RESEARCH ARTICLE

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2018, 9, 946Live-cell fluorescence imaging: assessment of
thioflavin T uptake in human epidermal carcinoma
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Thioflavin T (ThT), a positively charged heterocyclic small molecule, is a widely used fluorescent marker of amyloid pathophysiology to confirm the cause of death in *post mortem* brain tissue of Alzheimer's disease (AD) patients. Literature precedents indicate that current positron emission tomography (PET) agents, such as ¹¹C-PIB and ¹⁸F-flutemetamol, share significant structural similarity with ThT, a lipophilic dye which does not traverse the blood-brain barrier (BBB) to enable the detection of A β plaques *in vivo*. While vital for maintaining normal physiology and healthy brain function, the BBB comprises brain endothelial cells sealed via paracellular protein complexes, bound by an extracellular matrix forming tight junctions thus controlling the delivery of molecules into the brain. The human P-glycoprotein (Pgp/ABCB1, 170 kD plasma membrane protein), belonging to the ABC family of efflux transporter proteins, also lines the luminal surface of brain endothelial cells thus poised to secrete its recognized substrates into the blood. Herein, we postulate that thioflavin T (ThT), due to its physico-chemical attributes, such as moderate lipophilicity and protonated nitrogen, could very well be recognized as a transport substrate of Pgp (P-glycoprotein, ABCB1) thus restricting its permeation into the brain. To evaluate whether or not ThT is indeed recognized by Pgp as its transport substrate thus limiting its BBB permeability, herein, we evaluate cellular accumulation profiles of ThT and PiB (a similar structural uncharged mimetic) in human epidermal carcinoma KB-3-1 (Pgp⁻) and MDR KB-8-5 (Pgp⁺) cells, using live-cell fluorescence imaging. While ThT penetrates KB-3-1 cells, it gets excluded from KB-8-5 cells, and also indicates LY335979-induced uptake in Pgp-expressing cells. Furthermore, the cellular uptake profiles of PiB are not impacted by the expression of Pgp under identical conditions. These data show that uptake profiles of ThT have been modified by the expression of Pgp in these cells, and are inversely proportional to the expression of the transporter protein located on the plasma membrane of these cells. Combined data demonstrate that ThT is efficiently recognized by Pgp as its transport substrate.

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Introduction

Thioflavin T (ThT) (Fig. 1), a water soluble and cell permeable heterocyclic molecule, is widely used for identification and quantification of amyloid fibrils *in vitro*.^{1–6} Compared with its weak fluorescence in the free state, showing lower (blue-shifted) excitation and emission maxima at 350 and 440 nm, respectively, it fluoresces strongly with excitation and emission maxima at approximately 440 and 490 nm,^{7,8} respectively, following its addition to samples comprising β -sheet-

rich deposits, such as the cross- β -sheet quaternary structure of amyloid fibrils.⁹ Presumably, ThT has also been widely employed as an A β pathological stain in *post mortem* brain sections of patients with Alzheimer's disease (AD). However, it has a limited ability to permeate the brain. To overcome this

Fig. 1 Chemical structures of thioflavin-T, flutemetamol, and PiB.

