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The Role of P-Glycoprotein in Intestinal Transport versus the BBB Transport of Tetraphenylphosphonium

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Abstract: Tetraphenylphosphonium (TPP), a phosphonium cation, is a promising means for tumor imaging. A major contributor to the pharmacokinetics of phosphonium cations is the efflux transporter P-glycoprotein (P-gp). For this application it is important to ascertain the influence of the multidrug resistance system on TPP. Therefore, our aim was to characterize the interaction of TPP with P-gp, *in vitro* and in *in vivo* models. P-gp-mediated transport of [^{3}H]-TPP was assessed in Caco-2 cells and *ex vivo* in rat intestinal wall by the use of a diffusion cell system. The distribution of [^{3}H]-TPP across the blood−brain barrier (BBB) was studied in rats and mice treated with P-gp modulators and in Mdr-1a/b$^{(-/-)}$ knockout mice. The *in vitro* permeability coefficient of basolateral-to-apical transfer (PappB-A) of [^{3}H]-TPP was 8-fold greater than apical-to-basolateral (PappA-B) coefficient, indicative of net mucosal secretion. A concentration dependent decrease of this secretion was obtained by the P-gp substrate verapamil, while no effect was evident by the MRP2 inhibitor MK-571 and the BCRP inhibitor FTC. [^{3}H]-TPP transfer across rat jejunum wall was directional and concentration-dependent. 2,4-Dinitrophenol, cyclosporin A (CsA), verapamil and PSC-833 enhanced A−B transport of TPP 3.6-fold, 4-fold, 4.6-fold and 5.3-fold respectively. Likewise, PappA-B of [^{3}H]-TPP was 5-fold greater in P-gp knockout mice than in controls. *In vivo*, PSC-833, P-gp inhibitor, significantly increased the uptake of [^{3}H]-TPP in the liver, heart, small intestine and the lungs but not the brain. Similar results were obtained in P-gp knockout mice. Our study demonstrates that P-gp mediates TPP efflux *in vitro* and *in vivo*; however, the consistently poor BBB permeation of TPP in all *in vivo* studies including P-gp knockout animals indicates that it is most likely mediated by other mechanisms. These findings are important for optimized clinical application of TPP as an imaging agent in cancer.

Keywords: TPP; intestinal permeability; P-glycoprotein; BBB uptake; MDR; P-gp knockout mice; imaging; tumor; Caco-2; Ussing chamber; verapamil; dinitrophenol; absorption; distribution

Introduction

Organic cations, such as ^{99m}Tc-sestamibi (MIBI) and ^{99m}Tc-ethylcysteinate dimer, have been utilized for the imaging of malignant tumors.[1] Phosphonium cations exhibit relative selectivity to malignant cells, compared to normal epithelial cells.[1,2] Furthermore, in malignant cells, their cellular uptake is rapid and correlates with the rate of cell proliferation[3−5] and its cellular retention is prolonged.[3−6]

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We have previously characterized the kinetics and the *in vivo* tumor selectivity of the phosphonium ion triphenylmethylphosphonium (TPMP) in canine brain gliomas using positron emission tomography.[7] TPMP exhibited excellent features as a tracer for tumor detection, including rapid uptake, prolonged retention and, most importantly, high tumor-to-nontumor contrast. Similarly, the accumulation of the TPMP analogue, tetraphenylphosphonium (TPP), in glioma cells inoculated in rat brain was 20-fold greater than in the intact contralateral brain tissue.[8,9] Compared to MIBI, [^{3}H]-TPP has a greater accumulation in malignant tissue and a better tumor-to-nontumor ratio.[2] Indeed, TPP has been used over the past three decades as an electrochemical probe for trans-membrane potential in cultured cells and in tissues and has been proposed as a promising means for tumor imaging due to its enhanced tumor-to-nontumor uptake ratio.[7,10] The *in vivo* tumor selectivity of TPP is corroborated by *in vitro* findings demonstrating tumor selectivity in a large array of cell lines including gliomas and adrenocarcinomas.[11,12]

A major limitation in the clinical use of phosphonium cations is their low permeability across intact blood−brain barrier (BBB).[1] For example, we have previously shown that less than 0.1% of an injected dose of [^{3}H]-TPP is detected in mouse brains 5 and 120 min after its intravenous injection.[2] It has been suggested that the distribution of these compounds into the brain may be limited by active efflux transport, in particular by P-glycoprotein (P-gp), a transmembrane ATP-dependent efflux pump.[13] In rodents, P-gp is encoded by two genes, mdr1a and mdr1b, whereas in humans it is encoded by a single gene, MDR1. The two rodent P-gp proteins together have overlapping tissue distribution and substrate specificity as a single human P-gp protein.[14−21] Mdr1a is highly expressed in the intestine, at the BBB and the blood−testis barrier, whereas mdr1b is expressed in the adrenal gland, the pregnant uterus, and the ovaries.[22] In naturally P-gp-deficient Collie dogs, but not in

