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Comparison of the blood-brain barrier and liver penetration of acridine antitumor drugs*

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Summary. The blood-brain barrier penetration of amsacrine and its analogs 9-({2-methoxy-4-[(methylsulfonyl)-amino]phenyl}amino)-,5-dimethyl-4-acridine carboxamide (CI-921) and M-[2-(dimethylamino)ethyl]-acridine-4-carboxamide (AC) was measured in the barbiturate-anesthetized mouse. After intracarotid administration, AC was almost completely extracted (90%) in a single transit through the brain capillaries, whereas CI-921 (20%) and amsacrine (15%) were moderately extracted. AC is retained in the brain; no loss of AC from the brain was apparent at 1, 2, 4, or 8 min after injection. In contrast, after intraportal administration, 75% of the AC, 94% of the CI-921, and 57% of the amsacrine was extracted in a single transit through the hepatic vasculature. Rather than being retained in the mouse liver, these acridine antitumor agents show time-dependent loss ($t_{1/2}$ = 10 min for amsacrine and AC, 24 min for CI-921). We conclude that unlike most antitumor agents, these acridine drugs appear to penetrate the blood-brain barrier readily.

Introduction

The restrictive properties of cerebrovascular endothelial cells (which comprise the blood-brain barrier) are regarded as one of the major phenomena responsible for the failure of chemotherapy in the treatment of central nervous system (CNS) tumors [16]. Alterations in the morphology of the brain microvasculature, including the presence of fenestrations and gap junctions as well as compromised tight junctions, are seen in both primary and secondary CNS tumors [8, 17, 33]. Despite the anatomically demonstrable loss in blood-brain barrier function, drug-distribution problems exist in CNS tumors [17]. Thus, the degree to which the blood-brain barrier is functional within brain tumors and the tumor-adjacent neuropil is somewhat controversial and has been discussed in detail elsewhere [16, 21, 31]. It is generally accepted that the blood-brain barrier, although of variable integrity, does indeed compromise the delivery of drugs to the brain and to brain tumors [16, 22, 23]. This observation in conjunction with the unique sensitivity of normal brain tissue to toxic agents limits the number of chemotherapeutic agents that are currently available to the oncologist. It is generally regarded that with the exception of the nitrosoureas, few of the antitumor agents presently in use possess the physicochemical properties required for adequate penetration of the brain [17].

A series of acridine derivatives were synthesized as potential antitumor agents by Cain and associates [2, 6, 7]. Amsacrine [NSC 249992; 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide] is clinically used for the treatment of acute myelogenous leukemia [1, 5]; it is currently classified as an investigational drug by the National Cancer Institute in the United States [18]. *N*,5-Dimethyl-9-[(2-methoxy-4-methylsulfonyl-amino)phenylamino]-4-acridine carboxamide (CI-921), an amsacrine derivative, was developed with the intent of providing a broader antitumor spectrum [4] and is currently undergoing clinical trials. Acridine carboxamide (AC; *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide, NSC 601316) is a third acridine antitumor agent that was synthesized in the same laboratory and has been found to show curative activity against Lewis lung carcinomas in mice [3]. These three acridine drugs (see Fig. 1 for structures) display variable activity against solid tumors. Amsacrine shows only marginal activity against Lewis lung carcinoma, whereas CI-921 exhibits high activity and AC is curative [15]. The objective of the present study was to compare the brain uptake and brain retention of these three acridine antitumor

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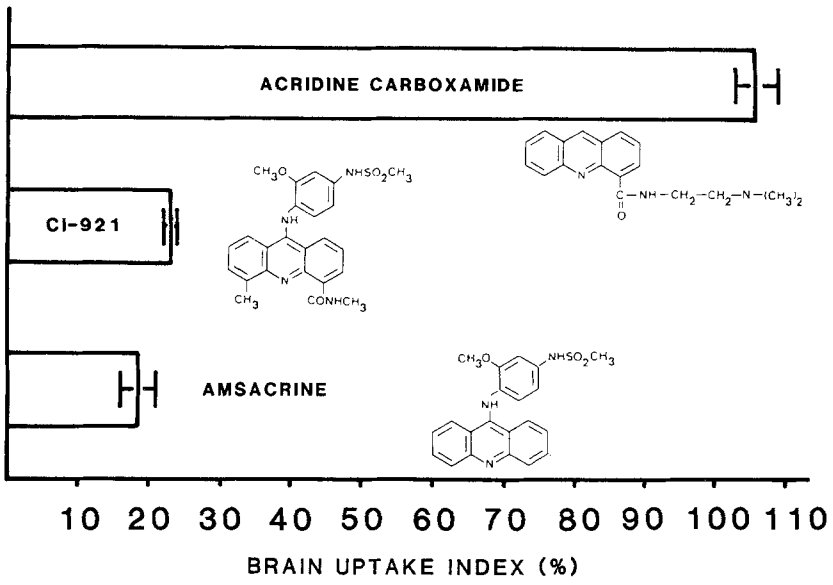

