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Quantitative fluorescence microscopy provides high resolution imaging of passive diffusion and P-gp mediated efflux at the *in vivo* blood–brain barrier

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Abstract

Quantitative fluorescent microscopy is an emerging technology that has provided significant insight into cellular dye accumulation, organelle function, and tissue physiology. However, historically dyes have only been used to qualitatively or semi-quantitatively (fold change) determine changes in blood–brain barrier (BBB) integrity. Herein, we present a novel method to calculate the blood to brain transfer rates of the dyes rhodamine 123 and Texas red across the *in situ* BBB. We observed that rhodamine 123 is subject to p-glycoprotein mediated efflux at the rat BBB and can be increased nearly 20-fold with p-glycoprotein inhibition. However, Texas Red appears to not be subject to MRP2 mediated efflux at the rat BBB, agreeing with literature reports suggesting MRP2 may lack functionality at the normal rat BBB. Lastly, we present data demonstrating that once dyes have crossed the BBB, diffusion of the dye molecule is not as instantaneous as has been previously suggested. We propose that future work can now be completed to (1) match BBB transfer coefficients to interstitial diffusion constants and (2) use dyes with specific affinities to cellular organelles or that have specific properties (*e.g.*, subject to efflux transporters) to more fully understand BBB physiology.

Keywords

Drug delivery; Stroke; Parkinson's; Alzheimer's; Blood–brain barrier; Quantitative fluorescent microscopy; Transport; Autoradiography

1. Introduction

Historically, dyes have been integral in studying the blood–brain barrier (BBB). Notably, the restricted movement of dyes between the blood and brain compartments was first demonstrated by Paul Ehrlich in the early 19th century (Ehrlich, 1885). In this work, water-soluble dyes injected intravenously stained all organs except the brain and spinal cord. This was attributed to either the dye's lack of affinity for brain parenchyma or the presence of a

physical barrier between the brain vasculature and brain tissue (Hawkins and Davis, 2005). Subsequent experiments by Goldmann demonstrated trypan blue injected into the cerebrospinal fluid (CSF) stained the brain and spinal cord but did not accumulate in the periphery, confirming the latter hypothesis (Goldmann, 1913). It is now known that trypan blue binds to albumin (Stopa et al., 2006), a 66kDa plasma protein, and the resulting complex is too large to appreciably move across an intact BBB. Moving ahead 50 years, in 1967, Reese and Karnovsky demonstrated on an ultra-structural level that the BBB was a physical barrier for the blood to brain passage of horse-radish peroxidase (MW 40,000) (Reese and Karnovsky, 1967), because the spaces between adjacent brain endothelial cells were effectively sealed together preventing paracellular movement (Brightman et al., 1971).

At approximately the same time, the literature shifts from predominantly using dyes to document the presence of the intact BBB, to using dyes to qualitatively measure BBB disruption in various pathophysiological states. For example, in 1956, trypan blue was used to show that the BBB could be disrupted by ultrasonic damage (Bakay et al., 1956) which worsened in the presence of angiography imaging agents (Shealy and Crafts, 1965). Dye accumulation was also seen in circulatory arrest with prolonged resuscitation (Lin and Kormano, 1977), significant acute arterial hypertension (Johansson et al., 1970), seizures (da Costa, 1972; Nemeroff and Crisley, 1975), and radiation (Schettler and Shealy, 1970).

It was recognized very early vital dye studies were only qualitative. To overcome this, radiotracers such as ^{203}Hg were concurrently administered. This method provided an initial visualization of dye extravasation followed by quantitative measurement of BBB disruption (Dereymaeker et al., 1970; Schettler and Shealy, 1970; Shealy and Crafts, 1965). It should be noted that these initial studies simultaneously injected two different tracers to demonstrate size selective openings at the BBB (Shealy and Crafts, 1965). However, spatial resolution of dye distribution was lost. Quantification of BBB disruption using autoradiography quickly became the gold standard (Blasberg et al., 1983) and has evolved into well-designed double or triple labeled studies where size dependent BBB permeability changes can be simultaneously measured (Miyagawa et al., 2003; Uehara et al., 1997). Though autoradiography does have limitations, two or three tracers require weeks to months of film development followed by the subtraction of multiple signals to obtain data (Miyagawa et al., 2003). In addition, while spatial resolution of the tracer can be maintained, image resolution with traditional film autoradiography is typically limited to $\sim 25\text{--}50\text{ }\mu\text{m}$ (Schmidt and Smith, 2005).

