Pharmacokinetics of Tumor Cell Exposure to [\textsuperscript{14}C]Methotrexate after Intracarotid Administration without and with Hyperosmotic Opening of the Blood-Brain and Blood-Tumor Barriers in Rat Brain Tumors: A Quantitative Autoradiographic Study

William R. Shapiro, Rand M. Voorhies, Emile M. Hiesiger, Peter B. Sher, George A. Basler, and Lauren E. Lipschutz

ABSTRACT

Using quantitative autoradiography, we investigated the entry over 90 min of [\textsuperscript{14}C]methotrexate (MTX) into \textit{C\textsubscript{6}} gliomas implanted bilaterally into Wistar rat brains. The [\textsuperscript{14}C]MTX was administered into the right carotid artery, yielding ipsilateral "arterial" brain and tumor concentrations and contralateral "systemic" concentrations. In a separate group of tumor-bearing rats, mannitol 1.6 M was given into the right carotid artery prior to administering the [\textsuperscript{14}C]MTX to disrupt the blood-brain barrier on the ipsilateral side. [\textsuperscript{14}C]MTX tissue concentrations were measured in regions of 50 x 50 x 20 \textmu m in tumor, peritumoral brain tissue (brain adjacent to tumor), and cerebral cortex. In the nonmannitol experiments, tissue concentrations from the rats at each time interval were fitted using a nonlinear curve fitting program, and the pharmacokinetic values of influx and efflux of [\textsuperscript{14}C]MTX into the three compartments were calculated. The influx rate constant \textit{K\textsubscript{i}} for [\textsuperscript{14}C]MTX ranged from 1.3 to 8.2 \mu g/min in the tumor. Influx rate constants in the cortex were 1.3-1.9 \mu g/min and in the brain adjacent to tumor were 1.7-2.8 \mu g/min. The efflux rate constant \textit{k\textsubscript{e}} was approximated for each tissue but was less reliable than the \textit{K\textsubscript{i}} values. The \textit{K\textsubscript{i}} for tumor, brain adjacent to tumor, and cortex was always higher than the corresponding \textit{K\textsubscript{i}}. Peak [\textsuperscript{14}C]MTX concentrations in the tumor were highest after arterial infusion with hyperosmolar barrier disruption, lower after arterial administration without barrier modification, and lowest after systemic administration. However, cortical [\textsuperscript{14}C]MTX concentration was also highest after arterial administration with barrier modification and higher than the highest tumor concentration. Furthermore, tissue exposure (concentration \times time) was also higher in the cortex after barrier disruption. The [\textsuperscript{14}C]MTX concentration \times time (\mu g/min/g \times 90 min \pm SEM) ratio between tumor and cortex after systemic administration was 33.4 \pm 4.1:15.7 \pm 1.9; after arterial administration it was 96.3 \pm 11.7:30.3 \pm 3.1; after arterial administration with barrier disruption it was 266.6 \pm 28.8:311.2 \pm 15.9. The greatest tumor:cortex ratio (3.1:1) occurred with arterial drug administration without barrier disruption. Disrupting the barrier enough to permit increased tumor exposure actually increased cortical exposure to a greater degree. The resulting poorer therapeutic ratio would not appear to support this technique in humans, at least for neurotoxic drugs.

INTRODUCTION

A major cause of chemotherapy failure in brain tumors is thought to be the emergence, because of inadequate drug entry, of resistant cell populations. Several laboratories have reported chemotherapy drug measurements in whole fragments of tumor in animal models of brain tumors (1-5); early studies were qualitative or, at best, semiquantitative and compared drug entry in the tumor to that in the distant brain tissue or BAT. Recent reports have described quantification of drug entry into human brain tumors using positron emission tomography (6). There have also been attempts in patients to improve drug entry by modifying the BBB with intracarotid hyperosmolar mannitol prior to the administration of methotrexate (7).

Using animal models of brain tumors, several laboratories have studied blood-brain and blood-tumor barrier physiology and blood flow with QAR (8). QAR permits one to make measurements in tissue regions of interest as small as 50 x 50 x 20 \mu m, thus allowing studies at the macrocellular level. Such studies have improved our understanding of barrier function, blood flow, and glucose metabolism and have allowed theoretical modeling of chemotherapeutic drug entry.