Fig. 1. Comparison of BUI values and structures of AC CI-921, and amsacrine. Note that the absence of the anilino group results in a markedly increased brain extraction of AC

agents in the CNS, a barrier tissue. Contrasting studies were also performed in the liver, a nonbarrier tissue with fenestrated capillaries that provide plasma-borne drugs direct access to hepatocyte membranes.

Materials and methods

Radiochemicals. [^{14}C]-Amsacrine (NSC 249992) was obtained from Research Triangle Institute (Research Triangle Park, N. C.) and prepared under a National Cancer Institute contract (NO1-CM-6-7703). [^{14}C]-CI-921 was kindly supplied by Warner-Lambert Parke-Davis (Ann Arbor, Mich.). The sodium salt of acridine-4-carboxylic acid was labeled by catalytic exchange in tritiated aqueous media by Amersham International (Buckinghamshire, England) and was further conjugated with 2-dimethylaminoethylamine to form the di-HCl salt of AC (sp. act., 164.6 μCi/μmol) by Dr. W. A. Denny (Cancer Research Laboratories, Auckland Medical School).

The chemical and radiochemical purity (>98%) of AC was also confirmed by high-performance liquid chromatography using a C18 Bondapak stainless-steel column (90.5 mm × 30 cm; Waters, Milford, Mass.) with a mobile phase of acetonitrile/water (32 : 65, v/v) containing 5 mM heptanesulfonic acid and 10 mM triethylamine phosphate for concurrent fluorescence (358 and 475 nm as excitation and emission wavelengths, respectively) and radiochemical detection (Raytest Ramona LS Radioactivity Monitor, FRG). The tin-indium TFC3 generator was obtained from Amersham International (Buckinghamshire, England). Other isotopes (methotrexate, vincristine, vinblastine, cytosine arabinoside, and diazepam) were obtained from Amersham or New England Nuclear Corporation (Boston, Mass.; butanol and tritiated water). The radiochemical purity of the test isotopes was confirmed by thin-layer chromatography (TLC) on glass-backed silica-gel plates (Whatman LK6D, 20 × 5 cm) using a butanol : acetic acid : water solvent system (4 : 1 : 1 by vol) or the solvent system recommended by the isotope supplier.

Isotopic scanning was performed on a Tracemaster Model LB285 linear analyzer (Berthold Analytical Instruments Inc., Nashua, N. H.). TLC separation of AC was also confirmed under exposure to ultraviolet light, where the position of the AC on the thin-layer plate could readily be identified by its characteristic fluorescence.

Partition coefficients. The partition coefficients of radiolabeled antitumor drugs were determined in an octanol : buffered saline triplicate-wash system [26]. The partition coefficients of amsacrine, CI-921, and AC were additionally determined in an octanol : saline system (without buffer) to determine the possible effects of physiological pH.

Injection solutions. A mixture was prepared that contained about 0.5 μCi (18.5 kBq) [^{14}C]-butanol and 5 μCi (185 kBq) test metabolite (tritiated antitumor drug) in the presence or absence of approx 50 μCi (1.85 MBq) ^{113m}In [chelated to ethylenediaminetetraacetic acid (EDTA)] per 100 μl saline [9] buffered with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Sigma Chemical Company, St. Louis, Mo.) to a final concentration of 10 mM (pH 7.55). Studies employing a [^{14}C] test isotope ([^{14}C]-CI-921) were performed using tritiated water or [^{3}H]-diazepam as the diffusible reference at the same (10-fold) tritium : ^{14}C ratio.

