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Influence of schedule of administration on methotrexate penetration in brain tumours

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Abstract

The influence of the administration schedule (intravenous (i.v.) bolus versus i.v. infusion) on the pharmacokinetics of methotrexate (MTX) in plasma and extracellular fluid (ECF) of a brain C6-glioma was investigated in rats. MTX concentrations were determined by high performance liquid chromatography (HPLC)-ultraviolet radiation (UV). MTX (50 mg/kg) was administered by i.v. bolus or i.v. infusion (4 h). Concentration–time profiles were fitted to a two-compartment open model. Maximum MTX concentrations ranged between 178 and 294 μg/ml (i.v. bolus), and between 11 and 24 μg/ml (i.v. infusion) in plasma. MTX rapidly entered the tumour tissue although its concentrations in the ECF were much lower than those observed in plasma for both modes of administration. In spite of an important interindividual variability, AUC_{ECF} was approximately 5-fold higher and mean MTX penetration in tumour ECF (AUC_{ECF}/AUC_{Plasma}) was approximately 3-fold higher after i.v. bolus than after i.v. infusion administration. These results indicate that i.v. bolus administration schedules promote MTX delivery in brain tumour tissue.

Keywords: Tissue distribution; Microdialysis; Pharmacokinetics; Glioma; Methotrexate

1. Introduction

Chemotherapy of brain tumours is still far from ideal, resulting in therapeutic failure with some of the most common anticancer agents [1]. As such, delivery through the blood–brain and the blood–tumour barriers is a critical determinant of drug efficacy and often restricts the choice of an effective drug to a few specific therapeutic agents. Over the past 20 years, much attention has been devoted to the study of lipophilic drugs possessing a very high therapeutic index, and which could be given by prolonged or intermittent infusion (e.g. nitrosoureas, procarbazine) [2]. These drugs have the advantage of a greater penetrability into tumour cells and preferential partitioning in lipid membranes and cellular structures. Their penetration in brain tumour tissue was thus thought to be unrestricted by the blood–brain tumour barrier. Yet, delivery of lipophilic drugs to brain tissue is often limited due to oedema, changes in cerebral blood flow and intracranial hypertension due to the presence of the tumour [3]. Moreover, clinical use of these drugs has only permitted modest gains in terms of therapeutic efficacy. These combined factors resulted in a renewed interest for hydrophilic drugs in the treatment of brain tumours [4]. In order to make the best use of available hydrophilic drugs, there is, however, a need to establish optimum doses, schedules and routes of administration that could improve delivery to brain tumour tissue. Therefore, the objective of this study was to characterise and compare the penetration of methotrexate (MTX) (used in this study as an hydrophilic model compound) into brain tumours after i.v. bolus or i.v. infusion administration in an experimental C6 glioma in rats by intracerebral microdialysis. This approach offers an unique opportunity to monitor the extracellular environment of the intact brain, and can lead to a better understanding of the time course of drug disposition in tumour tissue. This, in turn, may

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help in the design of highly effective drug dosing regimens that could improve efficacy and/or reduce toxicity.

2. Materials and methods

2.1. Drugs and chemicals

MTX was obtained from Lederlé (Oullins, France). Purified water was obtained from an Alpha-Q purification system (Millipore, Saint-Quentin en Yvelines, France). Cell culture products were obtained from Biomedia (Boussens, France), and all other chemicals were reagent or analytical grade.

2.2. Animals

Male Wistar rats weighing 260–300 g (Elevage Dépré, Saint Doulchard, France) were individually housed in a controlled environment (20±2°C; 65±15% relative humidity) and maintained under a 12:12 h light:dark cycle. They were allowed to adapt to the housing environment for at least one week prior to study and had access to food (U.A.R., Villemoisson sur Orge, France) and tap water *ad libitum*. All animal procedures adhered to the 'Principles of laboratory animal care' (NIH publication no. 85-23, revised 1985).