Recently, dye distribution across the BBB was completed using an *in situ* rat brain perfusion method (Hawkins and Egleton, 2006; Takasato et al., 1984). This work brought up an intriguing possibility that free dye movement across the BBB could be quantified in the absence of factors normally present in the blood which may alter apparent permeability coefficients. However, similar to previous reports using dyes, spatial resolution for quantification was not maintained even though microscopy was completed which allowed for qualitative visualization of permeability changes in different brain regions.

Given the recent developments in the field of quantitative microscopy (Dorn et al., 2008; Lockman et al., 2010), and that BBB disruption can be very heterogeneous depending on the

insult (Baumbach and Heistad, 1985; Belayev et al., 1996; Brown et al., 2004; Nitsch and Klatzo, 1983), we set out to determine if we could expand previous studies of dye movement across the BBB using a quantitative approach, in which we couple *in situ* brain perfusions with quantitative fluorescence microscopy using post mortem analysis techniques similar to autoradiography. We hypothesize that using the brain perfusion technique, we could take advantage of differing dye properties [*e.g.*, dyes being a substrate for efflux transporters (Bachmeier and Miller, 2005; Fontaine et al., 1996; Wang et al., 1995)] and that we could resolve permeability variances in brain at $\sim 1 \mu\text{m}$ increments.

2. Methods

2.1. Chemicals

Sulphorhodamine 101 (Texas Red: TxRd), Rhodamine 123 (R123) were purchased from Molecular Probes Invitrogen (Eugene, OR, USA). Probenecid, furosemide and verapamil were purchased from Sigma (St. Louis, MO). Cyclosporine A was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). MK571 was purchased from Cayman chemicals (Michigan, USA). All other chemicals used were of analytical grade and were used as supplied.

2.2. Animals

Male Fischer-344 rats (250–320 g) were purchased from Charles River Laboratories (Kingston, NY, USA) and were used for all the perfusion experiments done in this study. All studies were approved by the Animal Care and Use Committee and were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

2.3. Cell culture

Drug-sensitive MCF-7, adriamycin-resistant MCF-7/AdrR cells were kindly donated by Dr. US Rao, Texas Tech University Health Sciences Center, Amarillo, TX (Fairchild et al., 1990; Rao et al., 2006). MDCK-MPR2 cell line (Evers et al., 1998) was a kind gift from Dr. Borst (Netherlands). The cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal bovine serum and antibiotics (penicillin, 100U/mL; and streptomycin, 100 $\mu\text{g/mL}$). Cells were grown in a 37 °C humidified incubator with 5% CO₂.

2.4. In vitro transport studies

For fluorescent microscopy experiments, cells were seeded in 24-well microplates at seeding density of 50,000 cells per plate. After 24 h, cells were washed in PBS and then incubated with R123 (1 μM) in the presence or absence of known P-gp inhibitors (verapamil, cyclosporin A) along with DAPI to stain the nuclei of cells. After a 30-min accumulation period cells were washed four times in PBS and then 0.5 mL PBS was added to each well. To prevent efflux of fluorescent dyes the plates were kept on ice until microscopic evaluations were performed. Cells were visualized with an inverted fluorescence microscope (Olympus IX81). Total intracellular R123 was determined using Slidebook 4.2 and normalized per 100 cells, counted with binary voxel masking using DAPI fluorescence as the reference.

Using nonlinear regression (Prism 5.0), the Hill equation was fit to the fold increase in intracellular fluorescence (relative to that observed in the absence of the inhibitor) as a function of increasing inhibitor concentration. The mean IC_{50} of each inhibitor was determined from at least 6 independent experiments. Unless otherwise stated, data are presented as mean $\pm$ S.E.M. Similar methodology was utilized for TxRd transport studies, where cells were incubated with TxRd (5 μ M) in the presence or absence of MRP inhibitor MK571 along with DAPI.