Our laboratory has used QAR to measure blood-brain and blood-tumor barriers and blood flow in rat brain tumors, quantifying changes in barrier function and blood flow induced by hyperosmotic barrier modification (9). We found that intracarotid mannitol at 1.37 M did not significantly increase tumor capillary permeability but did so at 1.6 M. Based on these studies, we have begun to measure the entry of radiolabeled chemotherapeutic drugs using QAR to determine how much drug enters the different tissues. We wished to address several questions. (a) How much drug enters the brain tumor, the cortex, and the BAT? (b) Can we quantify the pharmacokinetics of drug entry and exit, the C\textsubscript{T}X of drug exposure? (c) How do different classes of agents behave with respect to barrier function and blood flow within the tumor, i.e., water-soluble versus lipid-soluble drugs? (d) How is drug entry altered by modifying the method of administration? Is there a difference in drug entry after intracarotid administration or after barrier modification? To answer these questions, we have investigated the entry of several drugs and have presented preliminary reports describing entry of labeled methotrexate (10), 1-(2-chloroethyl)-3-(2,6-dioxo-l-piperidyl)nitrosourea (11), and cisplatin (12). This report will detail studies on the entry of [\textsuperscript{14}C]MTX administered via the carotid artery without and after hyperosmotic barrier modification in brain tumor-bearing rats.

MATERIALS AND METHODS

In rats harboring bilateral \textit{C\textsubscript{6}} gliomas, [\textsuperscript{14}C]MTX concentration as measured by QAR was determined in tumor, BAT (a 0.5-mm peritumoral zone), and in cerebral cortex. The drug was administered via the right carotid artery either without or 2 min after hyperosmolar mannitol (1.6 M). Measurements of [\textsuperscript{14}C]MTX in the brain ipsilateral to the drug infusion therefore represented post-"arterial" administration (without...
or with barrier modification); measurements of [14C]MTX in the brain contralateral to the infusion represented drug entry following "systemic" administration. We used a concentration of 1.6 m mannitol because in our previous study (9) this concentration increased capillary permeability in rat brain tumors while 1.37 m did not.

**Tumors**

C6 rat glial tumor was purchased from the American Type Culture Collection (Rockville, MD) and maintained at 37°C in a humidified environment of 5% CO2 with 10% fetal calf serum in McCoy's 5A medium. The cells were harvested with 0.5% trypsin and 0.2% EDTA in Hanks' balanced salt solution, centrifuged, and resuspended on 0.5% agar in McCoy's 5A medium for inoculation.

**Animal Inoculation**

Male Wistar rats (Charles River Breeding Laboratories, Wilmington, MA) weighing approximately 450 g were anesthetized with chloral hydrate (0.35 g/kg, given i.p.) and ketamine (5 mg, given i.m.). The skull was exposed by a midline scalp incision, and two shallow 26-gauge punctures were made 3 mm to either side of the sagittal suture and 1 mm posterior to the coronal suture. A 2% lidocaine gel was applied to the rat's ears and the head was affixed in a stereotaxic apparatus (Model 900; David Kopf Instruments, Inc., Tujunga, CA). A 10-μl suspension containing 5 x 10⁴ viable C6 cells was inoculated bilaterally to a depth of 7 mm through the prebored holes.

**Animal Experiments**

At about Day 20, when signs of tumor growth (weight loss, lethargy, hunching, and ataxia) appeared, the rats were anesthetized with 9% ethrane and oxygen and maintained with 1 to 2% ethrane, 70% nitrogen, and 30% oxygen. The right common external and internal carotid arteries were exposed and clamped. A vinyl catheter (V2; Bolab Inc., Lake Havasu City, AZ) was inserted into the right external carotid artery in a retrograde fashion with the cannula tip facing but not occluding the bifurcation with the internal carotid artery. This cannula was used to sample arterial blood in the right carotid circulation. A T-shaped catheter was constructed from V2 vinyl tubing glued to a V2 catheter with a 26-gauge cannula for support and installed into the right common carotid artery for the infusion of the [14C]MTX and mannitol. The right and left femoral arteries and the right femoral vein were catheterized with V2 vinyl tubing for blood pressure measurements, systemic arterial blood sampling, and saline and dextran administration. All rats were allowed to awaken from anesthesia and were studied 2 h later while awake.

Rats undergoing barrier modification received 1.6 m mannitol as an infusion (2.5 ml over 30 sec) through a 0.2-μm filter into the right carotid artery. The 1.6 m mannitol concentration was made from mannitol obtained from Sigma (St. Louis, MO). Two min elapsed between the end of the mannitol infusion and the start of the [14C]MTX administration.

The [14C]MTX was supplied by Dr. Robert Engle of the Developmental Therapeutics program of the National Cancer Institute, Bethesda, MD, as [N-methyl-14C]methotrexate (14C in the N10 position), specific activity 21.2 μCi/mmol (43.4 μCi/mg). The [14C]MTX (25–50 μCi, 0.56–1.13 mg) was dissolved in 0.04 ml of 0.1 N NaOH and 0.96 ml of 0.1% trypan blue in 0.9% NaCl solution and infused over 2 min into the right common carotid artery by pump (Harvard Apparatus Co., South Natick, MA). After the experiment, the radiochemical purity of the [14C]MTX was determined to be greater than 93% pure by thin layer chromatography.