2.3. C6 cell line

The C6 glioma cell line (ECACC European Collection of Cell Cultures, Salisbury, UK) was initially produced by means of weekly injections of N-methylnitrosourea [5]. The cells were maintained in Ham F12 minimum essential medium containing 10% fetal calf serum (FCS), 0.5% streptomycin, 0.5% penicillin, 0.05% amphotericin B and 0.5% L-glutamine. The cells were grown to confluence in a humidified atmosphere of 5% CO_2 at 37°C. Exponential growth cultures were harvested with a solution of 0.05% trypsin and 0.02% ethylene diamine tetra-acetic acid (EDTA) and resuspended in Ham F12 medium. C6 cells were washed three times in Ham F12 medium supplemented with 10% FCS, and the suspension of viable cells was counted by a trypan blue dye exclusion method. Finally, the cells were suspended in Ham F12 minimum essential medium to a final concentration of 5×10^6 cells per 10 μl for intracerebral inoculation.

2.4. Intracerebral inoculation

Rats were anaesthetised by intraperitoneal (i.p) injection of 10 mg/kg of xylazine (Bayer, Leverkusen, Germany) and 100 mg/kg of ketamine (Parke-Davis, Courbevoie, France). The head was mounted into a stereotaxic head holder in a flat-skull position. The scalp was cleaned with 70% ethanol, and the skull exposed by a midline scalp incision. Tissue covering the skull was gently everted and the bregma identified. A small burr hole was drilled into the right side of the skull at a position 3 mm lateral from the midline and 1 mm anterior to the bregma. Four additional small holes were drilled for bone anchor screws several millimetres away from the burr hole. After inserting the bone anchor screws, a CMA/11 guide cannula (Phymep, Paris, France) was slowly lowered at a depth of 4 mm as determined from the brain surface. These stereotaxic coordinates, which correspond to the caudate nucleus, were chosen in order to minimise extra-cerebral extension of the tumour [6]. The skull was blotted dry and dental cement (Autenal Dental, Harrow, UK) applied to anchor the guide cannula. Ten minutes later, 10 μl of the tumour cell suspension was slowly injected over 2 min by means of a 25 μl Exmire microsyringe (Poly Labo, Strasbourg, France) equipped with a needle that extended 1 mm beyond the tip of the guide cannula. After a 3 min wait, the needle was removed and the guide cannula capped with a dummy stylet that extended 0.5 mm beyond its tip.

2.5. Experimental procedure

Fourteen days after tumour implantation, rats were anaesthetised with isoflurane (induction: 5% and maintenance: 1–1.5%) by means of an Isotec 4 evaporator (Ohmeda, Maurepas, France) and ventilated with a small animal respirator (Harvard Biosciences, Les Ulis, France). The animals were then placed onto a heating pad set at 38±0.5°C (Homeothermic Blanket System, Phymep, Paris, France). The end-tidal CO_2 was monitored on a CO_2 analyser (Engström Eliza, Cambro, Sweden) and respirator settings were constantly adjusted to maintain arterial blood gas values within the normal range. A polyethylene catheter (No. 3, Biotrol, Paris, France) filled with heparin-saline solution (25 000 UI/l, Héparine Choay, Paris, France) was inserted into the right carotid artery and used for blood sampling. A second catheter (No. 1, Biotrol, Paris, France) was inserted into the left jugular vein for drug administration.

Microdialysis was carried out with a CMA/100 microinjection pump (Phymep, Paris, France) and CMA/11 microdialysis probes (membrane length: 2 mm, tip diameter: 240 μm, cutoff: 6000 Daltons ; Phymep, Paris, France). Before the start of the experiments, microdialysis probes were flushed with Krebs–Ringer's solution at 15 μl/min to purge membranes and tubing of air bubbles. The perfusate flow rate was reduced to 7 μl/min 30 min before drug administration, and microdialysis was performed at this flow rate over the duration of the experiment.

According to a parallel design, animals were given 50 mg/kg of MTX either as a fast i.v. injection (flow rate of

1 ml/min over ~2 min) or a slow i.v. infusion (flow rate of 16.7 μl/min over 4 h) by using a CMA/100 microinjection pump. After correction for dead volume, dialysates were continuously collected every 15 min for 4 or 6 h on a CMA/140 microfraction collector (Phymep, Paris, France). Arterial blood samples (200 μl) were drawn at the midpoint of dialysis samples collection periods (i.e. 2.5, 7.5, 15, 22.5, 37.5, 67.5, 127.5, 172.5 and 232.5 min after i.v. bolus and 37.5, 82.5, 127.5, 172.5, 232.5, 247.5, 262.5, 277.5, 292.5, 322.5 and 352.5 min after i.v. infusion) and replaced with an equal amount of heparinised saline. Blood was centrifuged immediately after collection, and plasma and dialysate samples were stored at −20°C until analysis.