Single-injection methods. Prior approval of the Animal Ethical Committee of the University of Auckland was obtained. Locally bred CDl mice of either sex weighing 20–35 g were maintained on a 12-h light-dark cycle at 21° ± 1°C on a bed of wood shavings and were provided access to food and water ad libitum. Animals were weighed and anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal; Bomac Labs, New South Wales, Australia; 50–60 mg/kg) delivered from a graduated 100-μl syringe (Hamilton Company, Reno, Nev.).

Drug penetration into the CNS. For brain-injection studies, the animal was placed in the supine position and the right common carotid artery was isolated and separated from the vagosympathetic nerve trunk. A 25- or 30-μl volume of the injection solution was rapidly injected into the carotid artery through a 30-gauge needle (Terumo Corporation, Tokyo, Japan) without manipulation of the carotid arterial branches supplying the head and neck. It has been previously demonstrated that because the rate of injection exceeds the rate of carotid blood flow, the injection solution traverses the brain microcirculation in the ipsilateral hemisphere as a bolus without significantly mixing with the circulating blood [29]. The mouse was decapitated at 5 s after the injection. This period is sufficient for a single pass of the bolus through the brain but short enough to minimize both efflux of the labeled compound from the brain and recirculation of the labeled substance.

In brain-retention studies, the test antitumor drug was injected alone into the carotid artery, and mice were decapitated at various times ranging from 5 s to 8 min after the injection. The brain was then dissected into tared vials and prepared for liquid scintillation spectrometry. From the resulting data, the brain concentration-time profile was derived for each of the antitumor drugs studied.

Drug penetration into hepatic tissues. Liver-injection studies in anesthetized mice have been described elsewhere [10]. A laparotomy was per-

formed and the small intestine was reflected to expose the portal vein. A 30-µl volume of the isotope injection solution was injected as a bolus into the portal vein through a 27-gauge needle. At various times after the injection, hepatic blood flow was abruptly halted by simultaneous decapitation and severing of the thorax from the abdomen at the level of the diaphragm.

Tissues were quickly dissected, blotted dry, and placed in tared scintillation vials. To each vial, 1–1.5 ml of an organic base (Soluene, Packard Instruments) was added and rapidly digested at 50°–55°C. To decolorize the digested liver samples, isopropranol (0.5–1.0 ml) was added, followed by the dropwise addition of 30% hydrogen peroxide. Scintillation counting was performed in a Packard 2200 CA liquid scintillation spectrometer as described previously [9]. The injection solution (10 µl) was similarly prepared for radioisotope analysis.

Chromatographic analyses. Brains containing radiolabeled AC were homogenized in milli Q water at a ratio of 1 g tissue to 4 ml water, and 0.25 ml homogenate was extracted with 1 ml methanol. The sample was centrifuged at 2000 *g* for 10 min, the supernatant was aspirated, the pellet was again washed with 1 ml methanol and recentrifuged, and the supernates were combined. The pellet was washed a third time when the total supernates contained >95% of the total radioactivity. The methanol extracts were evaporated under nitrogen to a small volume (approximately 50 µl) for further TLC isotopic scanning as described above.

Data analysis. The brain uptake index (BUI) of each tritiated antitumor drug was determined from the ratio:

$$\text{BUI (\%)} = \frac{^{3}\text{H dpm}/^{14}\text{C dpm in brain}}{^{3}\text{H dpm}/^{14}\text{C dpm injected}} - \frac{^{113m}\text{In dpm}/^{14}\text{C dpm in brain}}{^{113m}\text{In dpm}/^{14}\text{C dpm injected}}.$$

The BUI is a comparison of the brain extractions (E%), since E = (dpm in brain)/(dpm injected). Thus, BUI = $[E_T/E_R - E_{In}/E_R] \times 100\%$, where E_T represents the brain extraction of the test compound, E_R indicates the extraction of the reference isotope, and E_{In} represents the extraction of the indium-EDTA chelate.

Indium quantitation in a beta counter has been described in detail by Oldendorf and Szabo [28]. This short-half-life (100 min) isotope is eluted from a tin-indium generator. It emits both internal conversion electrons and gamma rays and can be counted with equal efficiency in either beta or gamma counters [28]. Because of its short half-life, the activity of ^{113m}In must be determined within hours after its administration, and days later (after all of the ^{113m}In activity has decayed), samples are routinely assayed for tritium and ^{14}C.