Timed blood samples were collected during [14C]MTX infusion simultaneously from the right external carotid and femoral arteries. Samples were drawn at approximately 0.07, 0.3, 1.0, 1.3, 1.5, 2, 2.5, 2.7, 3.0, 4.0, 6.0, 8.0, 10.0, 20.0, 40.0, 60.0, and 90.0 min for the longest experiment, and for a correspondingly shorter time for the shorter experiments. A tape recorder and electric timer were used to keep track of the time. Samples collected from the femoral artery were used in the calculations of [14C]MTX after systemic administration. Samples collected from the external carotid artery from the beginning of the experiment until approximately 2 min after the termination of the drug infusion (when external carotid blood values equaled femoral blood values) were combined at that time with femoral artery values to calculate drug concentrations after arterial administration. Correcting for administered [14C]MTX dose/kg, there was no difference in the plasma [14C]MTX concentrations of the nonmannitol and mannitol rats.

Blood pressure was monitored from the left femoral artery; before intracarotid mannitol administration, mean blood pressure was 108 ± 11 (SD) mm Hg. The blood pressure rose to 161 ± 11 mm Hg during the first 30 sec of mannitol infusion, then fell over the next 30 sec, and was maintained at 114 ± 11 mm Hg for the duration of the experiment, using small quantities (~2.5 ml) of dextran (Dextran 75, 6%; Abbott Laboratories, North Chicago, IL) administered i.v. in some of the animals in the longer experiments. Body temperature was monitored with a rectal probe and maintained at a mean value of 37.4 ± 0.7°C by mounting the rat on a thermostatically controlled warming block. Other physiological measurements, which did not change significantly during the experiments, were as follows: pH, 7.41 ± 0.08; pO2, 94.5 ± 6.0 mm Hg; pCO2, 37.1 ± 4.6 mm Hg; and hemoglobin, 17.5 ± 1.6 g/dl.

Experiments were terminated at different time points (5, 10, 20, 40, 60, and 90 min) by killing the rats by decapitation. Nineteen rats killed before the mannitol died earlier than the planned experiment or were killed earlier because they became so ill; several were included in the experiments at the earlier times because these corresponded to one of the other planned intervals.

Blood samples were immediately centrifuged and 20 μl of the plasma pipetted and weighed in scintillation vials. Plasma protein was solubilized with 0.3 ml of NCS (Amersham Corp., Arlington Heights, IL), and 10 ml of OCS (Amerham) scintillation counting solution was added to each vial. Plasma radioactivity was measured with a Packard liquid scintillation spectrometer (Packard Instruments Co., Downers Grove, IL). After the animals were decapitated, the brains were dissected within 1 to 2 min and placed in Freon-12 (DuPont, Wilmington, DE) on dry ice, mounted with embedding matrix, and stored at −70°C. Frozen coronal sections, 20-μm thick, were cut on a cryostat with a Bright rotary retracting microscope (Hacker Instruments, Inc., Fairfield, NJ).

**Autoradiography**

Adjacent 20-μm brain sections were either mounted on coverslips and immediately dried at 60°C for autoradiography or mounted on glass slides and fixed for histological staining with hematoxylin and eosin. The dried tissue sections were placed on mat boards and exposed to single coated X-ray film [SB-5, MR-1, or OM-1 (Eastman Kodak Co., Rochester, NY)] in a cassette along with [14C][methyl methacrylate standards (Amersham Corp., Arlington Heights, IL) previously calibrated to reference 20-μm brain sections of known radioactivity. After an appropriate time interval, the films were developed and the radioactivity was determined by QAR.

The QAR equipment consisted of an EyeCom II image processor and Vidicom TV tube (Spatial Data Systems, Inc., Goleta, CA) and a PDP 11/73 computer. Individual coronal brain sections on the autoradiograms were digitized along with the standards. To convert the film images into units of radioactivity, the absorbance of images produced by the 14C standards was determined and a standard curve relating absorbance to tissue radioactivity was generated for each film. Based on this curve the stored absorbance data of autoradiographic images could be converted to radioactivity values (nCi/g tissue). The Vidicom TV tube was set at such a height above the film that 1 mm of length in the tissues equaled 24 pixels of length on the 512 x 536 pixel matrix of the image on the display. The minimal resolution was therefore 1 pixel = 41.67 μm.