2.5. In situ rat brain perfusion technique

In situ rat brain perfusion across the BBB was utilized to evaluate brain uptake of TxRd and R123 (Allen and Smith, 2001; Lockman et al., 2003b; Takasato et al., 1984). Rats were anesthetized with ketamine/xylazine, the left common carotid artery was exposed and pterygopalantine artery was left open (Allen and Smith, 2001). A PE-50 catheter filled with heparinized saline (100U/mL) was placed into the left common carotid artery after ligation of the left external carotid, occipital, and common carotid arteries. Body temperature was monitored and maintained at 37 °C using a heating pad attached to a YSI temperature controller (Yellow Springs, OH). Before animal perfusion, the heart was stopped by nicking the cardiac muscle to circumvent flow from the systemic circulation (Smith, 1996). Perfusion fluid was then delivered to the common carotid artery by a cannula at a constant rate of 10mL/min (perfusion pressure, 75–100 mm Hg) using a Harvard Model 944 dual channel pump (Harvard Apparatus, South Natick, MA). As the fluid is infused into the left hemisphere, it receives the majority of the fluid flow and so the data are for the left brain regions.

The perfusion fluid consisted of HCO_3 buffered physiological saline, containing 128mM NaCl, 24 mM $NaHCO_3$, 4.2 mM KCl, 2.4 mM NaH_2PO_4 , 1.5 mM $CaCl_2$, 0.9 mM $MgSO_4$ and 9 mM glucose (pH $\sim$ 7.35; $[Na] = 154.4$ mM). All solutions were filtered, oxygenated, warmed to 37 °C and adjusted to pH 7.35. To determine initial brain uptake of TxRd or R123 perfusion fluid containing either TxRd or R123 (50 μ g/mL) was infused into the cerebral circulation for 15–60 s or 30–120 s, respectively. At the completion of each perfusion time point, rats were decapitated, and the brain was removed from the skull. The brain was flash frozen in isopentane (-60 °C). Concentration of the fluorophore (TxRd or R123) in brain was determined using fluorescence microscopy and regional permeability was expressed by the unidirectional transfer constant, K_{in} (mL/s/g).

2.6. Brain sectioning

Cryostat sections of 20 μ m were obtained at -23 °C (Shan-don Cryotome®), mounted on glass slides, and air dried. Data were analyzed using quantitative fluorescence microscopy and all images were obtained using 15 ms exposure, through a 2 $\times$ objective with an additional optical zooming factor (Olympus MVX-10). Slidebook® 4.2 software was utilized to determine sum intensity per gram of brain which was then converted into concentration of dye per gram of brain using the brain homogenate standards. All the data was obtained from imaging of cortex regions.

2.7. Quantification of TxRd/R123 using fluorescent microscopy

Fluorescence was observed with an Olympus MVX-10 stereo microscope (objective: 2×, NA 0.5). The optical zoom range is from 0.63 to 12.6. Excitation and emission of TxRd was obtained using the Texas Red chroma filter (peak fluorophore excitation is 596 nm and emission is 620 nm); excitation/bandpass filter of 560/55, emission/bandpass filter of 645/75 and dichromatic mirror at 595 nm (Olympus America Inc., Center Valley, PA). The excitation and emission of R123 was obtained using the GFP chroma (excitation/band pass filter of 470/40, emission/band pass filter of 525/50 and dichromatic mirror at 495 nm).

The voxel by voxel sum intensity of fluorescence for brain homogenate samples was obtained with the 2× objective. The optical zoom range was maintained at 4× for a total optical magnification of 8×. The sum intensity per gram of brain homogenate was obtained using an exposure time of 15 ms with gain settings of 615 (Slidebook 4.2, Olympus Imaging Systems). The total fluorescence intensity signal for each concentration was then plotted as a function of grams of brain which was calculated using the area in microns squared multiplied by the thickness of the brain sample (20 μm) to obtain a total brain volume that was analyzed. The brain volume (μm³) was multiplied by the density of brain tissue (1.04 g/cm³) as similarly reported by (Tengvar et al., 1983) to obtain a weight of brain tissue.

