The Effect of P-glycoprotein on Paclitaxel Brain and Brain Tumor Distribution in Mice

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ABSTRACT

It may be inferred from the presence of P-glycoprotein (Pgp) in brain capillaries that this drug efflux pump is a factor in limiting the penetration of certain agents into brain tumors. However, by contrast with normal brain capillaries which constitute the blood–brain barrier, brain tumor capillaries are compromised or “leaky,” and the extent to which Pgp expression in brain tumor neovasculature retains its capacity to limit drug penetration has not been determined. To address this question, we studied the normal brain and brain tumor distribution of paclitaxel (PAC), a known Pgp substrate, using steady-state PAC dosing regimens in wild-type and Pgp knockout (mdr1a−/− and mdr1b−/−) mice bearing an intracerebral B-16 melanoma. At comparable steady-state PAC plasma concentrations of ∼5 µg/ml, steady-state PAC brain concentrations in Pgp knockout mice were ∼3-, 1.8-, and 1.7-fold greater in left brain, right brain, and brain tumor, respectively, than in wild-type mice and statistically different (P < 0.05) in each brain region. Determination of the steady-state brain/plasma concentration ratios or partition coefficients, which take into account any differences in plasma concentrations between each group, indicated a similar pattern as did the absolute brain concentrations. It is concluded that even in the neovasculature of brain tumors, Pgp has the facility to limit drug penetration, although somewhat less so than in normal brain.

INTRODUCTION

Pgp5 is an established factor in the pharmacokinetics of anticancer agents (1–6). As determined from studies of Pgp-deficient mice, and from investigations of the effects of Pgp inhibitors on normal rodents and humans, Pgp is capable of reducing the p.o. bioavailability, and facilitating the hepatobiliary excretion, of drugs that are substrates of the pump, such as PAC and digoxin (2, 7–10). The involvement of Pgp in these processes is based on its expression in gut enterocytes and bile canalicula. Another site at which Pgp could affect the distribution of anticancer agents is the brain, a site at which the pump is known to function as a component of the BBB (1, 2, 11). Expression of Pgp at the apical surfaces of brain capillary endothelial cells has been determined to limit the penetration into this organ of agents, such as ivermectin, cyclosporin A, and loperamide (1, 2, 7–10). The involvement of Pgp in the pump may be markedly diminished. These issues pertaining to the role of Pgp as a determinant of PAC distribution into the CNS and, thus, lead support to our investigation that sought to characterize the role of Pgp in PAC distribution, not only in normal brain, but also in brain tumors.

MATERIALS AND METHODS

Chemicals. PAC was obtained from Mead-Johnson (Taxol injection; Bristol-Myers Co., Princeton, NJ). The Pgp [Ab-1] antibody was purchased from Oncogene (Boston, MA). FVB parental and mdr1a/b−/−ko mice were provided by Taconic (Germantown, NY). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

In Vivo Intracerebral Tumor Models. To establish a syngeneic mouse brain tumor model, three cell lines (i.e., B-16, A20, and 4C8) were screened for Pgp expression and in vivo growth characteristics. The B-16 mouse melanoma cell line was kindly provided by Dr. Klein-Szanto, Fox Chase Cancer Center. The A20 mouse glioma cell line was kindly provided by the Brain Tumor Research Center, University of California (San Francisco, CA), and the 4C8 cell line was obtained from Dr. Linda Parysek, Children’s Hospital of Philadelphia. Western blot analyses were completed to assess Pgp expression in each cell line using KB-3–1 and KB-C2 human epidermoid carcinoma cell lines, provided by Dr. G. Kruh, Fox Chase Cancer Center, as Pgp-positive and -negative controls, respectively. Each cell line (i.e., B-16, A20, and 4C8) was evaluated for their ability to form brain tumors in male FVB mice (25–30 grams). Mice were anesthetized with an i.p. dose (0.1 ml/10 grams body weight) of a 3:2:1 (volume:volume:volume) mixture of ketamine hydrochloride (10 mg/ml); acepromazine maleate (1 mg/ml); xylazine hydrochloride (2 mg/ml), secured in a stereotoxic apparatus, and had implanted 3 µl of a tumor cell suspension (106 cells/ml) into the right (2.5 mm lateral from the bregma) thalamic region at a depth of 3 mm. The hole was sealed with bone wax, and the skin was sutured. The mice were then returned to cages and received a standard rat diet and water ad libitum. Animals were monitored daily for symptoms and body weight.