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shortcoming, several derivatives of ThT have been reported. Noticeably among those molecules, the neutral derivatives such as ^{11}C -PiB¹⁰ and ^{18}F -flutemetamol^{11,12} have been specifically developed for tracking A β *in vivo*. Due to the similar structural homology of ^{11}C -PiB to that of thioflavin T, the tracer has also shown the ability to detect abnormalities mediated by other aggregated proteins comprising β -sheet structures, such as AL-amyloidosis in cardiovascular diseases¹³ and myelin binding protein in multiple sclerosis.^{14,15} Within the specific context of delivery of drugs and imaging probes, across the blood–brain barrier (BBB), among various biochemical pathways controlling the delivery of molecules into the brain, P-glycoprotein (Pgp, ABCB1) and BCRP (breast cancer resistance protein, BCRP, ABCG2), the well-characterized members of the ABC family of transporters, are recognized as prominent drug transporter proteins lining the luminal surface of brain endothelial cells. These transporter proteins also play a vital role in regulating normal physiological functions,^{16–19} while also limiting the delivery of their recognized substrates across the blood–brain barrier. Previously, others and our laboratory have shown that Pgp (ABCB1)-recognized substrates possess nitrogen atoms in their scaffolds that can be protonated under physiological conditions and are also moderately hydrophobic.^{16,17} Therefore, we postulate that ThT, a positively charged molecule could potentially be recognized by Pgp as its transport substrate, while PiB, a neutral and similar structural mimetic, could penetrate the brain due to a lack of its recognition by the transporter protein. Although the physicochemical traits of ThT suggest that it could potentially be recognized by Pgp as its transport substrate,¹⁶ this biochemical process has not been ascertained at a functional level in a single cell. To test a hypothesis, whether or not ThT is recognized by Pgp as its transport substrate at a single cell level, cellular accumulation assays have been performed in human epidermal carcinoma KB-3-1 (Pgp–) and colchicine-derived MDR KB-8-5 (Pgp+) cells, and uptake profiles have been evaluated *via* live-cell fluorescence imaging. Additionally, the intracellular localization of ThT was also compared with that of Mitotracker-Red (a positive control) under identical conditions. Herein, the data show that cellular uptake profiles of ThT are modified by the expression of Pgp in human epidermal carcinoma cells and LY335979 (a highly specific and potent Pgp inhibitor)-induced accumulation of ThT in KB-8-5 (Pgp+) cells. The combined results indicate that ThT is recognized by Pgp as its transport substrate.

Methods

All reagents were purchased from Sigma-Aldrich (St. Louis), unless otherwise stated.

Cell culture

Monolayers of cells were grown at 37 °C under a 5% CO₂ atmosphere. Human epidermal carcinoma non-MDR KB-3-1 (Pgp–) cells and MDR KB-8-5 (Pgp+) cells were grown in media (DMEM (GIBCO, 11965) supplemented with heat-

inactivated fetal bovine serum (10%) and L-glutamine (2 mM)). Human epidermal carcinoma colchicine-derived MDR KB-8-5 cells (Pgp+) were grown in media supplemented with colchicine (25 nM).^{19–21}

Cell transport studies and live cell fluorescence imaging

Evaluation of cellular accumulation of thioflavin T (ThT) and PiB in human epidermal carcinoma cells using live cell fluorescence imaging. For evaluation of cellular transport assays *via* microscopy, human epidermal carcinoma KB-3-1 (non-MDR, Pgp–) and KB-8-5 (MDR, Pgp+) cells were plated onto borosilicate 8-well chambered (25 000 cells per well) slides (Nalge Nunc International, Rochester, NY) and allowed to grow to approximately 70% confluence at 37 °C under a 5% CO₂ atmosphere. Prior to imaging, media were replaced with imaging-media (phenol-red free DMEM (Gibco 31053) supplemented with heat-inactivated fetal bovine serum (10%) and L-glutamine (2 mM)). KB-3-1 (non-MDR, Pgp–) and KB-8-5 (MDR, Pgp+) cells were incubated with either ThT (10 μM) or PiB (1 μM) for 30 min at 37 °C under a continuous influx of 5% CO₂ atmosphere in either the absence or presence of LY335979 (zosuquidar trihydrochloride, 1 μM), a potent and specific Pgp inhibitor, and examined *via* live cell fluorescence imaging. The cellular accumulation of ThT and PiB was assessed using a Nikon Ti-E PFS inverted microscope equipped with a Nikon 20 $\times$ 0.3 NA Plan APO objective, Prior H117 ProScan flat top linear encoded stage, and Prior Lumen 200PRO illumination system with standard DAPI (4',6-diamidino-2-phenylindole) and FITC (fluorescein isothiocyanate), and Texas Red (sulforhodamine 101 acid chloride) filter sets. Images were acquired using a Photometrics CoolSNAP HQ2 digital camera and MetaMorph microscopy and imaging analysis software (version 7.7.0.0, Molecular Devices). Images were processed and analyzed using the ImageJ software package (NIH).

Assessment of intracellular localization of thioflavin T in human epidermal carcinoma cells and its correlation using Mitotracker Red. A stock solution of Mitotracker® (Mitotracker® Red CMH2XRos, Life Technologies, M7512) (1 μM) in DMSO was diluted to a final concentration of 25 nM in imaging-media and kept at 37 °C for 40 min, prior to experiments. Literature precedents indicate that higher concentrations (50 nM) of Mitotracker® can potentially impair mitochondrial function. Therefore, all experiments were conducted using 25 nM Mitotracker®. Cells were incubated with ThT (10 μM) and Mitotracker® (25 nM) for 30 min and then visualized using appropriate filter sets (DAPI excitation and FITC emission; Mitotracker®: Texas Red).