The ^{113m}In chelated to EDTA does not cross intact cell membranes. Thus, in the above ratio, the minuend (i.e., the term on the right side of the minus sign) indicates the proportion of the test isotope (tritiated drug) remaining in the brain vasculature. This is often a very small (<2%) fraction. BUIs are often determined without indium, and the BUI is simply defined as the subtrahend (E_T/E_R) of the above ratio [25, 27]. When a test compound is radiolabeled as a ^{14}C isotope, tritiated water is the most commonly used reference isotope; for determination of the uptake of a tritiated test compound, [^{14}C]-butanol is the most commonly used reference [25, 27]. Indium was eluted from a tin-indium generator using a dilute hydrochloric acid solution; because of the limited solubility of amsacrine in chloride-containing solutions, the brain uptake of this antitumor agent was determined without indium correction.

All data are presented as mean values ± SD. A modification of Student's *t*-test was employed to compare control and treatment groups [14]. The slopes of the log radioactive concentration : time profiles were analyzed by linear regression, and the half-life ($t_{1/2}$) was calculated as log 2/slope.

Results

In the barbiturate-anesthetized mouse, the BUI of butanol relative to diazepam was determined to be 81% ± 3%. This constant was used to express the brain uptake of CI-921 relative to butanol and to determine single-passage brain extraction (E%) from butanol-based BUI measurements (diazepam is cleared 100% in a single transit through the brain microcirculation [34]). BUI values for amsacrine, CI-921, and AC are shown in Fig. 1. Nearly all of the AC (BUI, 105%) delivered to the brain vasculature penetrates into the brain tissue, whereas the brain uptake of amsacrine (17.9% ± 2.6%) and CI-921 (22.5% ± 1.0%) is moderately extracted during a single passage through the brain.

Table 1. Comparison of the in vitro partition coefficient (octanol : buffered saline) and in vivo blood-brain barrier permeability (brain extraction) in the mouse

Drug	Partition coefficient log *P*	Brain extraction (E%)
Amsacrine	1.12 ± 0.08	15.0 ± 2.2
CI-921	2.01 ± 0.18	18.8 ± 0.8
Acridine carboxamide	0.70 ± 0.02	88.5 ± 2.7
Methotrexate	−1.16 ± 0.08	5.4 ± 2.4
Vinblastine sulfate	2.30 ± 0.03	3.3 ± 1.0
Vincristine	2.15 ± 0.04	2.3 ± 1.6
Cytosine arabinoside	−0.65 ± 0.11	2.0 ± 1.5
Butanol	0.85 ± 0.01	83.6 ± 2.3

Since most of the radioactive drugs studied were obtained as tritiated isotopes, brain extractions were calculated from BUI measurements ($n = 3–8$) using [^{14}C]-butanol as the reference (except for butanol and [^{14}C]-CI-921, where by [^{3}H] diazepam was the reference). Unger et al. [37] have shown that aminoglutethimide (log $P = -0.23$), a drug used in the treatment of metastatic breast cancer, crosses the rat blood-brain barrier (BUI, 31% ± 2%; i.e., E = 19%). Thus, there are other examples whereby log *P* measurements apparently do not always correlate with brain entry

To determine whether lipophilic properties might explain the high uptake of AC, octanol : buffered saline partition coefficients were measured in vitro. Amsacrine (log $P = 1.12 \pm 0.08$) and CI-921 (log $P = 2.01 \pm 0.18$) were more lipophilic than AC (log $P = 0.70 \pm 0.02$). Although relationships between the cerebral vascular permeability of anticancer agents [17] and that of other compounds [11] with octanol : water partition coefficients are well documented, such a relationship was not apparent in the present study (Table 1). The polar drugs vincristine, cytosine arabinoside, methotrexate, and vinblastine minimally penetrated the blood-brain barrier in the present study (Table 1). Moderate BUI values (about 20%, comparable with those reported for CNS-active drugs such as phenytoin and phenobarbital [12]) were found for amsacrine and CI-921 (Fig. 1).