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wild type dogs, MIBI did not cross the BBB.[23] However, in humans MIBI was excluded almost completely from cerebral tissues inside normal BBB.[13] Another phosphonium cation, [^{99m}] Tc-tetrofosmin, showed 3.5-fold greater brain distribution in mdr1a (−/−) mice, compared with the wild type.[24]

TPP is known to be a substrate for P-gp[25−27] although the role of P-gp in the pharmacokinetics of TPP has not yet been fully characterized. Gros and collaborators[25] reported that hamster cells transfected with mdr1a or mdr1b genes exhibit high levels of resistance to TPP, but the resistance was reversed by verapamil. In this study we evaluated TPP as a P-gp substrate *in vitro*, in human Caco-2 cells; *ex vivo*, in perfused rat intestine; and *in vivo*, in rodent models with P-gp genetic and chemical ablation. We also assessed whether P-gp restricts the brain distribution of TPP, in order to gain more information on the role of P-gp in the transfer of phosphonium-containing imaging agents across BBB.

Materials and Methods

Chemicals and Reagents. Cyclosporin A (CsA) as Sandimmune ampules (250 mg/5 mL) was purchased from Sandoz (Basel, Switzerland). [^{3}H]-TPP (28 Ci/mmol) and [^{3}H]-digoxin (37 Ci/mmol) were obtained from DuPont NEN (Boston, MA). MK-571 was purchased from Alexis Biochemicals (Lausen, Switzerland). PSC-833 and KZI (65:35 w/w of polyethoxylated castor oil and ethanol) were purchased from Novartis (Nuremberg, Germany). Dulbecco's modified Eagle medium (DMEM), heat-inactivated fetal bovine serum (FBS), nonessential amino acids (NEAA), and L-glutamine were purchased from Biological Industries, Beth-Haemek, Israel. All other reagents were from Sigma-Aldrich (Rehovot, Israel).

Cell Lines and Culture Conditions. Caco-2 cells were obtained from ATCC. Cells were grown in 75 cm^2 flasks with approximately 0.5 × 10^6 cells/flask at 37 °C in a 5% CO_2 atmosphere and at relative humidity of 95%. The culture growth medium consisted of DMEM supplemented with 10% heat-inactivated FBS, 1% NEAA, and 2 mM L-glutamine. The medium was replaced twice weekly.

Animals. Female mdr1a/b$^{(-/-)}$ and wild type mice weighing 26 ± 3 g (mean ± SD) were purchased from Taconic (Germantown, NY). Male C-57-Bl/6 mice weighing 27 ± 3 (mean ± SD) g and male Wistar rats weighing 275 ± 20 g (mean ± SD) were purchased from Harlan laboratories (Rehovot, Israel). Mice and rats were kept in a light-controlled room (light from 7:00 to 19:00) and were maintained on laboratory chow and water *ad libitum*. The research adhered to the principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985).

***In Vitro* Permeability Studies.** Cells (passage 60−66) were seeded at density of 25 × 10^5 cells/cm^2 on untreated culture inserts of polycarbonate membrane with 0.4 μm pores and surface area of 1.1 cm^2. Culture inserts containing Caco-2 monolayer were placed in 12 mm transwell plates. Culture medium was replaced every other day. Transport studies were performed 21−23 days after seeding, when the cells were fully differentiated and the transepithelial electrical resistance (TEER) values became stable (300−500 Ω·cm^2).

Transport studies were initiated by medium removal from both sides of the monolayer and replacement with apical and basolateral buffers,[28] both prewarmed to 37 °C. The cells were incubated for 30 min at 37 °C with shaking (100 cycles/min).

0.6 mL of [^{3}H]-TPP solution (27 μM; 1 μCi/ml) in the apical buffer, with or without inhibitor, was added to the apical side of the monolayer in the A−B direction studies, and 1.5 mL of basolateral buffer was added to the receiver compartment on the basolateral side of the monolayer. In the B−A direction studies, 1.5 mL of [^{3}H]-TPP solution (1 μCi/ml), with or without inhibitor, was added to the basolateral side of the monolayer, and 0.5 mL of blank buffer was added to the receiver compartment on the apical side of the monolayer. Three efflux transporter modulators, each in 3 different concentrations, were investigated: (1) verapamil (100, 50, and 10 μM); (2) MK-571 (100, 50, and 10 μM); and (3) fumitremorgin C (20, 10, and 5 μM). At fixed time points (30, 60, 90, 120, and 150 min), samples were taken from the receiver side (100 μL from basolateral side or 20 μL from apical side), and similar volumes of blank buffer were added following each withdrawal. At the last time point (150 min), samples were taken from the donor side as well, in order to confirm mass balance. Samples were immediately assayed for drug content. Caco-2 monolayers were checked for confluence by measuring the TEER before and after the transport study.

