SD from at least four independent experiments. Reaction medium and Pi determination are described at “Measurement of ATPase activity”. The data obtained in the presence of A β_{42} peptide were corrected by subtracting the corresponding data obtained with the solvent mixture employed for the preparation of A β_{42} solutions alone. The resulting points basically lay over the dotted line that indicates the level of the ATPase basal activity of P-gp. A β_{42} actually did not affect the ATPase activity (**a**). A stimulatory effect of ATPase activity was observed at 5 μ M verapamil (**b**). For all concentrations, vanadate (**c**) shows an inhibitory effect of ATPase activity. Dotted lines indicate ATPase basal activity level. Where error bars are not visible, they are contained within the symbols

been shown to be suitable to study the transport of anthracyclines mediated by P-gp in intact cells (Frezard and Garnier-Suillerot 1990, 1991; Pereira et al 1994; Borrel et al 1994a,b;

Mankhetkorn et al 1996). This method allows to accurately measuring the free cytosolic concentration of anthracyclines under steady-state conditions, as well as the initial rates of uptake and the kinetics of anthracyclines active efflux. Thus, we investigated the effect of A β_{42} on the P-gp-mediated ATP-dependent PIRA efflux. Following incubation in a glucose-free HEPES buffer containing sodium azide to deplete internal ATP, cells were incubated for 30 min in media containing 1 μ M of the fluorescent probe PIRA to allow the internalization of PIRA, until reaching a steady intracellular concentration. The internalization process can be followed as a decrease in the fluorescence of the external medium and is shown in the initial phase of the records in Figs. 3 and 4. When PIRA reached a steady internal concentration, glucose was added to the medium in order to initiate the synthesis of ATP by the cells, and hence, the active PIRA efflux by the P-gp. This efflux process can be seen in Fig. 3a (control). The rate of the increase in the fluorescence in the external medium is a measurement of the extent of the PIRA efflux mediated by the P-gp. To test the effect of an inhibitor of

Fig. 3 P-gp-mediated efflux of pirarubicin (PIRA) in K562/ADR cells. Effect of verapamil. P-gp-mediated efflux of PIRA was studied in K562/ADR cells in control conditions (**a**) and exposed to verapamil (**b**). The incorporation of PIRA 1 μ M in K562/ADR cells followed after ATP depletion with sodium azide, and the active efflux rate was determined after restoration of the ATP synthesis by the addition of glucose. The rate of the PIRA efflux was assessed as the slope of the fluorescent change indicated with a straight line in the figure. Comparing the slopes of the fluorescence change recorded in **a** corresponding to control conditions, with the one in the presence of verapamil in **b**, verapamil remarkably reduced the slope of the P-gp-mediated PIRA efflux

Fig. 4 P-gp-mediated efflux of pirarubicin (PIRA) in K562/ADR cells. Effect of $A\beta_{42}$. P-gp-mediated efflux of pirarubicin was studied in K562/ADR cells exposed to $A\beta_{42}$ (a). The incorporation of PIRA 1 μM in K562/ADR cells followed after ATP depletion with sodium azide, and the active efflux rate was determined after restoration of ATP synthesis by the addition of glucose. The rate of the PIRA efflux was assessed by the slope of the fluorescent change indicated with a *straight line* in the figure. The ratios of slopes of the fluorescence changes measured in control and in various concentrations of $A\beta_{42}$, $V_{control}/V_{A\beta_{42}}$, are plotted in b. The ratios calculated were virtually unchanged for different concentrations of $A\beta_{42}$ and could be significantly fitted by a horizontal *straight line*. Means $\pm$ SD, $n=4$

the P-gp transport activity, a similar experiment was performed in the presence of verapamil, and the results are shown in Fig. 3b. A change in the slope indicates that verapamil affects the P-gp-mediated efflux of PIRA. Comparing the slope of the fluorescence change recorded in Fig. 3a, corresponding to control conditions, verapamil remarkably reduced the slope of the PIRA efflux.

