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Brain and plasma pharmacokinetics and anticancer activities of cyclophosphamide and phosphoramide mustard in the rat

Shigeru Genka[1], Joseph Deutsch[1], Paul L. Stahle[1], Umesha H. Shetty[1], Varghese John[2], Cynthia Robinson[2], Stanely I. Rapoport[1], and Nigel H. Greig[1]

[1] Laboratory of Neurosciences, National Institute on Aging, National Institutes of Heallth, Bethesda, MD 20892, USA
[2] Department of Drug Development, Athena Neurosciences, Inc. 800-F Gateway Blvd., South San Francisco, CA 94080, USA

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Summary. By a sensitive and quantitative fluorometric assay, brain and plasma time-dependent concentration profiles were generated for phosphoramide mustard (PM) and active alkylating metabolites derived from cyclophosphamide (CPA) administration to rats. Whereas PM rapidly disappeared from plasma, with a monophasic half-life of 15.1 min, equimolar administration of CPA generated active metabolites in plasma that disappeared monoexponentially, with a composite half-life of 63 min. As a consequence, the time-dependent concentration integral of active alkylating metabolites derived from CPA administration, calculated between 5 min and infinity, was 3-fold that of PM. Pharmacokinetic parameters were calculated for each compound. The brain/plasma concentration-integral ratios of PM and active alkylating metabolites derived from CPA were 0.18 and 0.20, respectively. The cerebrovascular permeability-surface area product of PM was 7.5×10^{-5} s^{-1}, which is similar to that of other water-soluble anticancer agents that are restricted from entering the brain. The activities of a range of daily doses of PM and CPA were assessed against subcutaneous and intracerebral implants of Walker 256 carcinosarcoma tumor in rats. Inhibition of subcutaneous tumor growth by 50% was caused by CPA and PM doses of 6.6 and 12.0 mg/kg (daily for 5 consecutive days, starting 36 h after tumor implantation), respectively. However, administration of daily doses of up to 40 mg/kg did not significantly increase the survival of animals with intracerebral tumor implants. These studies indicate that active metabolites of CPA are restricted from entering the brain and that only subtherapeutic concentrations are achieved in brain tissue after systemic administration of CPA or PM.

Abbreviations: CPA, cyclophosphamide; PM, phosphoramide mustard; 4-HC, 4-hydroxycyclophosphamide; AP, aldophosphamide; PA, cerebrovascular permeability-surface area product

Offprint requests to: Nigel Greig, Laboratory of Neurosciences, Bldg. 10, Rm 6C 103, National Institute on Aging, National Institutes of Health, Bethesda, MD 20892, USA

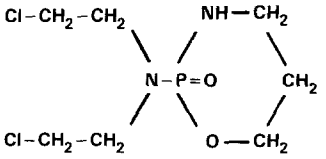
Introduction

Cyclophosphamide (CPA), 2-bis(2-chloroethyl)amino tetrahydro-2H-1,3, 2-oxazophosphorine-2-oxide monohydrate, is a cyclic phosphoramide ester of mechlorethamine that functions as an alkylating agent. It has a wide spectrum of clinical activity and is frequently included in combined regimens for the treatment of malignant lymphomas; acute leukemia; multiple myeloma; neuroblastoma; various sarcomas; and ovarian, breast, endometrium, and oat-cell carcinomas [5, 12]. Additionally, it is a potent immunosuppressive agent and is therefore useful in the prevention of graft-versus-host reactions following organ transplantations in a variety of nonmalignant diseases and in the treatment of rheumatoid arthritis and other autoimmune diseases [22].

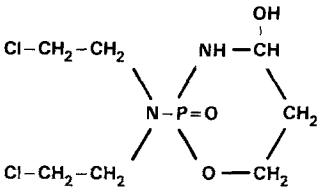
The biological activity of CPA requires metabolic activation. CPA is metabolized by hepatic microsomal (P-450 mixed-function oxidases) enzymes [4, 8, 19, 55] to two major intermediate products: (1) 4-hydroxycyclophosphamide (4-HC), by hydroxylation; and (2) aldophosphamide (AP), by subsequent aldehyde formation. 4-HC and AP appear in plasma and are ultimately further metabolized to at least two powerful intracellular alkylating metabolites, phosphoramide mustard (PM) and acrolein [6, 9, 43, 59] (Fig. 1). It has been suggested that the ultimate active alkylating metabolite of CPA is PM and that the important circulating metabolites are 4-HC and AP [9, 11, 47, 57]. As an alkylating agent CPA, through its active metabolites, prevents cell division by cross-linking DNA independently of the cell-cycle phase [5, 12].