Results and discussion

The fluorescence spectrum of ThT dissolved in ethanol (diluted to 5% ethanol in PBS) shows excitation and emission peaks at 350 and 438 nm, respectively. Therefore, we postulate that the fluorescence characteristics of ThT (Fig. 1) could also be employed to monitor its ability to interact with the ABC

family of transporters, such as Pgp, and track its intracellular localization using live cell imaging. Earlier, we have shown that this technique can be successfully employed to assess the functional expression of the ABC family of transporters, such as Pgp¹⁹ and BCRP.¹⁸ Therefore, the cellular accumulation of ThT, a cationic molecule, as a function of its concentration was assessed in human epidermal carcinoma cells to determine the optimal concentration of the probe needed for live cell imaging. While human epidermal non-MDR (KB-3-1) cells lack Pgp, the colchicine-derived MDR (KB-8-5) counterparts show immunodetectable levels of Pgp in human epidermal carcinoma cells, using monoclonal antibody C219 (Fig. 2). Literature precedents indicate a prevalence of multiple biochemical pathways, such as ion channels, receptor-mediated endocytosis, and diffusion across the bilayer, which could also mediate the cellular accumulation of moderately hydrophobic and cationic molecules. However, the net cell content of many hydrophobic cationic molecules transported by Pgp, the efflux transporter, has also been shown to be a net function of both passive membrane potential dependent influx and transporter-mediated efflux.^{20–22} Accordingly, cationic ThT would be expected to penetrate KB-3-1 (non-MDR, Pgp[−]) cells in response to inwardly directed electrochemical driving forces at the plasma membrane and mitochondrial membrane to localize within mitochondria, while excluded from Pgp-expressing KB-8-5 (MDR, Pgp⁺) cells. For the live cell imaging studies, human epidermal carcinoma KB-3-1 (non-MDR) cells were incubated with ThT at increasing concentrations for 30 min to optimize the concentration of ThT needed for fluorescence imaging. Based upon the initial results of the concentration-dependent output of the fluorescence signal, 10 μ M ThT was chosen as the optimal concentration needed for generating a sufficient fluorescence signal to visualize the probe in the intracellular compartments, using live cell imaging. Therefore, for evaluation, ThT (10 μ M) was incubated with KB-3-1 (non-MDR, Pgp[−]) for 30 min in media. Furthermore, ThT (10 μ M) was also incubated with KB-8-5 (MDR, Pgp⁺) for 30 min in media under identical conditions in either the absence or presence of LY335979 (Fig. 3), a potent and specific Pgp inhibitor.^{16,21,23} To further assess whether or not the inhibitor contributes to fluorescence output, LY335979 (1 μ M, control) was also incubated alone with KB-8-5 (MDR, Pgp⁺) for 30 min in media under identical conditions. The data demonstrated steady state accumulation of ThT in KB-3-1 (Pgp[−]) cells

Fig. 2 Western blot analysis of KB-3-1 and KB-8-5 cells using an anti-Pgp monoclonal antibody (C219). The arrow indicates the 170 kDa band corresponding to Pgp.

Fig. 3 Cellular uptake of thioflavin T in human epidermal carcinoma KB-3-1 (Pgp[−]) cells (top panel), LY335979 alone (1 μ M, control) in KB-8-5 (Pgp⁺) cells (2nd panel from the top), ThT alone in KB-8-5 (Pgp⁺) cells (3rd panel from the top), and ThT in KB-8-5 (Pgp⁺) cells in the presence of LY335979 (1 μ M, lower panel), a potent and specific inhibitor of Pgp, using live cell fluorescence imaging. Left: Transmission image; right: uptake following incubation of thioflavin-T for 30 min. Note: Exclusion of ThT from KB-8-5 (Pgp⁺) cells (3rd right panel from the top) and LY335979-mediated uptake of ThT in KB-8-5 (Pgp⁺) cells (lowest right panel).