To evaluate the effect of $A\beta_{42}$ on the P-gp-mediated ATP-dependent PIRA efflux, we measured the slope of the fluorescence increase in the presence of $A\beta_{42}$. Before the addition of glucose, cells were exposed for 5 min with increasing concentrations up to 2 μM of $A\beta_{42}$. Figure 4a shows the results obtained when cells were exposed to 1 μM of $A\beta_{42}$. Clearly, the rate of PIRA efflux in the presence of $A\beta_{42}$ is not much different to the one observed in control. The data plotted in Fig. 4b show that the slope ratios comparing control with the various concentrations of $A\beta_{42}$, $V_{control}/V_{A\beta_{42}}$, remain virtually unchanged. The values of the ratios measured at different concentrations of $A\beta_{42}$, and in control, were well fit by a

horizontal straight line. This finding indicates that $A\beta_{42}$ does not modify the efflux of PIRA by P-gp in K562/ADR cells.

$A\beta_{42}$ cytotoxicity remain unchanged in cells overexpressing P-gp

To evaluate the participation of P-gp in the transport of the $A\beta$ peptides across the plasma membrane, we evaluated the toxicity of $A\beta_{42}$ in the K562 cell line and in the same type of cells that overexpress P-gp, K562/ADR cells. Cells were incubated in media containing $A\beta_{42}$, and the viability of the cells was measured after 72 h using Trypan blue dye, as described in “Materials and methods.” Figure 5 displays the percentage of dead K562 cells (empty bars) and K562/ADR cells (filled bars) measured from cultures containing increasing concentrations of $A\beta_{42}$. The results showed that after 72 h, $A\beta_{42}$ displayed a remarkable cytotoxicity on both cells types. Under these conditions, $A\beta_{42}$ at a concentration of 4 μM had already produced almost total mortality of the two cell types. Furthermore, the magnitude of the mortality induced by each concentration of $A\beta_{42}$ was very similar and independent of P-gp overexpression. This result strongly suggests that overexpression of P-gp does not offer any protection against $A\beta_{42}$ cytotoxicity.

Discussion

$A\beta$ peptides could be removed from the brain extracellular fluids (BEF) by different clearance mechanisms not completely understood. It is now accepted that, when $A\beta$ peptides concentration in the BEF increases, either by overproduction by the neuronal tissue, or by defective clearance, $A\beta$ will form assemblies ranging from oligomers to protofibrils, fibrils, and amyloid plaques. Indeed, the accumulation of $A\beta$ peptides in

Fig. 5 Toxicity of $A\beta_{42}$ peptide in K562 and P-gp overexpressing K562/ADR cells. The toxicity of $A\beta_{42}$ peptide was evaluated by measuring the viability of cells after exposure to $A\beta_{42}$ for 72 h. Cell viability was assessed using the Trypan blue dye method. The K562 (empty bars) and K562/ADR cells (filled bars) were incubated with different concentrations of peptide during 72 h in the media without serum (means $\pm$ SD, $n=3$). No difference was observed in the percentage of death in both types of cells exposed to various concentrations of $A\beta_{42}$

senile plaques in the brain is a major pathological hallmark of AD.

Main clearance of A β peptides from the brain extracellular fluids is thought to occur at the level of BBB, which controls the exchanges of a variety of substances between blood and brain. Therefore, identification and functional characterization of receptors and transporters presents at the cerebral endothelium is important for understanding A β traffic. It is known that A β peptide influx to the epithelial cells is mediated by the receptor of advanced glycosylation products (RAGE) and by the low-density lipoprotein receptor-related protein (LRP1) (Shibata et al 2000; Deane et al 2004, 2008; Ito et al 2006; Nazer et al 2008; Fujiyoshi et al 2011). Overexpression of RAGE and a low expression of LRP1 may be related with A β peptide accumulation in the BEF and consequently with Alzheimer's disease (Weiss et al 2009).