2.8. Preparation of brain standards

To calculate the concentration of the dye in brain, standard curves were generated in rat brain homogenates. Briefly, 100 μL of standard solution of the dye was added to each of 500 mg of the brain and homogenized. The homogenized mass was flash frozen in isopentane (−60 °C) and sliced into 20 μm sections using a cryostat −23 °C and mounted onto glass, superfrost slides. The slices were analyzed using fluorescent microscopy and the sum intensity per gram of brain homogenate was plotted against concentration of the dye.

2.9. Kinetic analysis

Unidirectional uptake transfer constants (K_{in}) were calculated from the following relationship to the linear portion of the uptake curve:

$$Q^*/C^* = K_{in}T + V_0 \quad (1)$$

where Q^* is the quantity of fluorophore (TxRd or R123) in brain (μg/g) at the end of perfusion, C^* is the perfusion fluid concentration of fluorophore (μg/mL), T is the perfusion time (s) and V_0 is the extrapolated intercept ($T=0$ s; “vascular volume” in mL/g). After determination of a perfusion time that allowed adequate amount of fluorescent marker to pass into brain and yet remained in the linear uptake zone, K_{in} was determined in single time-point experiments as $K_{in} = [Q^* - V_0C^*]/C^*T$ (Smith and Takasato, 1986; Takasato et al., 1984).

2.10. Statistical analysis

The slope of the line (K_{in}) was determined with linear regression using least squares analysis. One-way ANOVA analysis followed by a Bonferoni's multiple comparison tests

was used for the comparison of the K_{in} values in presence of inhibitors. For all data, errors are reported as standard error of the mean unless otherwise indicated. Differences were considered statistically significant at the $p < 0.05$ level (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA).

3. Results

In this study, we evaluated the BBB transfer of TxRd and R123 since they are substrates of efflux transporters (Fig. 1) and their fluorescence intensity does not differ in standards prepared from brain over a range of physiological pH values (7.0–7.6) and various $\text{Na}^+/\text{Ca}^{2+}$ concentrations [data not shown; but is in agreement with previous reports (Ferguson et al., 1999; Gleva et al., 1990; Reichel et al., 2008)]. Further, TxRd and R123 appeared to have stable fluorescence (within $\pm 5\%$) with repeat fluorescent exposures (15–1500 ms) within tissue standards, provided that the excitation gain signal was maintained and visualization and focus of the sample was obtained in an alternate channel (e.g., TxRd fluorescence was visualized with the eGFP channel; excitation filter of 470 ± 40 nm) (Table 1). Lastly, the linear range of dye concentrations to fluorescent intensity within the brain tissue standards fell within concentrations of dye accumulation that were observed to be in brain at the end of 15–120 s perfusions. For both dyes, the correlation was linear for ~ 3 log units ($r^2 > 0.95$) (Fig. 2). Fluorescent images in Fig. 2A and B are representative brain homogenate sections ranging in concentration from zero to 50 $\mu\text{g/g}$, and zero to 8.33 $\mu\text{g/g}$ for TxRd and R123, respectively.

We then evaluated R123 uptake across the BBB using *in situ* rat brain perfusions. Concentrations of R123 were held steady in the perfusate and the experiment was stopped between 30 and 120 s, at 30 s intervals. Accumulation was linear ($r^2 = 0.898$) over 2 min (Fig. 3A) with a calculated transfer coefficient (K_{in}) for R123 of $2.0 \pm 0.5 \times 10^{-4}$ mL/s/g. Representative fluorescent images for the accumulation of R123 over time in brain are shown in Fig. 3B. Addition of the P-gp inhibitors, verapamil and cyclosporin A, into the brain perfusate increased the PS of R123 approximately 10- to 20-fold compared to control (PS for R123 in the presence of verapamil was $21.8 \pm 2.1 \times 10^{-4}$ mL/s/g and for cyclosporin A was $24.4 \pm 2.0 \times 10^{-4}$ mL/s/g). Representative fluorescent images of R123 brain accumulation in the presence and absence of P-gp inhibitors are shown in Fig. 2D, and is consistent with the literature (Neuhaus et al., 2010) and our *in vitro* data in adriamycin-resistant MCF-7/AdrR cells (Fig. 1A and B). The aggregate data suggests R123 is a substrate for P-gp that is sensitive to verapamil inhibition.