***Ex Vivo* Permeability Studies.** Transport experiments were performed in jejuni obtained from rats, mdr1a/b$^{(-/-)}$ mice or wild type mice. Animals were fasted for 16 h before

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the procedure. The entire small intestine was collected through an abdominal incision under ether anesthesia. Tissue was immediately rinsed with ice-cold saline, and the first 10 cm segment distal to the pylorus was discarded. Peyer's patches were identified visually, and sections containing them were not used in this study. Five segments, approximately 3 cm length each, were isolated from each rat. Intestinal segments were pulled onto a glass rod, and the fat adhering to the mesentery was removed. The epithelial surface was exposed and the intestinal sheet was mounted between the two chambers of a Grass and Sweetana diffusion cell.[29] Diffusion cells (exposed surface area of 0.5 cm^2) were filled with 3 mL of bicarbonate-Ringer buffer solution (pH 7.4), kept at 37 °C and circulated by gas lift (95% O_2−5% CO_2). Permeability studies to determine mucosal-to-serosal (A−B) and serosal-to-mucosal (B−A) permeability coefficients of [^{3}H]-TPP (27 μM) and [^{3}H]-digoxin (10 μM; ≈0.5 μCi/mL) were initiated 30 min after diffusion cells were set. For inhibition studies, verapamil, CsA, PSC-833 or 2,4-dinitrophenol (DNP; final concentration 0.2 mM each) was added 5 min before substrate administration. Samples were taken from the acceptor bathing solution at 15 min intervals for up to 150 min and replaced with drug-free buffer. At the onset and the end of the experiment samples were also taken from the donor side. The amount of drug in each sample was assessed by liquid scintillation counting with appropriate standard curves.

The Effect of PSC-833 on TPP Disposition *in Vivo*. Rats were divided into two groups: the first group was treated once daily for 4 days with intraperitoneal injection of 50 mg/kg/day PSC-833 dissolved in 1 mL of 50% KZI (KZI/saline 50:50, v/v), the second group received an IP injection of normal saline. Two hours after the last PSC-833 administration, 20 μCi of [^{3}H]-TPP (in 0.2 mL of normal saline) was injected to the jugular vein. Water and food were withdrawn. Two hours after [^{3}H]-TPP administration, the rats were sacrificed and the brain, heart, lungs, liver, small intestine and plasma samples were collected. The organs were weighed and transferred to scintillation vials.

C-57-Bl/6 mice were treated with 50 mg/kg/day PSC-833, as described for rats, or normal saline. Tissues and plasma samples were collected as described for rats.

To assess brain distribution of [^{3}H]-TPP in mdr1a/b$^{(-/-)}$ and control wild type mice, 2 μCi of [^{3}H]-TPP in 0.2 mL of saline was injected to the tail vein (n = 5, each group). 10 μCi of [^{3}H]-digoxin was injected to wild type and knockout mice (n = 5/group) as a positive control for BBB P-gp activity. Water and food were withdrawn. Two hours after [^{3}H]-TPP administration, mice were sacrificed and the brains were removed. Brains were weighed and transferred to scintillation vials.

Determination of Tissue [^{3}H]-TPP Concentrations. One milliliter of SOLVABLE, Packard, USA (aqueous based tissue solubilizer for a fast digestion of biological samples that were not minced), was added to vials containing the harvested organs. Vials were incubated overnight in a rotating bath at 60 °C and 100 rpm and then cooled to room temperature. EDTA (100 μL of 0.1 M) was added, followed by 100 μL of 30% H_2O_2. Vials were kept in room temperature for an additional 15 min and then transferred to a rotating bath of 60 °C at 100 rpm for 30 min. At the end of the incubation, vials were cooled to room temperature and 10 mL of scintillation liquid (Ultima Gold) was added. The vials were kept overnight at room temperature. [^{3}H]-TPP radioactivity was analyzed using a liquid scintillation counter (Tri-Carb 4530; Packard Instruments Company, Downers Grove, IL) with appropriate standard curves.