CPA has been used in the treatment of central nervous system tumors, most often in combination with other chemotherapeutic agents [29, 46, 51, 58]. As myelosuppression is the major side effect of CPA treatment, the agent is often given at high, lethal doses, followed by autologous bone marrow transplantation rescue [10, 42]. Recurrent pediatric medulloblastoma, glioma, and germ-cell tumors have responded to high-dose therapy [1]. Additionally, CPA has proved to be of value when delivered to the brain in combination with methotrexate and procar-

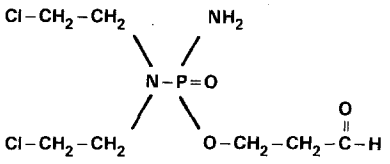
Cyclophosphamide



4-Hydroxycyclophosphamide



Aldophosphamide



Phosphoramide Mustard

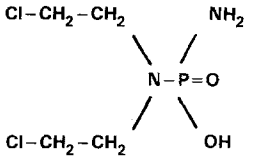


Fig. 1. Chemical structures of cyclophosphamide, 4-hydroxycyclophosphamide, aldophosphamide, and phosphoramide mustard

bazine following its required hepatic activation by i.v. administration prior to osmotic opening of the blood-brain barrier for brain tumor chemotherapy [37, 39, 40].

Although CPA has been used for brain tumor treatment, there are few quantitative reports concerning its delivery to and maintenance in the brain after its systemic administration. We therefore examined the brain and systemic anticancer activities of CPA and PM in rats and generated the time-dependent concentration profiles of alkylating activity following i.v. administration of these agents.

Materials and methods

Pharmacokinetics. For pharmacokinetic analyses, 2-month-old male Wistar rats (Charles River Laboratories Inc., Wilmington, Mass) weighing approximately 150 g were lightly anesthetized with halothane (Ayerst Laboratories, New York, N.Y.). The left saphenous vein was exposed, and 75 mg/kg CPA monohydrate (Sigma Chemical Co., St. Louis, Mo.) (equivalent to 70 mg/kg CPA free base) or PM cyclohexylamine salt, synthesized according to Friedman [20] (86.0 mg/kg, equivalent to 59.4 mg/kg PM and equimolar to 70 mg/kg CPA) in isotonic saline, was injected. Hereafter, all doses and concentrations are expressed in terms of the free base. At times between 5 min and 4 h following drug administration, blood was collected by cardiac puncture and the brain was removed and placed on ice-chillded 0.9% NaCl filter paper. A minimum of three animals were killed per time point. Blood was centrifuged (10,000 *g* for 1 min) and 2 ml plasma was removed, equal volumes being placed in two vials and immediately stored at –70° C. The brain was stripped of the cerebellum and the cerebral hemispheres were separated (each weighing approximately 0.5 g), immediately frozen to –70° C, weighed while frozen, and immediately stored at –70° C. Plasma and brain samples were quantitated for their alkylating activity by reaction with 4-(*p*-nitrobenzyl)pyridine (NBP) within 24 h).

Drug quantitation. Using a modified assay procedure based on those of Epstein et al. [17], Friedman and Boger [21], and Juma et al. [31, 32], concentrations of PM and of active alkylating metabolites derived from CPA administration were determined in samples by fluorometric measurement of their reaction product following incubation with NBP. Reaction of an alkylating agent with NBP generates a quaternary pyridinium compound that is transformed at basic pH into a highly colored pigment that absorbs at a wavelength between 540 and 575 nm [6, 21, 32]. Quantitation of the reaction product by fluorometric analysis, described below, proved to be highly sensitive and reproducible for determining low concentrations of PM and CPA alkylating metabolites in biological samples.

To plasma pharmacokinetic samples (1.0 ml) and standards (0.9 ml blank plasma plus 0.1 ml standard of PM), 10% trichloroacetic acid (0.2 ml) was added for deproteinization. The sample was then shaken vigorously and centrifuged (10,000 *g* for 1 min), and the supernatant (0.6 ml) was placed in a polypropylene tube provided with a cap. Acetate buffer (0.5 ml, 0.2 *M*, pH 4.6) was added together with 5% NBP (Sigma Chemical Co.) in acetone (0.2 ml) and the solution was shaken. It was boiled for 20 min and placed on ice for 10 min, and then octyl alcohol (2.5 ml), 1 *N* sodium hydroxide (1.0 ml) and acetone (1.0 ml) were added. Following vigorous shaking, the sample was placed in a dark environment on ice for a further 25 min. Finally, it was centrifuged (3,000 *g* at 6° C for 5 min) and the top layer was removed and kept on ice in a dark environment prior to spectroscopic measurement. Fluorescence emission was measured by a fluorescence spectrophotometer (Model LS-5; Perkin-Elmer Co., Oak Brook, Ill.) at an excitation wavelength of 535 nm and an emission wavelength of 625 nm.