To assure that autoradiographic readings could be verified histologically, hematoxylin- and eosin-stained histological sections were digitized and stored in the image array processor in a plane adjacent to the autoradiogram image. A program was developed that permitted the two images to be aligned. The cursor could then be applied to the histological digitized image and the processor would read the autoradiographic digitized image on the adjacent plane.
Calculations

Two methods were used to normalize the \(^{14}\text{C}\)MTX concentrations for the rats. Method A was used for the nonmannitol experiments and estimated the pharmacokinetics of entry of the drug. Method B was used for the mannitol experiments.

Method A. Using a two-compartment (blood and tissue) pharmacokinetic model detailed elsewhere (8), the entry of \(^{14}\text{C}\)MTX was calculated in the nonmannitol experiments by

\[
C_i(T) = K_t \int_0^T C_P e^{-\beta T} + V_p C_p(T) \, dt + V_p C_p(T)
\]

where \(C_i(T)\) is concentration of \(^{14}\text{C}\)MTX at time \(T\); \(K_t\) is influx transfer rate constant (ml/g/unit of time); \(C_p\) is plasma concentration integrated from Time 0 to \(T\); \(k_j\) is tissue-to-blood efflux rate constant (min\(^{-1}\)); \(k_m\) is metabolic rate constant (min\(^{-1}\)); \(V_p\) is plasma volume space; \(C_p(T)\) is plasma concentration at Time \(T\).

For methotrexate, which is a water-soluble molecule whose entry rate is restricted by blood-brain and blood-tumor barriers, the influx rate constant \(K_t\) depends on the change in the capillary permeability induced by the growing tumor. \(k_m\) is the efflux constant; in these 90-min experiments, methotrexate metabolism was assumed to be nil and \(k_m\) was assumed to be zero. \(V_p C_p\), the amount of \(^{14}\text{C}\)MTX in the blood contained within the tissues being imaged, was calculated by combining the value of \(C_p(T)\) with values of \(V_p\) as noted below. \(V_p C_p\) is a correction factor for the \(^{14}\text{C}\)MTX in the vascular space visualized in the images and represents intravascular drug available to tumor cells (the first term) plus intravascular drug. Only drug available to tumor cells is of interest in defining entry.

To quantify drug entry for all animals and over the range of time points (and given the extraordinary heterogeneity of brain tumors), we performed a frequency distribution analysis of pixels containing the different drug readings within each rat’s tumor, constructing histograms of the number of pixels versus drug concentration. The histograms were then sorted into 10 decades of increasing drug activity and mean activities were obtained for each decade. The mean activity for each decade (e.g., Decade 1, Decade 2, etc.) for each rat was fitted using the same decade in all other rats and a nonlinear curve-fitting program (NONLIN84; Statistical Consultants, Inc., Edgewood, KY) to estimate a range of 10 \(K_t\)s and \(k_m\)s, one pair for each decade. The resulting \(K_t\) and \(k_m\) values were combined in the equation with a 90-min blood curve to produce a normalized tissue distribution. This was done for both the systemic and the arterial \(^{14}\text{C}\)MTX administrations. The cortex and BAT were analyzed separately for each animal and each time point. The value of \(V_p\) used for the cortex and BAT was 0.5%. For the tumor, the values of \(V_p\) were taken from data generated by Blasberg\(^*\) in RG-2 experimental brain tumors. Blasberg found \(V_p\) values ranging from 1.05 ± 0.4% in small tumors to 1.86 ± 0.8% in central regions of large tumors. Hence, \(V_p\) varies with tumor size from "normal" brain values to progressively larger values. Therefore, for each tumor decade, we increased \(V_p\) linearly from 0.5% (Decade 1) to 2.5% (Decade 10). The 2.5% value represented 1 standard deviation above the largest \(V_p\) value reported by Blasberg.

Method B. It was not possible to normalize the mannitol experiments by method A, because mannitol temporarily alters the capillary permeability and therefore the \(K_t\) values vary over time. Instead, the blood curves for each rat were normalized to a standard blood curve using the plasma \(C\times T\) of the first 5 min, and the tissue values were adjusted accordingly. The resulting modifications of the mannitol-related tissue concentrations were thus not derived from transfer rate constants. Their tissue values were compared to those after arterial administration by normalizing the tissue concentrations of each decade over time, finally averaging the decades to obtain the total tissue \(C\times T\). For the mannitol experiments the tissue \(C\times T\) values for each decade were calculated by normalizing the observed tissue concentrations for each animal to a standard 90-min blood curve by method B, averaging the normalized tissue concentrations for all animals at each time point, linearly interpolating between time points to obtain a function of tissue concentration over time, and integrating. The \(C\times T\) for each decade was averaged to obtain the total \(C\times T\). Both methods were used in the case of the nonmannitol experiments, and the values obtained were similar.