Data Analysis. The *in vitro* and *ex vivo* permeability coefficients (Papp) for each compound were calculated from the linear plot of drug accumulation vs time, using the following equation:

$$\text{Papp} = (dQ/dt)/(C_0 \cdot A)$$

where dQ/dt is the steady state drug appearance rate at the receiver side, C_0 is the initial concentration of the drug at the donor side, and A is the cell layer surface area, for *in vitro* studies, or the exposed tissue surface area, for *ex vivo* studies (1.1 cm^2 or 0.5 cm^2, respectively).

Permeation rate was calculated for intestinal tissues from the linear portion of the [^{3}H]-TPP amount vs time plot. The amount entrapped in the intestinal membrane was determined as the difference between the initial amount and the total amount of [^{3}H]-TPP in the donor and receiver compartments at the end of the permeation study.

Each experiment was performed in intestines collected from three rats. Results are presented as mean ± SD, unless indicated otherwise. The statistical significance was determined by unpaired Student t test. A value of $P < 0.05$ was required for statistical significance.

Results

To validate and characterize the transport of TPP by P-gp *in vitro*, we studied substrate transfer across Caco-2 cells. We compared the apical-to-basolateral and basolateral-to-apical permeability coefficients (PappA-B and PappB-A, respectively) of [^{3}H]-TPP in the absence of transporter inhibitors and in the presence of verapamil (a P-gp substrate), MK-571 (a MRP2 inhibitor) and FTC (a BCRP inhibitor). In this model, PappB-A of [^{3}H]-TPP was 8-fold higher than PappA-B, indicating net mucosal secretion (Figure 1). Concentration dependent decrease of this secretion was obtained by the P-gp substrate verapamil, while no effect was evident by the MRP2 inhibitor MK-571 and the BCRP inhibitor FTC.

We further assessed [^{3}H]-TPP transfer *ex vivo*, across rat gastrointestinal mucosa in the absence of modulators and in combination with P-gp substrate (verapamil) and P-gp inhibitors (CsA and PSC-833) and DNP, an uncoupler of

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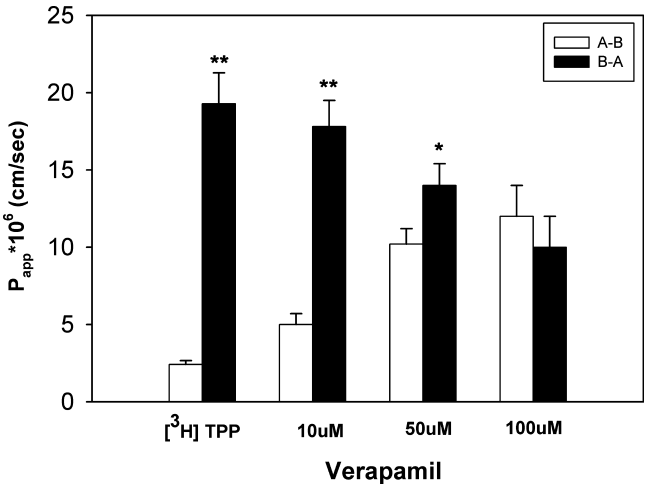

Figure 1. Permeability coefficients of [^{3}H] TPP in Caco-2 cells. Verapamil significantly enhanced the apical-to-basolateral transfer of [^{3}H]-TPP (27 μM). Values shown are mean Papp ± SEM, $n = 3$. Empty bars, PappA-B; filled bars, PappB-A. The apparent permeability (Papp) of [^{3}H]-TPP (27 μM) in the absorptive (A−B) and the secretory (B−A) directions, in the presence of different concentrations of verapamil (P-gp substrate): 100, 50, and 10 μM. Data presented as mean ± SEM; $n = 3$ in each experimental group. *$P < 0.05$, **$P < 0.01$.

oxidative phosphorylation.[23,30] In the absence of inhibitors, PappB-A was up to 15-fold greater ($P < 0.05$), compared to PappA-B, indicating net efflux of [^{3}H]-TPP from serosal to mucosal side of the jejunum (Table 1). However, these differences tended to disappear at higher [^{3}H]-TPP concentrations, probably due to saturation of a transport mechanism. The transfer of [^{3}H]-TPP across the intestinal membrane was characterized by a prolonged lag time (1 h) that was followed by an accelerated flux. The amount of [^{3}H]-TPP that was retained in the jejunal membrane at the end of the permeation studies was 14.9% ± 5.6 ($n = 32$) of the total amount introduced to the diffusion cells (data not shown).