To weighed brain pharmacokinetic samples and samples of blank brain (approximately 0.5 g weight, to which 0.1 ml of an appropriate concentration of PM was added for construction of a standard curve), sodium acetate buffer (1.5 ml, 0.2 *M*, pH 4.6) was added and the sample was then sonicated (Model 225; Heat Systems-Ultrasonics Inc., Farmindale, N.Y.) for 30 s at 4° C. Thereafter, 10% trichloroacetic acid (0.4 ml) was added for deproteinization and the sample was shaken vigorously and centrifuged (10,000 *g* for 1 min). The supernatant (0.9 ml) was pipetted into a polypropylene tube provided with a screw cap, acetate buffer (0.5 ml, 0.2 *M*, pH 4.6) and 5% NBP in acetone (0.2 ml) were added, and the solution was vigorously shaken. It was boiled for 20 min and placed on ice for 10 min, and then octyl alcohol (2.5 ml), 1 *N* sodium hydroxide (1.0 ml) and acetone (1.0 ml) were added. The sample was vigorously shaken, placed in a dark environment on ice for 25 min, and then centrifuged (3,000 *g* for 5 min at 6° C). The supernatant was separated and placed on ice in a dark environment prior to fluorometric measurement as described.

Concentrations were calculated from standard curves constructed for both plasma and brain using a minimum of five points, two samples per point, which were run daily and intermixed with the pharmacokinetic samples. The reliable limit of detection was 10 nmol/ml and nmol/g for PM in plasma and brain, respectively, and the mean correlation coefficient of six standard curves run over 1 month was >0.998 for both plasma and brain, with negligible intercept in all cases. Extraction of PM from plasma and brain samples was >75%.

Plasma protein binding. The percentage of binding of PM to plasma proteins in fresh rat plasma was determined at 100 and 1,000 nmol/ml by centrifugal ultrafiltration. Centrifree micropartition systems (Amicon Corp., Danvers, Mass.) were used for the rapid preparation of protein-free ultrafiltrates of PM as previously described [28]. Samples of plasma and ultrafiltrate were then immediately quantified for PM as described.

Calculations. Brain levels of PM and alkylating metabolites derived from CPA administration were calculated from net brain concentrations by subtracting the intravascular volume at the time of death (T) and were expressed as equivalents of PM. The intravascular concentration equaled the plasma concentration of alkylating metabolites (nmol/ml) at time T

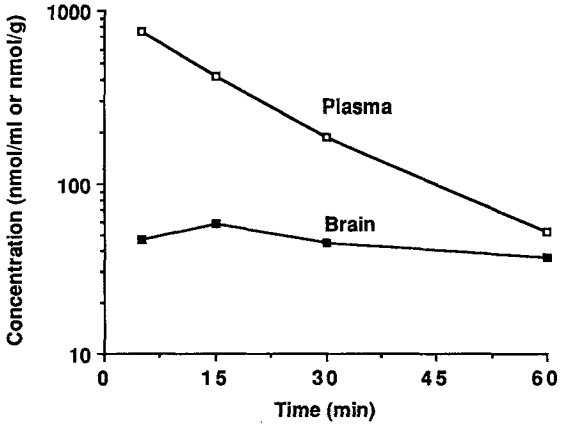


Fig. 2. Time-dependent concentration profiles of phosphoramide mustard (PM) in plasma and brain following its i. v. administration to rats (86 mg/kg PM cyclohexylamine salt, equivalent to 59.4 mg/kg PM free base)