The brain retention of the acridine drugs was studied after intracarotid administration of the drug. Although some of the amsacrine washes out of the brain, a significant fraction is retained, even for up to 4 min (Fig. 2). CI-921 is also retained in the brain, in sharp contrast to tritiated water, whose half-life is about 3 min (Fig. 2). Similarly, a significant fraction of the AC is retained in the brain (Fig. 2). A *t*-test [14] indicated that the quantity of AC remaining was not significantly different at 1, 2, 4, or 8 min after injection (Fig. 2), suggesting that this drug may bind within the CNS. Extracts of mouse forebrain were prepared for thin-layer chromatographic (TLC) analysis at

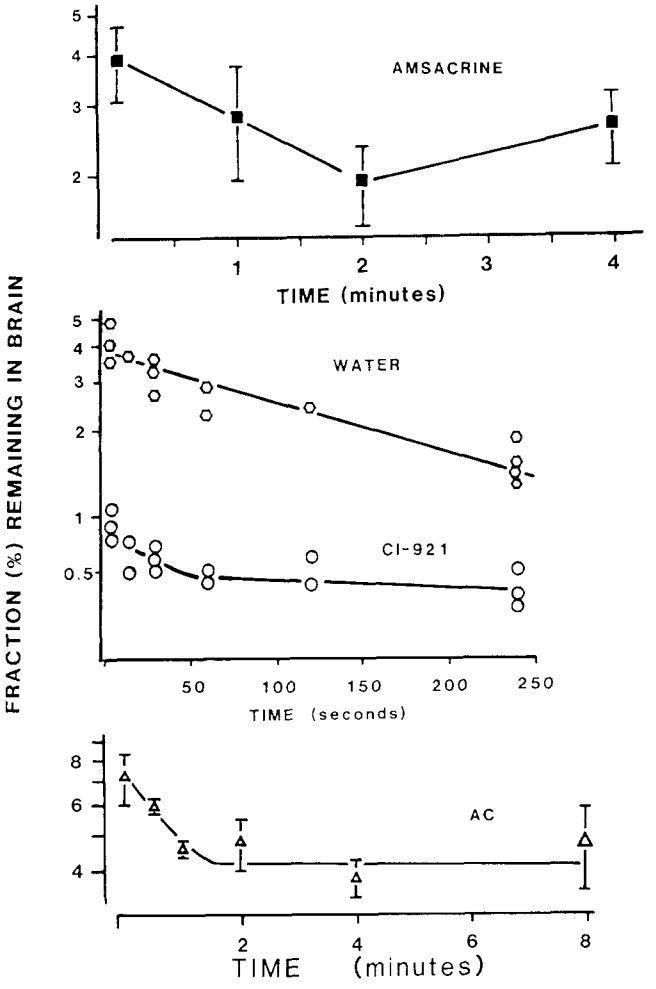

Fig. 2. *Upper panel:* Washout of radiolabeled amsacrine from mouse brain after intracarotid injection. *Vertical bars* represent ± 1 SD. *Middle panel:* Comparison of brain washout of tritiated water and [^{14}C]-CI-921 after intracarotid injection. Note that between 30 and 240 s, there is little, if any, loss of CI-921 from the brain; in contrast, tritiated water steadily washes out of the brain. Linear regression analysis ($r = 0.92$) indicates a slope of -0.20 min^{-1} and a half-time of water washout of 3.01 min. *Lower panel:* Brain retention of tritiated AC after intracarotid injection. Note that although there is some initial washout of this drug in the 1st min, brain AC levels remained constant between 1 and 8 min after drug delivery. *Vertical bars* represent ± 1 SD ($n = 3-4$ for each time point)