2.6. Probe calibration

The efficiency of the microdialysis probes was investigated by determining the *in vitro* recovery. Experiments were performed at 38°C in a constant temperature dry bath. The probes were placed in plastic vials containing an unstirred Krebs–Ringer's solution (122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 25 mM $NaHCO_3$, 0.4 mM KH_2PO_4) spiked with MTX (50 μg/ml), and perfused with the same Krebs–Ringer's solution at a flow rate of 7 μl/min for at least 30 min before the start of the experiment. Dialysate samples ($n=8$) were collected serially over 15-min intervals and the mean *in vitro* recovery (7.0±0.6%) computed from all the ratios of MTX concentrations in effluent dialysate to the probed reservoir according to the equation below:

$$\text{Recovery}_{in\ vitro} = \frac{C_{\text{dialysate}}}{C_{\text{sample}}} \times 100$$

In vivo recovery was estimated by reverse dialysis in a dedicated group of 5 tumour-bearing rats [7]. The probes were inserted into the guide cannula and perfused with Krebs–Ringer solution containing MTX (230 ng/ml) at a flow rate of 7 μl/min. After a 30-min waiting period necessary to attain a stable equilibrium, dialysates were collected every 15 min for 2 h ($n=8$). The mean *in vivo* recovery was then computed from all the ratios of the concentration lost to the initial concentration in the perfusate according to the following equation:

$$\text{Recovery}_{in\ vitro} = \frac{C_{\text{in}} - C_{\text{out}}}{C_{\text{in}}} \times 100$$

where C_{in} and C_{out} are the MTX concentrations in the perfusate inflow and outflow, respectively. Finally, MTX concentrations in dialysates were corrected by the mean *in vivo* recovery (8.0±3.8%) to yield estimations of extracellular fluid (ECF) concentrations.

2.7. Histological studies

Histological studies were performed in order to verify the placement of the microdialysis probe within the tumour tissue, and to relate MTX penetration with the sampling site environment. At the end of each experiment, animals were deeply anaesthetised. All blood from the circulation was removed by perfusing the heart with saline (120 ml) and severing the inferior vena cava. Subsequently, 120 ml of 10% neutral buffered formalin was injected to perform *in situ* fixation. The brains were removed from the cranial cavity and stored in 4% buffered formaldehyde solution at 4°C. After routine processing including dehydration in a graded alcohol series and paraffin embedding, the blocks were serially cut. Every tenth 5 μm section was routinely stained with haematoxylin-eosin saffron (HPS) and used for microscopic examination. The whole path of the semipermeable part of the microdialysis membrane and the tumour implantation site could thus be screened for morphological changes. All histological sections were semi-quantitatively examined for the severity and extent of oedema, necrosis and inflammation according to an empirical 3-point grading system (absent, moderate or severe). In an attempt to relate MTX penetration to tumour vascularisation, blood vessel density in and around the tumour tissue (excluding haemorrhagic and necrotic zones) was estimated by counting the number of vessels within five zones under ×40 and ×400 magnification. Results are expressed as the average number of blood vessels within the five zones.

2.8. Analytical procedure

MTX concentrations were quantified by an isocratic high performance liquid chromatography (HPLC) assay described in detail elsewhere [8]. Briefly, to 150 μl of plasma was added an equivalent volume of 0.8 M trichloroacetic acid. The mixture was then placed on a vortex mixer and centrifuged at 1000*g* for 10 min. The supernatant was injected in a chromatographic system where separation was achieved on an Hypersil ODS column C_{18} (150×2 mm; Interchim, Montluçon, France) using a mobile phase consisting of methanol and 40 mM dibasic potassium phosphate pH 7.0 buffer (20:80 (v:v); flow rate: 0.3 ml/min). The eluent was monitored on an Applied Biosystems 785A ultraviolet (UV) spectrophotometer (Eurosep, Cergy-Pontoise, France) at 307 nm. Calibration curves in plasma, prepared in the range of 0.125–125 μg/ml by spiking rat plasma, were linear over the range of concentrations studied ($y=78283x+15111$; $r=0.997$; $n=5$). Assay precision between days was characterised by a coefficient of variation ranging from 13.8 to 3.7% over an MTX concentration range of 0.125 and 125 μg/ml. No plasma sample was found with a concentration below