Similar experiments were carried out for the uptake of TxRd. TxRd brain accumulation increased in a linear fashion over 15–60 s ($r^2 = 0.897$) with a calculated PS of $1.08 \pm 0.14 \times 10^{-4}$ mL/s/g (Fig. 5A). Concurrent addition of the pan MRP2 inhibitor MK571 did not increase the K_{in} beyond control ($p > 0.05$; K_{in} : $1.29 \pm 0.19 \times 10^{-4}$ mL/s/g). To further confirm that TxRd was not subject to MRP2 mediated efflux in separate experiments we added probenecid (MRP2 and OATP inhibitor), furosemide (MRP1 and MRP2 inhibitor) or verapamil (a nonspecific inhibitor of efflux transporters) at various concentrations. In all three experiments there was not a significant change in the blood to brain transfer coefficient (K_{in}) of TxRd as compared to control. The observed K_{in} in the presence of the various

inhibitors ranged between from $0.79 \pm 0.18 \times 10^{-4}$ mL/s/g with verapamil to $1.29 \pm 0.19 \times 10^{-4}$ mL/s/g with MK571 (Fig. 4C). In contrast, our *in vitro* experiments demonstrated TxRd is a substrate of MRP2 with an $IC_{50} \sim 2.5 \pm 0.3 \mu\text{M}$ for MK571 (Fig. 1C and D). This data suggests that TxRd, while subject to MRP-2 efflux *in vitro*, may not be subject to efflux at the normal rat BBB.

4. Discussion

Fluorescent dyes have been commonly used to visualize and or detect cellular structures, intracellular ion changes, pH changes, hypoxia and localization of proteins in both *in vitro* and *in vivo* (Brismar and Ulfhake, 1997). Moreover fluorescent quantification has shown insight into intracellular and extracellular of doxorubicin (Shen et al., 2008), and BBB disruption (Hawkins and Egleton, 2006). Herein we have developed a method to study BBB transfer kinetics and mechanisms of transport using R123 and TxRd.

Initial *in vitro* control experiments confirmed that R123 is a substrate for P-gp mediated efflux and TxRd is subject to MRP2 mediated efflux. R123 accumulation into adriamycin-resistant MCF-7 cells increased in the presence of increasing concentrations of verapamil. The IC_{50} value obtained for verapamil is consistent with previous radiotracer experiments (Sharom et al., 2001). Similarly, TxRd showed increased intracellular concentrations with increasing concentrations the MRP inhibitor MK571 (Luna-Tortos et al., 2010; Zastre et al., 2009) in an MDCK cell line that significantly over expresses MRP2 (Evers et al., 1998). This data is in general agreement with previous studies demonstrating that TxRd is a substrate of MRP2 (Leader and O'Donnell, 2005; O'Donnell and Leader, 2006).

The first goal of this project was to quantify the *in vivo* brain uptake and potential BBB efflux of TxRd and R123. To accomplish this, we used the *in situ* brain perfusion technique, since it has several advantages in determining interactions of drugs with BBB transporters. Specifically, there is absolute control over the brain perfusate which helps determine accurate apparent permeability influx transport kinetics (Lockman et al., 2005a), efflux kinetics (Lockman et al., 2003b), inhibition constants for transporters (Lockman et al., 2001), and a direct measurement of the integrity of the BBB in healthy brain and in pathology (Lockman et al., 2003a, 2004, 2005b).

Using the *in situ* brain perfusion method, R123 appears to accumulate in a linear fashion over two minutes with a transfer constant that is ~ 10 -fold less than predicted by the octanol/water coefficient, which is indicative of compounds that are subjected to efflux at the BBB. A similar experiment to the *in vitro* data, addition of a P-gp inhibitor (verapamil or cyclosporine A) resulted in an increase in the endothelial and brain accumulation of R123. This data is consistent with parenchymal accumulation using microdialysis (Wang et al., 1995). It is of interest that the uptake of R123 was heterogeneous and spotty in distribution and appeared to primarily accumulate around blood vessels over the two minute period in the experiments without inhibitors. Brain distribution appeared to be very limited with little diffusion into the surrounding parenchyma.