RESULTS

Fig. 1 depicts qualitatively the computer-generated pseudo-color image of sections taken at 5 and 40 min after \(^{14}\text{C}\)MTX administration in rats bearing unilateral \(C_6\) gliomas. It is visually evident that there was more \(^{14}\text{C}\)MTX entry into the tumor on the side ipsilateral to the carotid administration than into tumor on the contralateral side. Drug concentration fell over time on both sides. On the left side of both mannitol and nonmannitol animals (i.e., that side receiving the systemically administered \(^{14}\text{C}\)MTX), there was very little entry of the drug into the cortex. Opening the BBB markedly increased the entry of \(^{14}\text{C}\)MTX into the cortex and peritumoral brain tissue on the side ipsilateral to the mannitol infusion. There was also increased entry into the arterial-sided tumor in the mannitol-administered rats as compared to the arterial-sided tumors of nonmannitol animals.

Quantitatively in the nonmannitol experiments method A was used to calculate the \(K_t\) and \(k_m\) transfer constants for each tumor decade and generate a calculated concentration at each time point. Table 1 lists the transfer constants for the cortex, BAT, and the 10 tumor decades for the brains receiving systemic \(^{14}\text{C}\)MTX and those receiving arterial \(^{14}\text{C}\)MTX. On both the systemic and arterial sides the \(K_t\) for the cortex was quite low, indicating slow or little transfer of \(^{14}\text{C}\)MTX into these regions. The \(K_t\) values for each of the decades within the tumor on the systemic and arterial side varied from little or slow entry to marked or rapid entry, presumably reflecting regions of low to high capillary permeability. There was good agreement between the \(K_t\) values on the systemic side and those on the arterial side. Because \(k_m\) is a measure of the efflux transfer constant, it is most reliably measured after there is considerable drug entry into the tissue (13). Therefore, the arterial tumor measurements were more reliable than those on the systemic side. However, the wide range of the standard deviations for the \(k_m\) values indicates that these numbers must be considered only approximations. Values of \(K_t\) and \(k_m\) for the BAT corresponded to those in the lowest 3 tumor decades.

Fig. 2A depicts the fit of the tissue concentrations of \(^{14}\text{C}\)-MTX to the normalized data for systemic drug administration for Decade 5 over 90 min. Fig. 2B depicts the fit of the tissue concentrations after arterial drug administration for Decade 5 over 90 min. Because the arterial plasma drug concentrations were higher after arterial administration than after systemic administration, the tissue concentrations were higher after arterial (Fig. 2B) than after systemic (Fig. 2A) administration.

Fig. 3 depicts in a histogram the tissue distribution of \(^{14}\text{C}\)MTX in the normalized tumors, comparing systemic with arterial administration 15 and 90 min after the drug was given. Arterial administration substantially increased the percentage of the tumor exposed to drug concentrations of \(\geq 1 \mu M \pm 0.5 \mu g/g\). On the systemic side the median drug concentration at 15 min was about 0.3 \(\mu g/g\), falling only to about 0.25 \(\mu g/g\) at 90 min. On the arterial side, the median drug concentration at

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\* R. G. Blasberg, personal communication.
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Intracarotid Methotrexate

Table 1 Transfer rate constants of entry (K₁) and efflux (k₂) for [¹⁴C]MTX into cortex, BAT, and 10 tumor decades following systemic and arterial administration of the labeled drug

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<th>Arterial (n = 12)</th>
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<td></td>
<td>K₁ ± SD (µg/g/min)</td>
<td>k₂ ± SD (min⁻¹)</td>
<td>K₁ ± SD (µg/g/min)</td>
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<tr>
<td>Cortex</td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>1.9 ± 0.6*</td>
<td>9.6 ± 9.6</td>
<td>1.3 ± 0.3</td>
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<tr>
<td>2</td>
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<tr>
<td>Tumor</td>
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</tr>
<tr>
<td>1</td>
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<td>6.9 ± 9.5</td>
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<tr>
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<td>6.2 ± 6.3</td>
<td>2.8 ± 0.4</td>
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<td>10</td>
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<td>7.3 ± 0.8</td>
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* All values are mean ± SD.

15 min was about 1.2 µg/g, falling to about 0.6 µg/g at 90 min. On the systemic side at 15 min, 12% of the tumor was exposed to 0.5 µg/g or more, while on the arterial side, 92% of the tumor was so exposed. By 90 min, 5% was exposed on the systemic side, while 60% was still exposed on the arterial side.