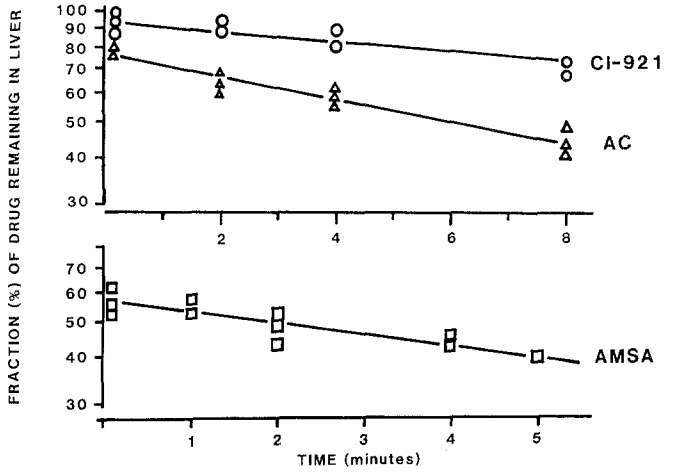

Fig. 3. Radioactive concentration-time profiles of amsacrine, AC, and CI-921 after portal-vein injection of the drug. *AMSA*, Amsacrine; *AC*, acridine carboxamide ($n = 3$ for each time point). In contrast to the situation in the brain (Fig. 2), washout of the acridine antitumor drugs from the liver is a linear function. Lines of best fit were determined by linear regression analysis and the correlation coefficients were 0.95, 0.96, and 0.88 for CI-921, AC, and AMSA, respectively

Table 2. Hepatic loss of radioactivity after the injection of acridine antitumor drugs

Drug	Correlation coefficient	Slope (min^{-1})	Intercept (E%)	$t_{1/2}$[a] (min)	Plasma $t_{1/2\alpha}$[b] (min)
AC	0.96	–0.0286	75	10	10
CI-921	0.85	–0.0127	94	24	36–72
Amsacrine	0.88	–0.031	57	10	10

Note that the maximal extractions (E%) are given by the intercepts; comparisons of brain (Table 1) and liver (Fig. 3) extractions indicate an AC value of E = 89% in the brain as compared with 75% in the liver, a CI-921 value of E = 19% in the brain vs 94% in the liver, and an amsacrine value of E = 15% in the brain vs 57% in the liver

[a] Hepatic half-life after portal injection (log 2/slope) of mice under barbiturate anesthesia

[b] Plasma drug half-lives determined from in vivo studies of AC [30], CI-921, and amsacrine [19]

5 min after the intracarotid injection of AC, and >95% of the total radioactivity was recovered. On silica-gel plates, TLC analyses of (a) the injected [^{3}H]-AC, (b) a mixture of tritiated and nonradioactive AC, and (c) brain extract, all of which comigrated in the butanol:acetic acid:water solvent system as a single peak, was further identified as a single fluorescent band displaying the same R_f (0.37) under ultraviolet irradiation.

Changes in the fraction of acridine antitumor drugs remaining in the liver at various times after portal injection are shown in Fig. 3. A high degree of correlation (suggested by the correlation coefficient's range of 0.85–0.96) in log-linear relationships between the drug remaining in the liver as a function of time was apparent (Table 2). The single-pass extraction (E) of these drugs is indicated by the intercept and amounted to 75% for AC, 94% for CI-921, and 57% for amsacrine. (This contrasts sharply to the situation in the brain [see Table 1], where E = 89% for AC, E = 19% for CI-921, and E = 15% for amsacrine, suggesting differential penetration in the liver vs the brain capillaries.) The half-life of radioactivity was derived (from data shown in Fig. 3) and seemed to be 10 min for AC, 24 min for CI-921, and 10 min for amsacrine (Table 2).

Discussion

In a recent discussion of factors that affect drug delivery to brain tumors, Greig [17] emphasized the importance not only of drug delivery to the brain but also of CNS retention of the various drugs. The neurotoxicity and seizures associated with amsacrine use [18] emphasizes that studies on the CNS penetration of acridine antitumor drugs may be

clinically important. The absence of an anilino group and minor substitutions on the carboxamide moiety result in maximal brain penetration (and enhanced retention) of AC. Lipophilic properties as indicated by the octanol:water partition coefficient have been demonstrated to be a good predictor of brain capillary permeability in a variety of previous studies [11, 17, 20, 26]. However, this characteristic was not apparent for the acridine antitumor drugs. AC was the least lipophilic but achieved maximal penetration into the brain. The degree of ionization is presumably an additional factor modulating the lipophilic membrane-transfer properties of these acridinyl compounds into the brain. The log P of these three acridinyl drugs was also determined in normal saline (i.e., without buffer) to compare the degree of ionization at physiological pH. Higher log P values were recorded for amsacrine, CI-921, and AC (log $P = 1.76 \pm 0.07$, 2.64 ± 0.06, and 1.47 ± 0.08, respectively). Table 1 indicates that significantly lower log P values (AMSA, 1.1; CI-921, 2.0; and AC, 0.7) were determined for these drugs when the saline phase was buffered to pH 7.55.