0.125 μg/ml. Dialysate samples were directly injected into the HPLC system. Calibration curves were prepared in the range of 3.1 to 125.6 ng/ml by spiking Krebs–Ringer solution and were linear over the range of concentrations studied ($y = 136x - 163$; $r = 0.999$; $n = 5$). The interday coefficient of variation ranged between 13.3 and 2.4% and no dialysate was found with a concentration below 3.1 ng/ml.

2.9. Pharmacokinetic and statistical analysis

Pharmacokinetic parameters of MTX were determined for each animal by fitting simultaneously plasma and brain tumour ECF concentration–time curves to a two-compartment open model by nonlinear least-squares regression (Ph/Edsim Professional, version 2.05, Mediware, Groningen, The Netherlands). The choice of the model was based on the Akaike information criterion. The area under the concentration–time curve from time zero to infinity (AUC), mean residence time (MRT), half-life of distribution and elimination ($t_{1/2}\alpha$ and $t_{1/2}\beta$, systemic clearance (CL) and volume of distribution (Vd_{SS}) were calculated from estimated rate-transfer microconstants according to standard pharmacokinetic equations [9]. The extent of drug transport into the tumour ECF (MTX penetration) was expressed as the ratio of AUC_{ECF} over AUC_{Plasma} established from time 0 to infinity. All results are expressed as mean±S.D. Statistical comparisons of pharmacokinetic parameters were carried out on a microcomputer with Prism for Windows 95/NT (version 2.01, GraphPad Software, San Diego, USA). Non-parametric Mann-Whitney *U* tests (Wilcoxon rank sum test) with the a priori level of significance set at $P < 0.05$ were used because the number of animals was small, and there was no certainty that the data were normally distributed.

3. Results

Following tumour cell implantation and recovery from anaesthesia, all animals rapidly resumed normal activity without showing ill effects. During the period of development of the tumour, no loss of appetite, gross behavioural disorders or neurological signs were observed.

3.1. Histological findings

Fourteen days after inoculation, C6 cells formed a tumour mass centred on the injection site in 92% of cases. There was no tumour spread to the contralateral hemisphere. The tumour shape was usually ovoid, with a higher vertical (6.7±1.7 mm) than horizontal diameter (3.7±1.2 mm). Microscopic analyses of brain sections adjacent to the microdialysis probe showed a narrow elongated space produced by the passage of the probe projecting into the central part of the tumour (Fig. 1). They also revealed the presence of moderate or severe oedema (2 and 3 cases, respectively), necrosis (1 and 4 cases, respectively) and haemorrhage (3 and 2 cases, respectively) in most animals. The average number of blood vessels in the tumour tissue was 2.4±1.0 (range: 1.0–4.8) versus 3.9±1.2 (range: 1.2–5.4) around the tumour tissue. No significant difference could be found between the two treatment groups (i.v. bolus and i.v. infusion) and no relationship could be established between histological findings and pharmacokinetic parameters.

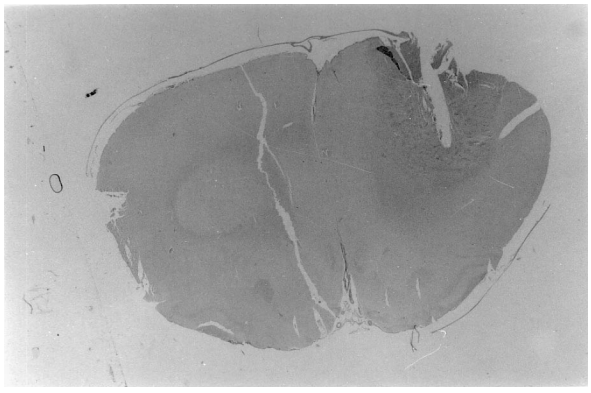

Fig. 1. Photomicrograph of a brain C6 glioma showing the narrow elongated space produced by the passage of the microdialysis probe projecting into the central part of the tumour.