Several studies have suggested that amyloid peptide could be a P-gp substrate, and thus, this protein could participate to its clearance from brain. Lam et al. (2001) reported that human synthetic A β ₄₀ and A β ₄₂ can bind the purified hamster mdrl (P-glycoprotein multidrug transporter), increase the ATPase activity of mdrl in plasma membrane vesicles derived from the multidrug ovary hamster cell line CH^RB30, and be transported across mdrl membrane vesicles. Cirrito et al. (2005) reported that, in Pgp-null mice, [¹²⁵I] A β ₄₀ and [¹²⁵I] A β ₄₂ microinjected into the CNS, clear at half the rate that they have in wild-type mice; therefore, they proposed that P-gp is involved in the transport of A β across the BBB in vivo and that ablation of P-gp at the BBB enhances A β deposition into the brain. However, it should be noted that, in this situation, LRP1 expression in the brain capillaries of P-gp knockout mice was also suppressed by 51 % compared with wild-type mice (Cirrito et al 2005). This suppression of LRP1 expression could also attenuate the elimination of A β ₄₀, and therefore, unexpected gene modulation and consequent alteration of systemic A β clearance in the knockout mice could not be ruled out (Ito et al 2006). Kuhnke and et al. (Kuhnke et al 2007), using MDR1-transfected LLC cells growing in a polarized cell layer, showed that when FITC-A β ₄₀ and FITC-A β ₄₂ were delivered from the basolateral to the apical compartment in the presence of an ABCB1 inhibitor, cyclosporine A, both peptides transport was significantly decreased. These results suggested impaired clearance of A β peptides by ABCB1 inhibition and lead them to conclude that ABCB1 played a key role in the clearance of A β ₄₀ and A β ₄₂ from the brain. Hartz et al. (2010) studied the accumulation of fluorescein-hA β ₄₂ in capillary lumens of isolated Tg2576 transgenic mouse brain capillaries. In this AD animal model, the brain capillary P-gp expression and transport activity were substantially reduced compared to wild-type control mice. These authors also showed that the restoration of P-gp expression and transport activity in brain capillaries significantly reduced brain A β levels compared to control mice. They remarked

that, although brain accumulation of A β is not the only major contributor to cognitive impairment in AD, reducing A β accumulation in the transgenic hAPP (Tg2576) mouse model does delay pathology (Iadecola 2004; Blennow et al 2006; Karlinski et al 2009). Most recently, reports from Kaddoumi's lab (Abuznait et al 2011; Qosa et al 2012) have proposed that ABCB1 plays a major role in A β transport.

On the other hand, other investigators have questioned the proposed role of P-gp in the transport of A β peptides. Evidence against the proposal of P-gp-mediated transport of A β has been reported by Ito et al. (2006). This group emphasized that LRP1 partially affects the cerebral elimination of hA β ₄₀ and argue that P-gp does not play a significant role in the brain-to-blood efflux transport of hA β ₄₀ at BBB in rats. They suggested that molecules, yet to be unidentified, may be involved in the cerebral A β ₄₀ uptake process in rats. In support to this idea, Nazer et al. (2008) reported that, in Madin Darby Canine Kidney (MDCK) cells, stably overexpressing P-gp did not exhibit any increased transport of [¹²⁵I] A β across their monolayers when compared with MDCK cells transfected with empty vector and identically cultured. They concluded that it was possible that P-gp functions in conjunction with another transporter or requires an as-yet-unidentified cofactor for A β transcytosis such that its inhibition or deletion in vivo inhibited A β clearance and increased cortical A β levels, but P-gp upregulation failed to promote A β efflux in their cellular model.

In this study, our purpose was to clarify if amyloid- β peptide is or not a substrate of P-glycoprotein. To that aim, we studied the interaction of human synthetic A β ₄₂ with P-gp in intact cells overexpressing P-gp and in P-gp containing membrane vesicles.

The ability of P-gp to transport substrates depends on ATP hydrolysis, and the transport of substrates by P-gp often stimulates ATPase activity (Lam et al 2001). Our results showed that A β ₄₂ monomers, dissolved in PBS solution, were indeed able to stimulate the ATPase activity. However, the same results were obtained when same volumes of the solvent of A β ₄₂ alone were used. In addition, A β ₄₂ did not modify PIRA transport by P-gp in K562/ADR cells. Moreover, the extent of mortality induced on K562/ADR cells overexpressing P-gp compared to A β ₄₂ toxicity on K562 cells was similar. Taken together, the experimental results described in this investigation strongly support the conclusion that A β ₄₂ is not a substrate for P-gp and that A β ₄₂ and P-gp do not interact.