All three of these acridine antitumor agents were to some degree retained in the brain after intracarotid administration. Further in vivo studies also indicated relatively high levels of radioactive AC equivalents in the brain, which remained unchanged at 1, 8, and 24 h after i.v. administration [30]. Elevated brain:plasma AC ratios were observed when tissue:plasma ratios for other organs showed a decrease over the 24-h study period [30]. These acridine antitumor drugs are known to bind to double-stranded DNA by intercalation [4, 13], but the mechanism responsible for their retention in the brain awaits definition.

It is apparent that the acridine antitumor agents behave differently in brain and liver with regard to extraction and disposition. In the liver, all were extracted by >50% during the first passage, which is in agreement with previous in vivo mouse studies showing that these acridine antitumor agents experienced high extraction from plasma [19, 30]. These in vivo studies indicated that the plasma concentration-time profiles of AC and amsacrine were more similar, whereas CI-921 exhibited higher plasma concentrations and a longer half-life at approximately equimolar doses. This appears to be in agreement with the radioactivity concentration-time profiles observed in the liver (Table 2). However, it should be kept in mind that in the present study, saline-borne drugs were delivered to both the brain and the liver, and other studies have shown that plasma proteins can alter (anticonvulsant, thyroid, and anticoagulant) drug uptake into tissues [12, 35, 36]. The mechanism responsible for the relatively higher brain vs liver uptake of each of the acridine antitumor drugs is not understood at this time. It may have some relevance to the neurologic side effects observed following the administration of these acridine drugs.

Of the antitumor agents currently in use, only the nitrosoureas adequately penetrate the blood-brain barrier [17]. Bischloronitrosourea (BCNU), one of the drugs of choice in the treatment of brain tumors, exhibits a log P (octanol) = 1.5 and a BUI value of 100% (W. H. Oldendorf, personal communication). AC (log P, 0.7; BUI, 105%) is remarkably similar in its ability to gain access to the brain, suggesting that exploration of the possible localization of AC-like drugs in the brain and in experimental CNS tumors may be warranted. In preclinical studies of AC in mice, acute neurotoxicity appeared to be dose-limiting after i.v. administration and correlated with elevated brain levels of AC immediately after the injection [30]. This neurotoxicity could be avoided by using an alternative route (i.p.) of administration [30]. Studies to test for possible regional binding sites of AC in the brain seem to be warranted.

In a study of patients presenting with metastatic brain tumors, low CSF levels of AMSA were observed, which led Zhengang et al. [38] to suggest that amsacrine would not likely cross the blood-brain barrier. In contrast, a subsequent study of patients undergoing amsacrine treatment by these authors reported that brain concentrations of the drug were not unusually high but measurable [32]. Furthermore, elevated brain levels of amsacrine was correlated with neurotoxicity and seizures [32], suggesting that amsacrine gained access to the CNS. The present study confirms the blood-brain barrier penetration of this drug as well as a moderate brain uptake in the mouse, and Stewart and associates [32] have demonstrated that amsacrine-distribution studies in the mouse correlate with the clinical situation.

Intra-arterial administration of chemotherapy is by no means uncommon in the treatment of gliomas. Intracarotid drug infusion is hypothesized to be more efficacious than systemic drug administration, but this has not yet been proven [31]. The observation that some patients who fail systemic chemotherapy have responded to similar or lower intracarotid doses of the same drugs [31] is often cited in favor of this treatment method. Drugs that are readily taken up and retained by the brain but exhibit short systemic half-lives (such as AC) might be suited to intracarotid administration modalities, providing that neurotoxicity is avoided at the doses used.

The blood-brain barrier is known to impair drug delivery to CNS tumors [16], and the low brain uptakes observed for vincristine, vinblastine, methotrexate, and cytosine arabinoside in the present study (Table 1) exemplify this concept. For drugs that do not readily penetrate the blood-brain barrier, intra-arterial delivery of hyperosmotic agents such as mannitol or arabinose has been experimentally used to open the blood-brain barrier transiently, enabling the passage of nonneurotoxic drugs into the brain. Intra-arterial chemotherapy (methotrexate, cyclophosphamide, and procarbazine) in conjunction with osmotic opening has shown promise [22, 24]. However, the heroic nature of this treatment emphasizes the acute need both for better CNS antitumor agents and for antitumor agents that can readily penetrate the blood-brain barrier.

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