DNP increased the permeation coefficient of TPP by 3.6 fold, from 0.063 ± 0.016 to 0.2283 ± 0.1581 cm/s ($P < 0.05$) (Figure 2, Table 2). Similarly, CsA, verapamil and PSC-833 increased the mean permeability rate constant of [^{3}H]TPP 4.0, 4.6 and 5.3-fold, respectively ($P < 0.05$, Table 2).

To evaluate the contribution of P-gp to [^{3}H]-TPP distribution *in vivo*, we compared the disposition of [^{3}H]-TPP in rats pretreated with PSC-833 or saline. In the two study groups, [^{3}H]-TPP achieved lesser brain to plasma concentrations, compared to heart, lungs, liver and small intestine. PSC-833 did not affect brain to plasma concentration ratio of [^{3}H]-TPP compared to saline while it caused a significant increase in tissue to plasma concentration ratio. As can be seen in Figure 3, PSC-833 increased the tissue to plasma concentration ratio in the heart, liver, lungs and small intestine 3.4-, 3.3-, 1.9- and 2.5-fold, respectively, while the brain to plasma concentration ratio was very low. Similar results were obtained when [^{3}H]-TPP distribution was studied in PSC-833 treated wild type mice (Figure 4). In mice, PSC-833 caused a significant increase in tissue to plasma concentration ratio in the lungs, liver, small intestine and the heart when compared to saline treated mice. However, the distribution of [^{3}H]-TPP into the brain was not affected by PSC-833 (Figure 4).

The *ex vivo* intestinal permeability of [^{3}H]-TPP was also assessed in mdr1a/b$^{(-/-)}$ and wild type controls. [^{3}H]-Digoxin, an established P-gp substrate,[31] served as the positive control. PappA-B of [^{3}H]-digoxin was doubled in the knockout compared to wild type mice, from $8.4 \times 10^{-6} \pm 1.2$ to $15 \times 10^{-6} \pm 2.3$ cm/s ($P < 0.05$), whereas PappA-B of [^{3}H]-TPP was 5-fold greater in the knockout mice, compared to controls: $19 \pm 3.1 \times 10^{-6}$ vs $4.2 \pm 0.9 \times 10^{-6}$ cm/s, respectively, $P < 0.01$ (Figure 5). In contrast to the gastrointestinal system, the *in vivo* [^{3}H]-TPP brain to plasma concentration ratio was not significantly different between mdr1a/b$^{(-/-)}$ and control, wild type mice, while the brain to plasma concentrations ratio of [^{3}H]-digoxin were significantly higher (9 fold) in the mdr1a/b$^{(-/-)}$ mice (Figure 6).

Discussion

TPP has been suggested as an *in vivo* probe for characterizing molecular changes during tumor evolution, based on its strong accumulation in malignant tissue and a good tumor-to-nontumor ratio. However, in common with MIBI, TPP crosses the intact BBB very poorly.[2] Our aim was to assess the contribution of P-gp to the pharmacokinetics of TPP, in particular to its distribution across the BBB.

The important role that P-gp plays in drug pharmacokinetics has been recognized in a recent guidance document developed by the US Food and Drug Administration (FDA).[32] According to this document, the bidirectional transport assay across cell monolayers, including those of Caco-2 cells, is regarded as the definitive assay for identifying P-gp substrates. By using an *in vitro* system, we demonstrated that the transport of [^{3}H]-TPP across these polar cells is directional and can be inhibited by verapamil, an established P-gp substrate.[32] Furthermore, similar results have been obtained when the transfer of [^{3}H]-TPP was assessed *ex vivo*, across rat jejunum. The transport exhibited concentration-dependence and could be inhibited by the P-gp modulators verapamil, CsA, PSC-833 and by the oxidative phospholeylation inhibitor DNP (Tables 1 and 2, Figure 2). The concentrations of verapamil (10, 50, and 100 μM) were

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Table 1. Effect of [^{3}H]-TPP Initial Concentration in the Donor Chamber on Its Mucosal-to-Serosal (A−B) and Serosal-to-Mucosal (B−A) Permeation Rate across Rat Jejunum[a]

	permeation rate (mean ± SD) %/(cm^2 h)		
initial [^{3}H]-TPP in donor compartment (mM)	A-to-B	B-to-A	permeation rate ratio (B−A/A−B)
2	0.727 ± 0.149	0.587 ± 0.162	0.81
0.4	0.457 ± 0.018	1.044 ± 0.387*	2.28
0.08	0.063 ± 0.016	1.069 ± 0.455*	17.0
0.02	0.075 ± 0.092	1.165 ± 0.37*	15.53

[a] Transport experiments were performed *ex vivo*, in jejuni obtained from untreated rats. * indicates significantly different from the A-to-B PR ratio ($P < 0.05$).