(Fig. 3, top panel), while it was excluded from KB-8-5 (Pgp⁺) cells (Fig. 3, 3rd panel from the top). Of note, LY335979 did not show any significant fluorescence signal in KB-8-5 (Pgp⁺) cells (Fig. 3, 2nd panel from the top). Importantly, LY335979 (1 μ M), a Pgp inhibitor, induced cellular accumulation of ThT into KB-8-5 (Pgp⁺) cells thus indicating specific recognition of the optical probe by the transporter protein (Fig. 3, lower panel).

To further assess the effect of a lack of charge on cell uptake profiles of a given probe, PiB, a similar structural mimetic of ThT, was synthesized and analytically characterized. The fluorescence spectrum of PiB dissolved in ethanol (diluted to 10% ethanol in PBS) shows excitation and emission peaks at 380 and 445 nm, respectively. Following incubation of PiB (1 μ M) in both KB-3-1 (Pgp[−]) (Fig. 4, upper panel) and KB-8-5 (Pgp⁺) (Fig. 4, lower panel), the probe penetrated and indicated cell uptake independent of Pgp expression. These data demonstrate that PiB is not recognized by Pgp as its transport substrate under identical conditions. These results are also consistent with the penetration of PiB into the brain to enable β amyloid imaging.

Numerous cationic probes have been shown to distribute electrophoretically into the mitochondrial matrix in response

Fig. 4 Cellular uptake of PiB in human epidermal carcinoma KB-3-1 (Pgp⁻, top panel) and KB-8-5 (Pgp⁺, lower panel) cells using live cell fluorescence imaging. Left: Transmission image; right: uptake following incubation of PiB for 30 min. Note: Uptake of PiB in both KB-3-1 (Pgp⁻) and KB-8-5 (Pgp⁺) cells.

Fig. 5 Assessment of intracellular localization of thioflavin T (10 µM) in human epidermal carcinoma KB-3-1 (Pgp⁻) cells and its correlation with MitoTracker Red (10 nM), a well-validated marker for staining of mitochondria using live cell fluorescence imaging.

to favorable negative transmembrane electrochemical gradients across the inner mitochondrial membrane.^{24–28} The accumulation of these molecules is a net function of the overall charge, solubility in inner membrane lipids, and the matrix aqueous space. Previously, others²² and our laboratory have shown that hydrophobic and monocationic metalloprobes also penetrate non-MDR cells in response to favorable negative transmembrane potential gradients, existing at the plasma membrane and across mitochondrial membranes to localize within the mitochondria.^{16,17,19,20,29,30} To directly evaluate intracellular localization, ThT was also incubated in non-MDR cells in either the presence or absence of MitoTracker® (Mito-thioflavin T (ThT) Mitotracker Red Merge Tracker® Red CMH₂XRos), a well-known fluorescent marker for staining mitochondria. ThT penetrated KB-3-1 cells, demonstrating a close correlation with the MitoTracker Red (Fig. 5). Overall, these data indicate that ThT is recognized by Pgp as its transport substrate.

Conclusions

Cellular uptake profiles of ThT obtained through live cell imaging were inversely proportional to the expression of Pgp

(indicated by LY335979-induced uptake) in human epidermal carcinoma cells. In the absence of Pgp expression at the plasma membrane, ThT penetrated KB-3-1 (Pgp⁻) cells to localize within the mitochondria of these cells. Combined data indicated that ThT is an efficient transport substrate of Pgp, thus it would not traverse the BBB. Additionally, uptake profiles of PiB in both non-MDR and MDR cells were not modified by expression of Pgp, indicating that PiB is not recognized as a transport substrate. While cellular uptake data in human epidermal carcinoma cells provided direct evidence for recognition of ThT by Pgp as its transport substrate at a single cell level, the correlation studies using MitoTracker Red indicate its intracellular location within the mitochondria. Further studies for the interaction of ThT with other ABC families of transporters, such as BCRP (ABCG2), are in progress. Finally, FDA guidelines make it mandatory that the status of new therapeutic drugs (discovered for treating CNS diseases) be verified in terms of their recognition profiles with the ABC family of transporters. Therefore, the described *in cellulo* transport live cell imaging bioassay could also have an implication as an indirect screening protocol in the context of drug delivery across the BBB, since this cost-efficient methodology could be deployed for the verification of the cross-reactivity of mildly fluorescent drugs with the ABC family of transporters at a single cell level.

Conflicts of interest

There are no conflicts to declare.

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