The discrepancy between our results and those proposed by others could be due to the use of difference cell lines. This possibility was already suggested by Abuznait and Kaddoumi (2012) and also by others (Ohtsuki et al 2010), who have proposed that the mechanism of transcellular transport of A β depends on the cell line. To this possibility, we like to add that the source and the preparation of the A β solutions could also affect the results. A β is prone to form a complex variety of

aggregates, and the aggregation pattern of synthetic peptide depends on the dissolving protocol followed to prepare the A β solutions. Therefore, A β preparation could introduce a lot of variability in the results, and most of the studies concerning the conceivable P-gp-mediated transport of A β do not provide precise description of the preparation of A β solutions, neither the state of aggregation of the peptide used (i.e., monomers, dimers, etc.). Furthermore, binding of A β with fluorescein or fluorescein isothiocyanate (FITC) could also modify the peptide behavior in solution and in membranes.

Our results also can be analyzed from the point of view of the molecular interaction of the substrate required for the transport process by P-gp. Various works suggest that P-gp substrates are usually organic molecules with sizes ranging from <0.2 kDa to almost 1.9 kDa. Additionally, the substrates of P-gp should be electrically neutral or positively charged (Spoelstra et al 1992; Sharom et al 1998; Schinkel and Jonker 2003; Fromm 2004; Sharom 2008; Eckford and Sharom 2009; Hill et al 2013). A β_{42} peptide is a hydrophobic molecule of about 4.5 kDa and is negatively charged at physiological pH. Therefore, from this criterion, A β peptide does not meet the characteristics of molecular weight and charge required for P-gp substrates. Sharom et al. (1996) studied the interactions of a series of synthetic hydrophobic peptides with P-gp. They found that the maximal interaction corresponds to small peptides made of three residues, and the interaction decreases with increasing the peptide length. Interestingly, no cytosolic peptides occurring naturally in cells have yet been described as P-gp substrates in the literature.

In order to be transported by P-gp, A β should be located in the substrate side of the protein. All models proposed for the function of the P-gp describe that substrate binding site of P-gp is localized in the cytoplasmic leaflet of the membrane bilayer (Higgins and Gottesman 1992; Varma et al 2003). Following these models and hypothesizing that A β could be a P-gp substrate, the transport of this peptide should first involve its passage through the membrane in order to be localized in the cytoplasm. It is very unlikely that the A β peptides will cross the cytoplasmic membrane, like the known cell penetrating peptides (CCPs), to localize in the cytoplasmic leaflet of the membrane. Moreover, the A β peptides are highly hydrophobic and form aggregates in an aqueous medium. Therefore, if found in the cytoplasm, A β would likely aggregate, making it difficult to be transported by P-gp.

The size of the active site of P-gp is ranked between 5 and 50 Å (Loo and Clarke 2001; Sauna et al 2004). The size of A β aggregates could be in the range of 50–100 Å, and the size of one single alpha helix of A β_{42} is about 63 Å (Roychaudhuri et al 2009; Morgado and Fändrich 2011).

As it was mentioned above, to be transported by P-gp external A β needs to cross the membrane to the cytosol to reach the substrate binding site of P-gp. So far, there is not a defined answer to the question of how external A β can insert

and cross the plasma membrane, either in its monomeric or the aggregated forms. To the extent that the crossing through plasma membrane of the cell penetrating peptides (CCPs) has been largely studied, one could try to consider A β peptides like CPPs. A thoughtful analysis shows that this consideration is groundless. CCPs are defined as peptides with a length between 5 and 30 amino acids, able to pass through the plasma membrane by mechanisms dependent or independent of energy, and producing low cellular toxicity (Lindgren et al 2000; Lundberg and Langel 2003; Mussbach et al 2011; Milletti 2012; Bechara and Sagan 2013). The A β_{42} peptide, on the other hand, has a 42 amino acids sequence and is highly toxic to cells through interaction with membranes (Verdier et al 2004). Furthermore, most CCPs contain positive charges (Schmidt et al 2010), whereas A β_{42} is negatively charged at physiologic pH. Although hydrophobic peptides are able to fit into the membranes, most probably they must remain attached to the membranes reducing the efficiency of internalization (Walrant et al 2012). It has been generally presumed that peptides insertion in the membrane could modify the stability of the membranes and the functioning of other membrane proteins, such as membrane enzymes and transporter. Under our experimental conditions, no modification of the transport activity of P-gp was observed when cells were exposed to different concentrations of A β_{42} .

In summary, the results obtained here show that, under our experimental conditions, the transport activity of P-gp was not modified by the presence of A β_{42} . In addition, P-gp does not offer any protection to cells from the damage induced by A β . All these data strongly support the idea that A β_{42} is not transported by P-gp.

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