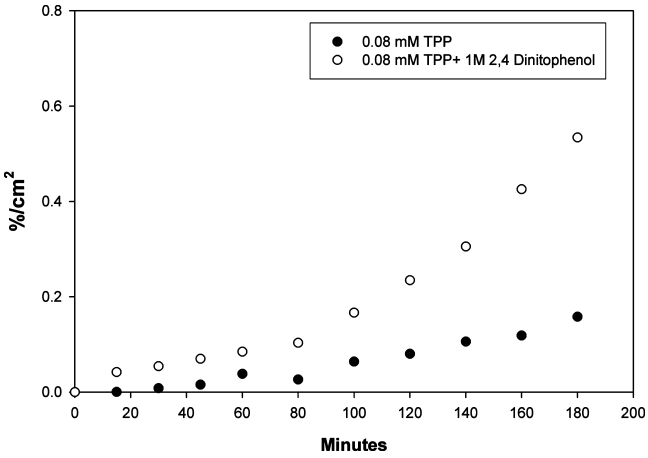

Figure 2. The effect of 1 M 2,4-dinitrophenol (DNP) on 0.08 mM [^{3}H]-TPP transport across the intestine wall (A-to-B).

Table 2. Effect of Transport Modulators on Mucosal-to-Serusal Permeation Rate of [^{3}H]-TPP across Rat Jejunum[a]

modulator	permeation rate (mean ± SD) %/(cm^2 h)	ratio of permeation rates in the presence and absence of a modulator
control	0.06 ± 0.016	1.00
verapamil	0.29 ± 0.022*	4.65
CsA	0.25 ± 0.022*	4.01
PSC-833	0.33 ± 0.0298*	5.3
DNP	0.23 ± 0.1581*	3.64

[a] Transport experiments were performed *ex vivo*, in jejuni obtained from untreated rats. * indicates $P < 0.05$. [^{3}H]-TPP initial concentration in the donor compartment was 27 μM. Initial modulator concentration in the donor compartment was 0.2 mM ($n = 5$). CsA, cyclosporin A; DNP, dinitrophenol.

greater than their *in vitro* IC_{50} (2.1 μM and 1.3 μM, respectively),[32] indicating complete P-gp inhibition. The finding was substantiated by the inhibitory effect of DNP on [^{3}H]-TPP transfer, given that P-gp is ATP-dependent.

The *ex vivo* permeation results on mdr1a/b (−/−) and wild type mice were consistent with the preceding results. In the P-gp knockout mice there was a significant increase in the A−B permeation values of [^{3}H]-digoxin, a known P-gp substrate and [^{3}H]-TPP, compared to the wild type mice, confirming that [^{3}H]TPP is a P-gp substrate.

Taken together, our results show that [^{3}H]-TPP is a P-gp substrate. As such, P-gp may affect the tissue distribution of TPP. To assess this possibility, we studied the distribution

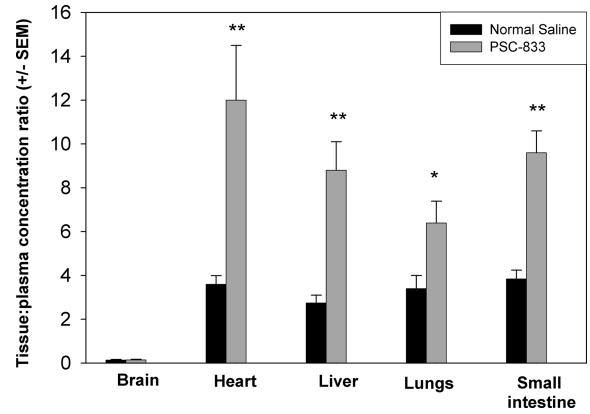

Figure 3. [^{3}H]-TPP uptake after intravenous injection into male Wistar rat tissues *in vivo* following pretreatment with 50 mg/kg/day PSC-833. CsA significantly increased [^{3}H]-TPP uptake into the lungs, liver, small intestine and spleen, but not into the brain. Values shown are mean ± SEM; $n = 5$ each group.*$P < 0.05$, **$P < 0.01$.