Pharmacokinetic Studies. The goal of the pharmacokinetic investigations was to compare PAC brain and brain tumor distribution in wt and Pgp ko mice under steady-state conditions. Both wt and Pgp ko mice were implanted with B-16 melanoma cells as described above. In ∼10 days, animals showed CNS concentrations that occur as a result of loss of Pgp function in liver, the results suggested that Pgp influenced normal brain penetration of vinblastine (1, 3). Another unsettled issue regarding Pgp, and of direct significance to cancer treatment, is whether the pump is able to reduce the penetration of anticancer agents into brain tumors. The notion that Pgp might function in this capacity is supported by studies showing that the pump is expressed in brain tumor neovasculature (12–14). However, on the other hand, tumor blood capillaries are known to be “leaky,” a feature that is thought to contribute to the enhanced penetration of anticancer agents into brain tumors (15), and it is possible that under these pathological circumstances, the activity of the pump may be markedly diminished. These issues pertaining to the function of Pgp in anticancer drug penetration into normal brain and brain tumor also have a bearing on whether Pgp inhibitors have a role in treating brain cancers.

Studies of PAC distribution into the CNS of both rodents and humans have indicated cerebrospinal fluid, and normal brain concentrations are only a small fraction of concomitant plasma concentrations (16–18). Limited data from patients have indicated higher PAC brain tumor concentrations than in normal brain consistent with the disruption of the BBB (17). These investigations did not address the role of Pgp as a determinant of PAC distribution into the CNS and, thus, led support to our investigation that sought to characterize the role of Pgp in PAC distribution, not only in normal brain, but also in brain tumors.

5 The abbreviations used are: Pgp, P-glycoprotein; BBB, blood–brain barrier; LC-MS, high-pressure liquid chromatography-mass spectrometry; CNS, central nervous system; ko, knockout; PAC, paclitaxel; wt, wild-type.
symptoms (i.e., arched back and lethargy) and about a 10% weight loss. At this time, animals were anesthetized as described above and had implanted both a right carotid artery and jugular vein catheter. To achieve steady state PAC plasma concentrations, two simultaneous constant rate infusions, an initial 15-min high-rate infusion (0.42 mg/kg/min) followed by a 165-min slow-rate infusion (0.021 mg/kg/min), were administered through the jugular vein canula. These PAC administration regimens were designed to achieve steady-state plasma concentrations of 5 μg/ml and were based on preliminary pharmacokinetic studies in both wt and Pgp ko mice. During the PAC administrations, ~40-μl blood samples were collected from the right common carotid artery at 15, 30, 60, 120, and 180 min. Plasma was harvested from each blood sample and stored at −80°C until analyzed for PAC by LC-MS (see below). Immediately after the 180 min blood samples were collected, mice were anesthetized with ether and sacrificed by cervical dislocation, and the whole brain was removed and frozen in dry ice before storage at −80°C and subsequent analysis by LC-MS. PAC plasma and brain concentrations and partition coefficients, calculated as the ratio of steady-state brain/plasma concentrations measured at 180 min, were compared statistically using either a two-sided ANOVA or t test. A value of P < 0.05 was considered statistically significant.

Quantitation of PAC by LC-MS. An LC-MS assay was developed to measure PAC in plasma and brain samples because it afforded improved sensitivity with small sample volumes. Plasma samples were thawed to room temperature, and 20-μl aliquots were added to 150 μl of methanol and 50 μl of a 3 μM internal standard (cephalomannine) solution. After vortex and centrifugation, the supernatant was collected and added to 750 μl of water that was passed through a preconditioned (washed with 1 ml of methanol and 3 ml of water) solid phase C2 cartridge. The resultant eluent containing PAC was diluted twice with water, and 10-μl aliquots were injected onto the LC-MS system (Hewlett Packard 1100 series and MS: Finnigan, Navigator) that consisted of an octadecyl silane chromatographic column (Hypersil, 5 μm, 2.1 × 100 mm) and mobile phase of 50% acetonitrile, 0.1% formic acid in water pumped at a flow rate of 0.2 ml/min. PAC and the internal standard were detected at m/z ratios of 854.3 and 832.4, respectively.

For brain and brain tumor samples, whole brains were thawed, and ~60 mg each of left brain (contralateral hemisphere from the tumor), right brain, and brain tumor were collected by gross dissection. To each sample, five times the volume of water was added and homogenized under ice-cold conditions for 10 s. To each 200-μl aliquot of tissue homogenate, 50 μl of the internal standard solution and 600 μl of methanol were added. The mixture was vortexed for 1 min, maintained at room temperature for 5 min, and then centrifuged (15,000 × g, 5 min). The resultant supernatant was collected, and 3 ml of water were added. The diluted supernatant was passed through a preconditioned C2 cartridge, which was then washed with 3 ml of water and finally eluted with 250 μl of acetonitrile. The eluted samples were diluted 2-fold with water and injected onto the LC-MS system described above. The assay was both accurate and precise with coefficients of variation of <15%.