Fig. 4 depicts the normalized (method B) [¹⁴C]MTX concentrations over 90 min in the cortex, BAT, and 10 tumor decades following arterial drug administration with hyperosmotic mannitol barrier manipulation. During the first 15 min, the cortical and BAT concentrations were in the same range as the tumor values. The half-time of decline for tumor was approximately 52 min, the same value as that obtained using the k₂ from the arterial infusion without mannitol (Table 1, Decade 5). The half-time of decline for cortex was about 18 min, approximately twice as fast as the value obtained from the k₂ of cortex after arterial infusion without mannitol (Table 1, Cortex).

Fig. 5 compares examples of the tumor and cortex concentrations after arterial drug administration with those after arterial administration plus barrier manipulation using method B to normalize the mannitol-related data. There was a pronounced increase in tumor tissue [¹⁴C]MTX concentration at 15 min; the median concentration increased almost 4-fold compared to concentrations after arterial drug administration without mannitol. At the same time, the drug concentrations in the cortex increased even more than those in tumor. At 15 min the concentrations in the cortex after mannitol were over 8-fold greater than those without mannitol, in part exceeding those in the tumor; by 90 min the relationship was reversed. Table 2 tabulates the CxT values over 90 min (calculated from normalized data) for all three methods of [¹⁴C]MTX administration, systemic, arterial, and arterial plus mannitol, and for three tissues, tumor, BAT, and cortex. Arterial drug administration...
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Fig. 2. A, nonlinear least squares fit of tissue concentrations of [MC]MTX to the normalized data generated by using method A. The equation, and \( k_1 \) and \( k_2 \) values shown in Table 1 for Decade 5 of the tumor after systemic administration of drug, including the plasma volume in the respective tissues. ---, plasma curve; –––, calculated tumor concentration; •, imaged tissue concentrations. B, similar fit for Decade 5 after arterial administration. The correlation coefficients for these two decades were 0.51 and 0.75, respectively.

Fig. 3. Normalized histograms of the tissue distribution of ["C"]MTX in the tumors, comparing systemic (left) with arterial administration (right) 15 (upper) and 90 (lower) min after drug was given. The histograms were generated from curves using the \( K \) values in Table 1. Arterial administration substantially increased the percentage of the tumor exposed to "cytotoxic" drug concentrations. Almost tripled the tumor CXT over that following systemic administration; adding barrier opening almost tripled it again. While arterial administration almost doubled the cortical CXT, the major change in the cortex occurred after mannitol, increasing the CXT in the cortex more than 10-fold over that following arterial drug administration. The tumor:cortex ratio with systemic administration of 2.1 was increased to 3.1 by arterial administration and reduced to 0.9 by opening the BBB.

DISCUSSION

The goal of these studies was to define in detail the pharmacokinetics of entry of a chemotherapeutic agent into an experimental brain tumor. While the entry of a number of such drugs has been investigated in similar models, few studies reported detailed quantification, and even then drug entry was measured in small particles of tumor representing tens of thousands of cells. In contrast, QAR permits quantification of drug entry in regions composed of only tens of cells, thus allowing one to approach in vivo what was only previously possible in vitro. By this method we could determine the amount of tumor exposed...
to a given concentration of drug. Simply determining a mean and variance would have assumed a normal bell-shaped distribution. As is evident in Figs. 3 and 5, the distribution is not necessarily bell shaped. Minimal chemotherapeutic drug exposure is required to kill tumor cells and QAR permits a measurement of the proportion of cells underexposed.

We chose to study methotrexate despite its general ineffectiveness as chemotherapeutic for malignant glioma because (a) there is little metabolism of [14C]MTX during the 90-min time course of the experiment; (b) methotrexate is a water-soluble drug and its transfer across the blood-brain and blood-tumor barriers is limited; (c) we could model two different routes of drug administration, systemic (i.v.) and arterial; (d) we could measure drug entry into brain tumor and brain after purposely opening the BBB.