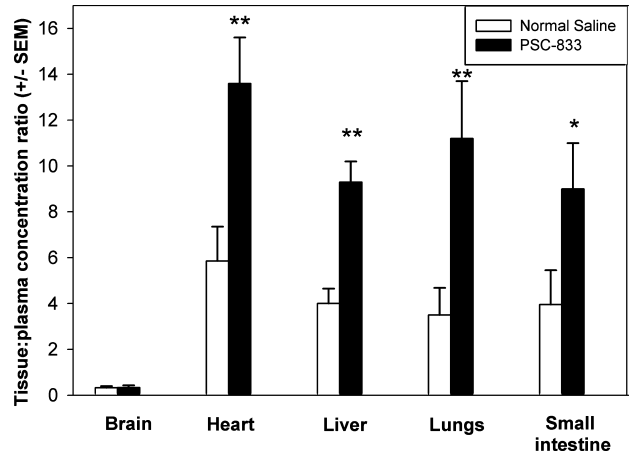

Figure 4. [^{3}H]-TPP uptake after intravenous injection into male C-57-Bl/6 wild type mice tissues *in vivo* following pretreatment with 50 mg/kg/day PSC-833. PSC-833 enhanced [^{3}H]-TPP distribution into multiple tissues, but not the brain. Values shown are mean ± SEM; $n = 5$ each group. *$P < 0.05$, **$P < 0.01$.

of [^{3}H]-TPP in rats and mice in the absence and the presence of PSC-833. In the absence of a modulator, greatest accumulation of [^{3}H]-TPP in mice was in the myocardium, which has a high density of mitochondria, as previously

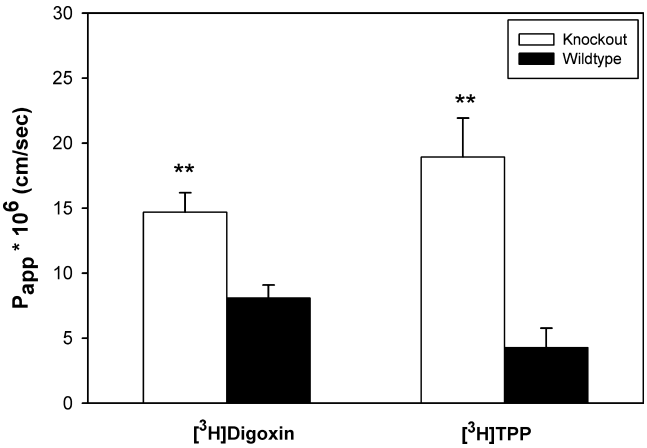

Figure 5. PappA-B of [^{3}H]-TPP (27 μM) and digoxin (10 μM) in intestines from mdr1a/b$^{(-/-)}$ mice and wild type controls. Transport experiments were performed *ex vivo*, in jejuni obtained from untreated mice. Filled bars, Papp in wild type mice; open bars, Papp in mdr1a/b (−/−) mice. Values shown are means ± SEM; $n = 6$ tissues. *$P < 0.05$, ** $P < 0.01$.

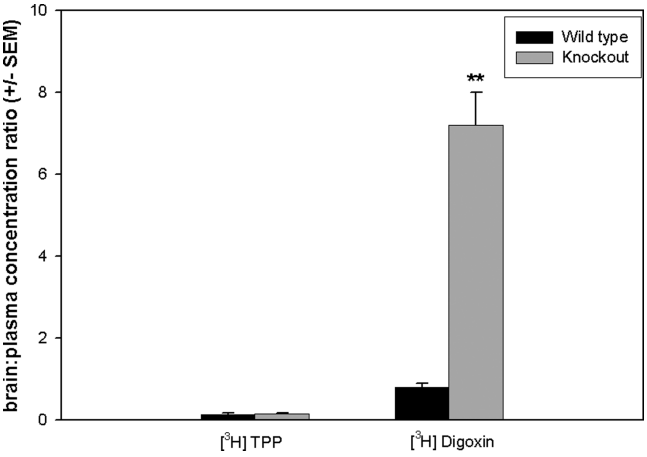

Figure 6. *In vivo* brain disposition of [^{3}H]-TPP and digoxin in mdr1a/b$^{(-/-)}$ mice and wild type controls. Values shown are brain to plasma concentration ratio. Values shown are means ± SEM; $n = 5$ mice/group. **$P < 0.01$.

demonstrated by us.[2] PSC-833 significantly enhanced [^{3}H]-TPP accumulation into multiple tissues as compared to plasma, including the heart (Figures 3, 4). These results are in line with data from previous studies demonstrating that TPP is a P-gp substrate[25−27] and suggest that P-gp is likely to limit the distribution of [^{3}H]-TPP into these tissues. Nevertheless, [^{3}H]-TPP accumulation in the CNS was very low in rats and mice and did not increase in the presence of PSC-833 (Figures 3, 4). Furthermore, the brain disposition of [^{3}H]-TPP was not greater in P-gp knockout mice than in wild type controls. These data are in line with the results obtained with P-gp inhibition and further suggest that the limited distribution of [^{3}H]-TPP across the BBB is not due to P-gp-mediated efflux (Figure 6).