RESULTS AND DISCUSSION

The area of membrane transporters is increasing in importance as more and more transporters are discovered and their potential role as drug efflux pumps is uncovered. The pharmacokinetic investigations that have been completed have considered the role of transporters in drug distribution in normal tissues (1–3, 6, 7), and thus, this how this role might be altered by a tumor have not yet been evaluated. A recent study by Fellner et al. (19), which appeared after submission of this study, used a nude mouse brain tumor model to show that inhibition of Pgp with valspodar did increase entry of PAC into normal brain and enhanced efficacy as measured by tumor volume. Our results, as presented below, are consistent with these observations and specifically quantitated the effect of Pgp on brain tumor concentrations of PAC. This latter contribution pertaining to how drug efflux pumps might alter drug distribution in the presence of a tumor is particularly relevant to brain tumors that are known to compromise the BBB, a pivotal site for expression of Pgp.

To examine the question of how the presence of a brain tumor might influence drug penetration via a BBB efflux pump, we analyzed the penetration of PAC into the normal brain and brain tumors of wt and Pgp ko mice bearing B16 melanomas. PAC was chosen for this investigation because it is a known Pgp substrate and possesses activity in brain tumors. In addition, because PAC is not a known substrate of other drug pumps, such as MRPI and ABCG2 (20, 21), the use of this drug eliminated these other pumps as confounding factors in our analysis. Because wt and Pgp minus mice are immuno-competent the use of a syngeneic tumor model, only a few described in mouse brains (22, 23) was required. We screened several of the cell lines that have been used previously for this purpose (i.e., 4C8, A20, and B-16) for their ability to form intracerebral tumors in wt mice and selected the B-16 melanoma. Two properties of this cell line were advantageous for our studies. Firstly, the dark brown/black tumors B-16 formed when implanted into brain greatly facilitated the separation of normal brain and brain tumor samples by gross dissection, a feature that enhanced the accuracy of measurements of PAC concentrations in normal brain and brain tumor. In addition, we found that B-16 had the lowest levels of Pgp expression of any of the cell lines examined, a feature which would minimize the contribution of the endogenous expressed pump to tumor drug levels.

Two alternate study designs could have been used to assess the tissue distribution of PAC, either a single dose regimen or a steady-state regimen. Each regimen can lead to the estimation of a pharmacokinetic parameter known as the partition coefficient to characterize drug distribution that is calculated as either the ratio of tissue:plasma area under the drug concentration-time curve values from single dose studies or as the ratio of steady-state tissue:plasma drug concentrations in the steady-state design (24). The current investigation used the steady-state approach not only to conserve on animal usage compared with a single dose or nonsteady-state regimen but also because it would more readily enable achievement of equivalent systemic exposures of PAC in both wt and Pgp ko animal groups. Equivalent systemic exposures of PAC in each animal group facilitated the comparison of PAC brain distribution, as well as minimize potential effects Cremophor EL, the standard vehicle used in the clinical formulation of PAC, might have on PAC pharmacokinetics (25).

Fig. 1 shows the PAC plasma concentrations in wt and Pgp ko mice after administration of steady-state dosing regimens of PAC. The steady-state PAC regimens consisted of two sequential continuous rate i.v. infusions, the first a 15-min high-rate infusion immediately followed by a slower rate infusion until 180 min. As shown in Fig. 1, PAC plasma concentrations became relatively constant after 15 min, with mean values ranging between 4.2 ± 1.05 μg/ml and 6.3 ± 1.76 μg/ml. From 120 to 180 min, plasma concentrations of PAC were not statistically different (P < 0.05) in the wt and Pgp ko groups and were 4.8 ± 1.4 μg/ml and 5.7 ± 1.6 μg/ml, respectively. These data confirm that steady-state conditions were achieved in each group and that the aforementioned potential confounding effects of dissimilar systemic exposures and Cremophor EL on brain distribution were nullified. In addition, our results may be relevant to PAC brain and brain tumor distribution in humans because the steady-state PAC plasma concentrations of ~5 μg/ml are within the range of maximum plasma concentrations obtained after different administration regimens of PAC to patients (26).