3.2. Methotrexate pharmacokinetics

Mean concentration–time profiles of MTX in plasma and tumour ECF following i.v. bolus or i.v. infusion of 50 mg/kg are shown in Fig. 2. Pharmacokinetic parameters and results of statistical analyses are reported in Table 1.

At the end of drug administration, MTX concentrations in plasma ranged between 178 and 294 μg/ml after

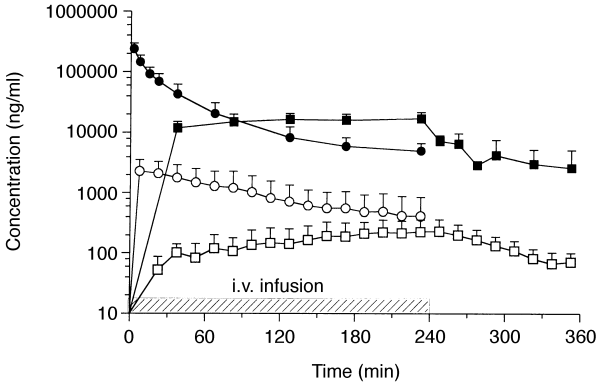

Fig. 2. Average methotrexate concentrations in plasma (●: i.v. bolus; ■: i.v. infusion) and in C6-glioma extracellular fluid (○: i.v. bolus; □: i.v. infusion) as a function of time after the administration of 50 mg/kg in rats (mean±S.D.).

Table 1
Pharmacokinetic parameters of MTX in brain tumour-bearing rats ($n = 6$) after i.v. bolus or i.v. infusion administration (50 mg/kg)[a]

Parameter	i.v. Bolus	i.v. Infusion
k_{10} (min)	0.0458±0.0132 (0.0320–0.0630)	0.0595±0.0529 (0.0110–0.1570)
k_{12} (min)	0.0400±0.0211 (0.0090–0.0600)	0.0288±0.0380 (0.0020–0.1010)
k_{21} (min)	0.0233±0.0111 (0.0110–0.0350)	0.0342±0.0450 (0.0070–0.1250)
$t_{1/2}\alpha$ (min)	8.3±3.9 (5.0–14.2)	10.2±6.7 (2.6–17.1)
$t_{1/2}\beta$ (min)	73.9±36.8 (42.8–141.1)	68.9±20.7 (49.3–105.3)
MRT (min)	47±7 (36–56)	164±12* (156–187)
V_C (ml)	69.1±10.7 (53.7–83.4)	128.8±102.6 (36.5–318.2)
Vd_{SS} (ml)	194.4±85.1 (133.5–362.4)	196.6±84.9 (115.5–361.4)
CL (ml/min)	3.157±0.975 (1.973–4.435)	4.374±1.075 (3.321–5.727)
AUC_{Plasma} (μg/min/ml)	6568.1±2213.3 (4058.3–9477.1)	4112.5±1104.7 (2915.9–5721.2)
AUC_{ECF} (μg/min/ml)	284.4±208.9 (67.4–642.4)	55.9±27.9* (20.5–89.0)
AUC_{ECF}/AUC_{Plasma} (%)	4.15±2.20 (1.01–6.78)	1.39±0.79* (0.59–2.82)

*$P < 0.05$, comparison i.v. bolus versus i.v. infusion. MTX, methotrexate; MRT, mean residence time; Vd_{SS}, volume of distribution; $t_{1/2}\alpha$ and $t_{1/2}\beta$, half-life of distribution and elimination; CL, systemic clearance; AUC, area under concentration–time curve from time 0 to infinity; AUC_{ECF}/AUC_{Plasma} (%), mean MTX penetration into the tumour; AUC_{ECF}, area under the tumour ECF concentration–time curve; AUC_{Plasma}, area under the plasma concentration–time curve. k_{10}, elimination rate constant from the central compartment; k_{12}, transfer rate constant from the central to the peripheral compartment, k_{21}, transfer rate constant from the peripheral to the central compartment; V_C, volume of the central compartment.