We then completed similar experiments using TxRd. Brain uptake of TxRd was linear over the first 60s, longer uptake times were not completed because parenchymal tissue between

the vasculature was filling with TxRd during the shorter uptake times. In contrast to the *in vitro* and R123 data, MK571 had no effect on the accumulation of TxRd, suggesting a lack of significant MRP2 mediated efflux at the normal *in vivo* BBB. To further confirm this lack of efflux, we used the MRP inhibitors furosemide, verapamil, and probenecid in separate experiments (Chikhale et al., 1995; Gerk and Vore, 2002; Janneh et al., 2005, 2007). Similar, to the MK571 data, we did not observe increased brain accumulation of TxRd.

There is controversy as to whether MRP2 is expressed at high enough levels at the BBB to actively prevent substrates from entering brain. Multiple studies have evaluated the expression of MRP2 at the BBB under normal physiological conditions and there has been variability in the results depending on species, strain of the species, and methodology (Dallas et al., 2006). For example, studies utilizing RT-PCR or western blotting did not detect MRP2 in both primary cultured bovine brain microvessel endothelial cells and the capillary-enriched fraction from bovine brain homogenates (Zhang et al., 2000). Similarly, MRP2 expression was not detected in western blots of isolated rat brain capillaries (Yousif et al., 2007). However, low level expression of MRP2 has been visualized by both immunohistochemistry and confocal microscopy in the luminal membranes of endothelial cells from isolated brain capillaries of fish, rats, and pigs (Miller et al., 2000).

While we did not observe TxRd MRP2 mediated efflux at the rat BBB, it should be noted that these experiments were performed in healthy animals with no evidence of pathology. Significant MRP2 expression and function was observed in brain capillaries in a preclinical pilocarpine-induced status epilepticus model (Hoffmann et al., 2006). Similarly, MRP2 is upregulated at the BBB after dexamethasone treatment (Bauer et al., 2008), similar to other dexamethasone exposure studies which have shown an increase in the expression and function of P-gp, and BCRP (Narang et al., 2008). The aggregate of this data suggests that if MRP2 is expressed at the BBB, it is at low levels under normal conditions, and has little to no impact on keeping relevant substrates from entering brain.

The second overarching goal of this project was to determine if we could fully utilize quantitative microscopy to resolve permeability differences across the BBB with a spatial resolution of $\sim 1 \mu\text{m}$. Fig. 5 demonstrates that after 45 s of TxRd uptake into brain, the dye has not completely diffused through the entire parenchymal space between the blood vessels. This data is similar to the low power magnification images in Figs. 3 and 4. Dye concentrations appear to be highest directly adjacent to vessels. The data suggests that after a molecule has traversed the BBB, diffusion is not instantaneous, and parameters such as size, lipophilicity, non-specific protein binding, blood flow, cellular structures between vessels, and interstitial space may all govern the rate of diffusion from the blood vessel and subsequently through the parenchymal tissue (albeit this is only a $30 \mu\text{m}$ distance). One limitation in this study is that diffusion constants cannot be calculated accurately from the data in Fig. 5, because of the potential post-mortem diffusion of dye between the time of sacrifice and brain isolation/freezing in isopentane (~ 30 s in this image). Further work should be done to determine if rates of brain uptake can be directly coupled to diffusion measurements accounting for influx and/or efflux mechanisms present at the BBB, neurons, and adjacent glial cells.

The advancements in quantitative fluorescence microscopy stand to significantly add to the literature with regard to sensitivity, specificity, and resolution of BBB permeability. Given the broad range of dyes and their specific affinities for various cell organelles or processes (*e.g.*, mitochondria, lysosomes, nuclei, apoptosis, *etc.*), it is hoped that using this method will allow investigators to more fully understand the BBB permeability and/or physiology in various experimental protocols (*e.g.*, stroke, cancer, Alzheimer's).