A pivotal motivation to conduct this investigation was to compare brain and brain tumor drug concentrations so as to determine whether Pgp altered drug distribution under conditions when the BBB was compromised by a tumor. To accomplish this comparison, PAC concentrations were measured under steady-state conditions at 180 min in three brain regions from each animal, normal right and left brain and brain tumor. It has been observed that “normal” brain samples collected in the ipsilateral hemisphere as the brain tumor can...
PACLITAXEL BRAIN AND BRAIN TUMOR DISTRIBUTION IN MICE

Fig. 1. Mean (±SD) PAC plasma concentrations in Pgp wt (closed symbols) and ko (open symbols) mice. PAC was administered as two consecutive constant rate infusions of 15 and 165 min designed to achieve plasma concentrations of 5 μg/ml. The administration method.

show enhanced permeability depending on the proximity of the sample to the tumor (27). It was, therefore, anticipated that normal right (the hemisphere of the tumor) brain PAC concentrations would be higher than normal left brain, yet less than brain tumor. Fig. 2 illustrates the mean (±SD) brain and brain tumor PAC concentrations in both wt and Pgp ko groups. Each difference between Pgp wt and ko groups in the same brain region was statistically significant (P < 0.05). Normal left brain PAC concentrations were ~3-fold greater (P < 0.05) in the Pgp ko mice (0.31 ± 0.09 μg/gram) compared with wt mice (0.11 ± 0.06 μg/gram), in accord with the notion that Pgp limits PAC penetration into brain. Right brain PAC concentrations were ~1.8-fold greater (P < 0.05) in the ko mice (0.33 ± 0.06 μg/gram) compared with the wt mice (0.18 ± 0.11 μg/gram), a difference that suggests the presence of the brain tumor in the right hemisphere did modulate drug penetration in the grossly defined normal brain region. The comparison of brain tumor PAC concentrations in wt (0.9 ± 0.67 μg/gram) and Pgp ko (1.56 ± 0.5 μg/gram) groups revealed an ~1.7-fold increase (P < 0.05) in the Pgp-deficient mice. This value was roughly comparable with the differences observed between normal brain in the wt and Pgp ko mice, suggesting that even under the conditions in brain tumor, in which enhanced drug penetration is readily apparent compared with normal brain, expression of Pgp was a limiting factor.

Comparisons of PAC concentrations within either the Pgp wt or ko groups indicated that PAC brain tumor concentrations were statistically greater (P < 0.05), ranging from ~5- to 7.5-fold higher, than either right or left brain PAC concentrations, consistent with the known “leakiness” of brain tumors. Right and left brain PAC concentrations were not statistically different from one another. Therefore, the analysis of PAC brain concentrations indicated that the rank order of concentrations was brain tumor > right brain > left brain in both animal groups, consistent with the effect of brain tumors on the BBB and that Pgp ko mice have between 1.7- and 3-fold greater PAC concentrations than in the corresponding brain region of wt mice.

Although steady-state PAC plasma concentrations enable direct comparisons of brain concentrations, analysis of partition coefficients, calculated as the ratio of steady-state tissue/plasma PAC concentrations, provides the most accurate assessment of drug distribution because any differences in plasma concentrations are accounted for in the ratio. Fig. 3 shows the mean (±SD) partition coefficients for each brain region in both wt and Pgp ko mice. The pattern of partition coefficient values with respect to both brain region and genetic type is similar to the pattern observed for PAC brain concentrations. There was a slight decrease in the differences between wt and Pgp ko mice in each brain region. These differences were 2.3-fold in left brain (0.023 ± 0.017 wt versus 0.054 ± 0.021 Pgp ko) and 1.6-fold in both right brain (0.036 ± 0.024 wt versus 0.059 ± 0.023 Pgp ko) and brain tumor (0.18 ± 0.134 wt versus 0.28 ± 0.112 Pgp ko) and were statistically different (P < 0.05) in both left and right brain but not in brain tumor (P = 0.09). The high variability of the partition coefficients in brain tumors may likely be attributed to the heterogeneous
nature of the tumor vasculature because PAC concentrations were more variable in brain tumor than in plasma and no doubt contributed to the lack of statistical significance (P = 0.09) between the brain tumor groups. The primary inferences from the partition coefficient data are that the presence of the brain tumor slightly augments, but does not abolish, the role of Pgp on PAC distribution in the tumor.

Investigations of drug penetration into brain tumors indicate that both physiological variables (i.e., blood flow, membrane integrity, and hypoxia), as well as drug-specific parameters (i.e., molecular weight and lipophilicity), are important determinants (28). Given the complexity of energy-dependent, plasma membrane-based systems for effluxing anticancer agents, and the poor results of chemotherapy in treating brain tumors, it is important to understand the extent to which ABC transporters are determinants of drug distribution into brain tumors. This is the first investigation that has addressed this question by simultaneously characterizing the role of Pgp on drug uptake into normal brain and brain tumors. Using wt and Pgp gene-disrupted mice under steady-state conditions, a number of important findings was made. We found that, with regard to a prototypical natural product anticancer agent (PAC), the BBB is compromised in brain tumors, leading to higher drug concentrations. The results also showed that under conditions in which the impact of Pgp on plasma levels is minimized, Pgp expression influences PAC concentrations in brain tumor, as well as in normal brain. Finally, we demonstrate the value of using a syngeneic brain tumor model to explore mechanisms underlying anticancer drug disposition in brain.

REFERENCES


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