[a] Values are presented as mean±S.D. (range).

i.v. bolus and between 11 and 24 μg/ml after i.v. infusion. They were characterised by a polyexponential decay that could be fitted to a two-compartment open model. As expected, all pharmacokinetic parameters derived from plasma concentration–time curves were not significantly different, except for the MRT which was much higher after infusion than after bolus administration. This result is easily explained by the fact that MRT after intravenous infusion takes into account the duration of the infusion.

In the tumour ECF, individual concentration–time profiles (estimated by taking into account *in vivo* recovery) were characterised by a very fast entry of MTX into the tumour tissue. After i.v. bolus administration, MTX concentrations reached a maximum within 15 min (2226±1274 ng/ml), then declined at a rate similar to that determined in the plasma. In contrast, MTX concentrations in the tumour ECF after i.v. infusion increased steadily during the duration of the infusion to reach a maximum of 234±129 ng/ml 4 h after the start of the infusion. Concentrations of MTX into the tumour ECF were much lower than those in the plasma for both modes of administration. In spite of an important inter-individual variability, mean MTX penetration into the tumour ECF (expressed as the AUC_{ECF}/AUC_{Plasma} ratio) was approximately 3-fold higher after i.v. bolus than after i.v. infusion administration (4.15±2.20 compared with 1.39±0.79). This increase in MTX penetration was mainly related to an approximately 5-fold increase of the area under the tumour ECF concentration–time curve (AUC_{ECF}) after i.v. bolus (284.4±208.9 compared with 55.9±27.9), whereas the area under the plasma concentration–time curve (AUC_{Plasma}) values were similar (6568.1±2213.3 compared with 4112.5±1104.7).

4. Discussion

The objective of this study was to characterise and compare the penetration of MTX (used in this study as an hydrophilic model compound) in brain tumours after administration of the drug by i.v. bolus or i.v. infusion in an experimental tumour model in rats by intracerebral microdialysis.

The choice of a C6 glioma was based on the fact that experimental brain tumours induced by transplantation of tumour cells, by using stereotaxic coordinates and a given number of cells, allow reproducible tumour localisation and growth. Moreover, the C6 brain tumour model fulfils the production rate, growth, predictable histology gradient, and reproducibility criteria of an *in vivo* experimental brain tumour and has been used in numerous studies [10,11]. The procedure for implantation of tumour cells derived from that described by Morreale and colleagues [12]. It offered an unique opportunity to perform both the inoculation of tumour cells and microdialysis in the central part of the tumour, whatever the exact size of the tumour mass. Moreover, injection of tumour cells in the caudate nucleus greatly reduces the risk of extra-cerebral extension [6]. In our study, all tumours originated in the right caudate nucleus, were well localised and no secondary masses were produced.

In order to minimise tissue damage, ECF samples were collected by using commercially available microdialysis probes with the smallest diameter. A high perfusion flow rate coupled with long sampling times was used to increase the absolute recovery (i.e. the total amount of drug that is collected within a given time interval). Although the relative recovery is reduced with a high flow rate, this approach allowed the collection of

a sufficient volume of dialysate as required by the analytical method, yet enabled the sampling interval to be compatible with the establishment of the ECF concentration–time profile of MTX.

Although sampling of tumour ECF was always performed at a precise and reproducible location within the tumour mass, *in vivo* recovery (determined in a separate group of animals) was highly variable between animals, suggesting anatomical differences in the tumour mass. As such, the heterogeneous nature of the cellular organisation of solid tumours may well have contributed to the important inter-individual variability since blood flow within the centre of a tumour is frequently lower than in the periphery due to the presence of oedema and necroses. An alternative approach would have required the determination of individual *in vivo* recovery before performing the pharmacokinetic experiment. However, preliminary MTX administration would have affected tumour growth and intratumoral disposition, and thus modified the subsequent pharmacokinetic profile of MTX. Moreover, this approach would have required a 6-h washout period between the determination of probe recovery and the pharmacokinetic experiment, a delay not compatible with our anaesthetic protocol.

Histological studies were performed after pharmacokinetic experiments to examine individual tumour development and to verify the exact placement of the probe within the tumour. Brain tissue reactions to the implantation of the microdialysis probe often revealed the presence of local haemorrhage along the path of the microdialysis probe. However, no relationship between the presence and/or the severity of the haemorrhage and MTX tumour penetration could be found. Likewise, MTX tumour penetration was not significantly correlated with other histological parameters.