Before commenting on the results, it is necessary to deal briefly with several technical aspects of the studies. The extraordinary heterogeneity of drug entry into the brain tumors evident in the display on the monitor of the computerized imaging system (Fig. 1) required that we devise a system of analysis that took advantage of the imaging power of QAR and did not simply average the values over the regions of interest. Furthermore, the diversity was amplified by the variations among the animals. The best method of analyzing drug entry is to determine the permeability-surface area product for the drug. Since the capillary surface area in brain tumors is unknown, the transfer rate constant of entry, $K_1$, was used as a measure of the rate of drug entry. In our system, we had already determined that there was a relationship between the size of the tumor and the value of $K_1$ for [14C]AIB, a marker for capillary permeability (9). Now we wished to determine $K_1$ for [14C]MTX in many areas of diverse capillary permeability. The task was further complicated by the fact that the drug we were using also moved back into the bloodstream, thus making it necessary to determine an efflux constant $k_2$ if we were to model the results over time and over many animals. Using method A, the calculation performed on the histograms of the concentrations of drug allowed us to sort all of the data into 10 tissue decades and fit the drug-entry equation for each decade. The tissue data could then be normalized to a single blood curve covering the time of the experiment and all of the animals compared as if the experiment had been done with a positron emission tomography scan. As is evident in the fitting of the transfer constants, there was considerable heterogeneity among the rats. Nevertheless, the fit of the raw data to the calculated results depicted in Fig. 2 was reasonable enough to normalize the drug entry in the nonmannitol experiments. Histograms of drug distribution could then be constructed to compare systemic with arterial administration, as exemplified in Fig. 3. A comparison to an "average" of $K$ values is instructive. For the entire tumor on the systemic side, the $K_1$ was $4.3 \pm 0.9 \mu l/g/min$, and $k_2$ was $7.6 \pm 5.7 \mu l/g/min$; the correlation coefficient was 0.54. For the arterial side, the $K_1$ was $3.8 \pm 0.5 \mu l/g/min$ and the $k_2$ was $14.4 \pm 4.8 \mu l/g/min$; the correlation coefficient was 0.83. (The intratumoral $V_P$ was assumed to be $15\%$.) The average $K$ values for the tumors fell near the midpoint of the $K$ values derived using method A. As noted, the proportion of cells exposed to drug concentrations was readily determined using this method and likely to yield a better estimate than that obtained by using only the average values.

Method B normalized the data for the mannitol experiments by adjusting the blood curve of each rat to a standard curve using the first 5 min, a method that was cruder than using the transfer rate constants but made necessary by the complex behavior of capillary permeability under the influence of BBB modification. Here, linearity was assumed between the blood curves and the tissues concentrations, an unlikely event. However, this method was used for both the arterial and the arterial-plus-mannitol experiments in order to compare them. The fact that the shape of the tissue concentration histogram for arterial nonmannitol delivered [14C]MTX derived from method A (Fig. 3) was similar to that derived from method B (Fig. 5) appears to justify this approach, permitting comparison of drug distribution after arterial administration with that after mannitol.

The second technical issue is the choice of the model represented in the equation. This two-compartment model makes no attempt to define intracellular drug entry. While such transport is best defined by in vitro experiments, Dedrick and colleagues have detailed in vivo experiments in animals for several tissues (but not brain) (14). The results suggest a saturable system consistent with folate-related active transport and irreversible binding to dihydrofolate reductase. While such an analysis might be possible in our system, it would require multiple doses of drug and was beyond our immediate interest. We wished to study transcapillary drug exchange in an experimental brain tumor and this was readily accomplished using the two-compartment model. We assumed that the [14C]MTX was reversibly bound to plasma proteins and was only bound about 15% (14).

Our results bear directly on the influence of the blood-tumor barrier on drug entry and therefore on the theoretical effectiveness of a drug in brain tumor chemotherapy. [14C]MTX entry within the experimental brain tumor was highly heterogeneous, ranging from little entry in regions of low $K_1$ to modest entry in regions of high $K_1$. Drug entry into the distant cortex was nil, and entry into the BAT was also low and may have represented diffusion from the tumor. Blasberg et al. (reviewed in Ref. 8) has modeled drug entry in experimental brain tumors using QAR and values of capillary permeability measured by [14C]AIB entry and values of blood flow measured by [14C]-iodoantipyrine entry. These models were based on average values for $K_1$ and flow and could not estimate how much of the tumor would fail to be exposed to any given drug concentration. Our experiments using [14C]MTX directly allowed us to detail regional drug entry.

The results of the measurements after systemic drug administration indicate that a large portion of the tumor would be underexposed to this drug (Fig. 3). As pointed out by Shapiro (15), when brain tumor cells are exposed to concentrations of chemotherapeutic agents that are too low to be cytotoxic, the
tumor cells readily become resistant to the agent. Assuming MTX could kill brain tumor cells, its water solubility makes its entry after systemic administration too poor to be effective. In contrast, the intraarterial route of administration clearly increased the drug exposure for a large percentage of the tumor, the C×T for 90 min being substantially higher than that following systemic administration (Table 2). Collins (16) has suggested that the advantage of intraarterial drug administration over systemic administration relates to transfer of the drug during the first pass in the artery supplying the tumor. The most advantageous circumstance occurs when the drug is rapidly metabolized (or cleared from the body) or when blood flow through the tumor is substantially slower than through normal tissues. For methotrexate, there was a clear advantage of the arterial route over the systemic route during the first 90 min, although it would be anticipated that the advantage would be less over a longer time interval. These considerations favor the intraarterial route for a drug like 1-3-bis(chloroethyl)-1-nitrosourea, which is rapidly metabolized. Nevertheless, the maximum early methotrexate exposure for much of the tumor was substantially better after arterial than after systemic administration. The cortical exposure also increased, but less so than in the tumor.