Similar to our results, Tahara et al. previously reported on different roles of the P-gp efflux system depending upon its location. In this work P-gp played a major role in the efflux of fexofenadine in the small intestine and BBB, but only a limited role in its biliary excretion.[33]

It has been previously shown that the BBB permeability of several ^{99m}Tc-labeled compounds, including MIBI and [^{3}H]-TPP, is limited, compared to other tissues and cell types.[1,2] Several explanations for this phenomenon have been suggested. First, these compounds may be recognized by P-gp, which is highly expressed at the BBB and pumps them from the brain back into the circulation. Indeed, most studies in animals with genetic depletion or chemical inhibition of P-gp indicated that its greatest impact on drug distribution is at the BBB.[34] On the other hand, it has been previously demonstrated in mice that P-gp at the BBB is more resistant to inhibition by tariquidar than in other tissues.[35] In our study both genetic and chemical ablation of P-gp activity did not increase the brain uptake of [^{3}H]-TPP despite enhanced uptake to other tissues in the presence of PSC-833. Since the limited accumulation of [^{3}H]-TPP in the CNS is not due to P-gp mediated efflux, we evaluated other efflux systems. Both the specific MRP2 inhibitor MK-571 and the specific BCRP inhibitor FTC showed no effect on the permeability of [^{3}H]-TPP. We have not assessed the impact of BCRP on brain penetration of [^{3}H]-TPP, since BCRP does not restrict the distribution of MIBI, a structurally related compound, into the brain.[1]

It has been suggested that the dipole membrane potential at the BBB restricts the transport of MIBI into the brain.[1] TPP exhibits greater cellular accumulation, compared to MIBI, despite its lower lipophilicity (log $P = 1.2$ vs 2.89).[1] Like MIBI, TPP may enter cells by passive diffusion and tends to concentrate in the cytoplasm and mitochondria due to the negative potential of plasma and mitochondrial membranes.[2] Thus compounds that modulate the dipole potential, such as verapamil and CsA, may enhance the uptake of Nernstian probes like MIBI, TPP and rhodamine 123 across the cell membranes. Nevertheless, Cattelotte et al. reported that verapamil decreased MIBI transport into mice brains, regardless of their P-gp status.[1] Thus, we cannot rule out the possibility that CsA affects [^{3}H]-TPP transfer across the BBB in opposite directions, by P-gp inhibition and modification of endothelial cell dipole potential.

Alternatively, the low uptake of [^{3}H]-TPP and other phosphonium cations into intact brain could result from an absence at the BBB of an uptake mechanism for these compounds, that exists in other tissues, such as organic cation

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transporters. However, although organic cation transporters are expressed in the CNS, so far they have been localized mainly to neurons and glial cells and not to endothelial cells.[36]

Although many P-gp substrates undergo metabolism by cytopchrome P450 3A (CYP3A) isoforms, we were unable to find data on CYP3A-mediated metabolism of TPP. The disposition of potential TPP metabolites in normal and tumor tissues should be further evaluated in future studies. Nevertheless, CYP3A4 and CYP2D6, that are together responsible for metabolism of approximately 50% of all therapeutic compounds, have not been detected at the human BBB[37] and have not been reported to be significantly expressed at the BBB from other species. Furthermore, the contribution of other drug metabolizing enzyme to the barrier function of brain capillary endothelial cells has not been established yet. The poor CNS permeability of TPP is unlikely to be explained by metabolism at the BBB.

We conclude that P-gp contributes to the distribution of TPP into the heart, lungs, liver and small intestine; however the poor CNS permeation may be mainly explained by the absence of passive transcellular diffusion across brain endothelial cells or the absence of an uptake mechanism. If TPP's modest permeability into normal rodent tissues such as the lungs and the small intestine is replicated in humans, its high tumor selectivity and good correlation with the rate of cell proliferation make it a promising clinical diagnostic utility as an imaging tracer for tumor staging in these tissues. However, TPP does not appear to be a good imaging probe for CNS tumors. Furthermore, TPP may be excluded from those tumors that highly express active P-gp. These findings are important for optimized clinical application of TPP as an imaging agent in cancer.

Abbreviations Used

P-gp, p-glycoprotein; TPP, tetraphenylphosphonium; BBB, blood−brain barrier; Papp, permeability coefficient; CsA, cyclosporin A; DNP, 2,4-dinitrophenol; MIBI, sestamibi; TPMP, triphenylmethylphosphonium; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; NEAA, nonessential amino acids; TEER, transepithelial electrical resistance; FDA, Food and Drug Administration; CNS, central nervous system.

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