Although used primarily for the treatment of primary central nervous system lymphomas [1], MTX has also been proposed for the treatment of primary and metastatic brain tumours [13]. In this study, MTX was chosen as a model compound for hydrophilic anticancer drugs despite its general ineffectiveness for the treatment of malignant glioma. Doses of MTX were selected arbitrarily over a range corresponding to those used in clinical practice. In rats, maximum plasma concentrations obtained after i.v. bolus administration were in the range of those observed in patients receiving high-dose MTX therapy in the treatment of solid tumours [14]. Their decay was best described by a two-compartment open model, in agreement with the findings of Bremnes and colleagues and Devineni and associates who reported biphasic elimination profiles in plasma after i.v. injection of doses ranging between 10 and 1000 mg/kg in rats [8,15]. Apparent volumes of distribution and total body clearances established in this study were very close to those previously reported in healthy rats [8]. These results suggest that the presence of a brain C6-glioma did not affect the disposition of MTX at the time of the experiment. The lack of abnormal clinical signs over the period of development of the tumour also seems to confirm this hypothesis. In agreement with the pharmacokinetic theory, AUC_{Plasma} from 0 to infinity after i.v. bolus was not significantly different from that obtained after i.v. infusion. The equilibration between MTX levels in brain tumour ECF and plasma was rapid. This result was not surprising since numerous microdialysis experiments have shown that the equilibration of drug concentrations between brain and blood is very fast, even for hydrophilic compounds such as MTX [8,16,17]. Concentrations of MTX in tumour ECF were much lower than those in plasma. Although the exact mechanism underlying this observation remains unknown, contributing factors may include protein binding, bulk flow (ECF drainage), and the presence of specific transport processes into and/or out of the brain tumour [18]. These results may also be explained by the fact that MTX is a water-soluble drug that is highly ionised at physiological pH (99.8%) which does not penetrate the blood–brain barrier to any great extent under normal conditions.

Intravenous bolus administration increased the exposure of brain tumour tissue (AUC_{ECF}) to MTX by a factor of approximately 5 compared with i.v. infusion with an average MTX concentration in tumour ECF 4 h after i.v. bolus administration not significantly different from that obtained by i.v. infusion. In addition, MTX penetration (AUC_{ECF}/AUC_{Plasma}) after i.v. bolus was increased 3-fold compared with i.v. infusion. These results indicate that high plasma concentrations, by enhancing the concentration gradient across the barrier, promote the penetration of MTX into brain tissue. Moreover, high plasma concentrations may also leave a far higher free fraction available for distribution into the brain in light of the concentration-dependent protein binding of MTX [19,20]. Since Synold and colleagues demonstrated that high extracellular concentrations but not long duration of exposure led to increased intracellular formation of MTX-polyglutamates, i.v. bolus injection may thus offer an interesting alternative to i.v. infusion to improve the efficacy of MTX [21]. Moreover, since MTX tends to accumulate less in tissues with high and medium vascularisation after i.v. bolus than after i.v. infusion [22,23], bolus administration schedules may help reduce MTX toxicity.

An attempt was made to predict MTX concentrations in tumour ECF after i.v. infusion from the pharmacokinetic parameters obtained after i.v. bolus administration. Our simulations (data not shown) constantly overestimated MTX concentrations observed *in vivo* in tumour ECF. These results from simulation studies when taken into consideration together with our experimental findings may suggest the existence of nonlinear drug transport processes into brain tumour tissue.

In summary, this study demonstrated that i.v. bolus administration increases MTX delivery in brain tumour extracellular fluid compared with i.v. infusion. In light of the lack of data on MTX cytotoxic activity in this type of brain tumour model, this approach should, however, only be considered advantageous if it can be shown that it increases the therapeutic index of methotrexate. Further studies thus remain necessary to better characterise the impact of administration schedules on MTX efficacy and toxicity *in vivo*. This approach, by enabling the direct measurement of drug concentrations in the target tissue ECF, may also be applied to the study of other possibly more effective anticancer agents used in the treatment of brain tumours, provided that microdialysis probe recovery is high enough and sensitive analytical assays are available.

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