Opening the BBB with 1.6 M intracarotid mannitol further increased [14C]MTX entry, and indeed the highest tumor exposure to the drug followed arterial drug administration plus barrier manipulation (Fig. 5). However, cortical drug exposure was also increased >1000% over that following arterial administration (Table 2). The highest drug concentration in the tumor with the lowest brain concentration occurred when the drug was given via the artery. Intracarotid mannitol actually reversed the ratio.

This result with barrier manipulation was predicted by our previous studies with mannitol in which we measured capillary permeability in two tumor models using [14C]AIB (9). As noted above, before mannitol administration, K values for [14C]AIB in both Walker 256 carcinosarcoma and C6 glioma were much higher than those in cortex. Mannitol at a concentration of 1.37 M did not increase the K values for either tumor. At 1.6 M, mannitol increased the K values for both tumors (1.7-fold in C6 glioma and 13-fold in Walker 256). At the other concentrations, mannitol markedly increased cortical K values in all rats by 48- to 72-fold at 1.37 M and by 90- to 105-fold at 1.6 M. Warnke et al. (17) reported similar findings in ethyl-2-nitrosourea-induced rat brain tumor, as did Nakagawa et al. (18) in RG-2 tumors. Inoue et al. (19) have examined the entry of 5-fluorouracil into RG-C6 gliomas in rats after barrier modification with 1.4 M mannitol; no significant increased tumor concentration was detected. Neuwelt et al. (20) reported increased methotrexate entry into avian sarcoma virus-induced rat gliomas after osmotic manipulation. In that study, the exact mannitol concentration was not given, but it is apparent that the capillary permeability (and therefore drug entry) varies both with the tumor type and the concentration of mannitol used to open the barrier.

Ohata et al. (21) determined [3H]methotrexate loss from rat brain after osmotic opening of the BBB. The half-time of decline in drug concentration prior to barrier manipulation was 20 min; after BBB opening it was 4.8 h. In our study, the half-time of decline in the tumor after arterial administration without barrier manipulation was approximately 50 min and was not changed by mannitol. Corresponding cortical values were 32 and 18 min. As noted, the nonmannitol figures were calculated from the k2 values in Table 1. Both the tumor and cortical values have a wide variance, however, because of the rather large standard deviations of about 50%. The measure of half-time after mannitol may be more reliable because there is more drug in the tissue. It is clear that the tumor half-time changed little and that drug washed out of the cortex faster than out of the tumor. This was especially true in the cortex where, once the capillaries were opened by the mannitol, the efflux was substantially faster than in tumor. The most likely explanation for the more rapid decline in the cortex is the higher blood flow in cortex than in tumor. This indicates that the pharmacological rule of "easy in, easy out" applies as well to brain tumor chemotherapy. After barrier opening, the high cortical concentration seen at 15 min declined rapidly, so that at 90 min it was less than in tumor. However, the C×T for the cortex still exceeded that for the tumor (Table 2).

Clinically, Neuwelt et al. have treated patients harboring brain tumors with methotrexate after hyperosmotic barrier manipulation using 1.37 M mannitol; they have not reported serious neurotoxicity with this regimen (7). Previous reports of brain toxicity from methotrexate (reviewed in Ref. 22) suggest that if patients live long enough, brain damage is likely if the drug is given by this means. The efficacy of hyperosmolar mannitol in a given patient would depend on the capillary permeability characteristics of the patient's tumor and on the concentration of the mannitol used. Higher mannitol concentrations are likely to be needed for some tumors (or parts of all tumors), but as seen in our experiments, more mannitol plus methotrexate proved to be lethal to 60% of the rats. Clearly, the choice to use mannitol in human brain tumor chemotherapy must take into account the potential neurotoxicity of the agent. The recent work of Neuwelt et al. with monoclonal antibodies in which blood-brain manipulation was necessary to get the large protein fragments into the tumor (23) may be a more appropriate use for this technique than is chemotherapy.

Finally, entry of a water-soluble drug like methotrexate clearly depends on breakdown of the blood-tumor barrier. Its entry into peritumoral tissue depends on both breakdown of the blood-tumor barrier and diffusion from the tumor itself. This may be of great importance in improving chemotherapy because tumor cells are likely to migrate (and grow) into the brain adjacent to tumor. QAR can be used to measure such diffusion and these studies are underway.

REFERENCES


Pharmacokinetics of Tumor Cell Exposure to $^{14}$C-Methotrexate after Intracarotid Administration without and with Hyperosmotic Opening of the Blood-Brain and Blood-Tumor Barriers in Rat Brain Tumors: A Quantitative Autoradiographic Study