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Highlights

- Quantitative fluorescence microscopy can determine the rate of dye movement across the blood-brain barrier.
- Rhodamine 123 is subject to efflux at the blood–brain barrier.
- Texas red appears not to be subject to efflux at the rat blood–brain barrier.
- Once past the blood–brain barrier, dye diffusion in parenchyma is not instantaneous.

Fig. 1.

In vitro accumulation studies demonstrating that R123 is a substrate for P-gp, and TxRd is a substrate for MRP2. (A) An increasing accumulation of R123 in MCF-7/AdrR cells in the presence of various concentrations of the P-gp inhibitor verapamil (representative images in B). Similarly, there is a greater accumulation of TxRd (C) into an MDCK cell line that significantly over expresses MRP2 in the presence of various concentrations of MK571 (representative images in D). All data represent mean $\pm$ S.E.M. for an $n = 6$ independent observations. IC_{50} was calculated using a non-linear regression of the sigmoidal dose-response curve.

Fig. 2.

Concentrations of dyes in tissue homogenates have a linear correlation to fluorescence emission. Standard curves of R123 (A) and TxRd (B) in brain homogenates. The standard curves (linear regression slope) have a linear relationship ($r^2 > 0.96$ for both R123 and TxRd) between the total sum fluorescent intensity and the concentration of R123/TxRd per gram of brain homogenate. Data represent mean $\pm$ S.E.M. for an $n = 3$ independent observations.

Fig. 3.

Linear brain accumulation of R123 (A) versus time, for the calculation of K_{in} . Image (B) shows representative accumulation of R123 at perfusion times of 3–120 s. The addition of P-gp inhibitors (Cyclosporine A, Verapamil) significantly ($**p < 0.01$ and $***p < 0.001$) increased brain uptake (C; representative images shown in D). Data were analyzed using one-way ANOVA followed by Bonferroni's test. Data are mean $\pm$ S.E.M.; $n = 3$. Scale bar represents 200 μm . Q_{br} represents the concentration of fluorophore in brain ($\mu\text{g/g}$) and $Q_{\text{perfusate}}$ represents the concentration of fluorophore in perfusion fluid ($\mu\text{g/mL}$).

Fig. 4.

Linear brain accumulation of TxRd (A) versus time, for the calculation of K_{in} . Images (B) show representative accumulation of TxRd at perfusion times from 15 to 60 s. Simultaneous inhibition of MRP2 did not alter ($p > 0.05$) the brain accumulation of TxRd (C; representative images are shown in D). Data were analyzed using one-way ANOVA followed by Bonferroni's test. All data represent mean $\pm$ S.E.M. for total brain; $n = 3-5$ for all points. Scale bar represents 200 μm .

Fig. 5.

(A) Representative image of the total accumulation of TxRd into brain parenchyma after 45 s of brain perfusion. Blood vessels were marked during perfusion using the protein bound impermeant marker indocyanine green. (B) is a magnified image showing two blood vessels. A histogram of fluorescent intensity *versus* distance is shown in C.

Table 1

Time for photobleaching of dyes and gfp.

Time (s)	Texas red	GFP
0	100	100
30	90.3 $\pm$ 5.5	97.0 $\pm$ 1.3
60	87.8 $\pm$ 4.8	96.2 $\pm$ 0.1
90	87.5 $\pm$ 3.8	95.5 $\pm$ 0.28
120	86.9 $\pm$ 4.4	94.5 $\pm$ 1.2
150	86.0 $\pm$ 4.3	95.1 $\pm$ 0.85

The percent of photobleaching of TxRd in brain homogenate samples exposed to either TxRd (excitation/emission: 560 $\pm$ 55/645 $\pm$ 75 nm) excitation or eGFP (excitation/emission: 470 $\pm$ 40/525 $\pm$ 50 nm) excitation. Photobleaching was minimal (<5%) if the sample containing TxRd was viewed with the eGFP filter. However, photobleaching was significantly higher if the TxRd sample was viewed with the TxRd filter. All data represent mean $\pm$ S.E.M. ($n = 3$).