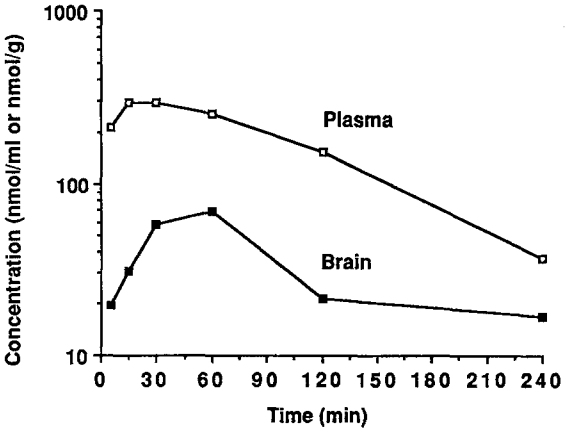


Fig. 3. Time-dependent concentration profiles of total alkylating metabolites generated from the i. v. administration of cyclophosphamide (CPA) monohydrate to rats (75 mg/kg, equivalent to 70 mg/kg CPA free base and equimolar to 59.4 mg/kg PM free base)

multiplied by the regional blood volume (ml/g brain). Regional blood volume was measured after i. v. injection of each of three anesthetized rats with [^{14}C]-methyl bovine serum albumin (17 μCi/mg; New England Nuclear Research Products, Boston, Mass.); the latter was determined to be approximately 99.5% pure by polyacrylamide slab electrophoresis. Blood and brain samples were collected at 2 min as described and digested overnight at 50° C in 1 ml Protosol (New England Nuclear). Then, 10 ml Ready Solv MP liquid scintillation cocktail was added and counting was performed in a liquid scintillation spectrometer (Model LS9000, Beckman Instruments, Palo Alto, Calif.). [^{14}C]-Methyl bovine serum albumin (mol. wt., 69,000 daltons) remains within the cerebral vasculature during the 2-min study [48]. Regional blood volume was calculated by dividing the radioactivity in the brain sample by that in blood (dpm g^{-1}/dpm ml^{-1}) and amounted to 1.7% for each cerebral hemisphere.

The apparent first-order rate constants, α, for plasma elimination of PM and of total alkylating metabolites following CPA administration were determined as being the absolute values of the slopes of the least-squares regression lines for the terminal portions of semilogarithmic plots of PM and of CPA-derived total alkylating metabolite concentrations (for PM, 15–60 min; for CPA, 30–240 min). Apparent half-lives were calculated using the formula $t_{1/2} = 1n\ 2/\alpha$. The areas under the curve (AUC) from 5 min to infinity for PM and for total alkylating agents derived from CPA were calculated as being the sum of the AUC from 5 to 60 min for PM and from 5 to 240 min for CPA using the trapezoidal rule, and the AUCs from 60 and 240 min to infinity, respectively, were estimated using the formula C/α, where C is the mean plasma concentration of PM or of total alkylating metabolites derived from CPA measured at 60 and 240 min, respectively. Clearance of agents from plasma was calculated according to the formulae dose/AUC and volume of distribution dose/(AUC × α).

Finally, the cerebrovascular permeability-surface area product (PA) of PM was calculated according to the two-compartmental model of Ohno et al. [44]. Unidirectional brain uptake of PM (excluding its back-diffusion from the brain into the blood, which was predicted to be negligible during the 60-min perirod of the experiment) is defined as

$$d^*C_{Br}/dt = PA.^*C_{Pl}.F, \quad (1)$$

where PA (s^{-1}) is the cerebrovascular permeability (P; cm/s); x, the capillary surface area (A; cm^2/g brain or cm^2/cm^3 brain); $^*C_{Pl}$ (nmol/ml), the plasma concentration of PM; t (s), time; and $^*C_{Br}$ (nmol/g), the brain parenchymal concentration of PM, corrected for intravascular drug as described. Finally, F represents the free fraction of drug present in plasma. Equation 1 can be integrated to the time of death, T, to give PA in terms of the plasma concentration integral:

$$PA = {}^*C_{Br}.(T)/{}^*C_{Pl}.dt.F. \quad (2)$$

Anticancer activity studies. For determination of the anticancer activity of CPA and PM in intracerebral tumors, 2-months-old male Wistar rats (Charles River Laboratories) weighing approximately 150 g were lightly anesthetized with halothane. Walker 256 carcinosarcoma tumor (250 cells in 5 μl Dulbecco's modified Eagle's tissue culture medium; Gibco Laboratories, Chagrin Falls, Ohio) containing 1% low-melting-point agar was then injected into the parietal cerebral cortex through a 30-gauge needle attached to a Hamilton syringe. With the exception of control animals, all rats received CPA or PM at doses of up to 40 mg/kg i. p. once daily for 5 consecutive days beginning 36 h after tumor implantation. This time has been reported to be sufficient for the reestablishment of blood-brain barrier integrity after an intracerebral injection [27]. The survival of animals was subsequently recorded, cumulative survival rates were calculated by the Kaplan-Meier test, and statistical significance was examined using a Cox-Mantel test [36].

For subcutaneous tumors, 0.1 ml freshly minced Walker 256 carcinosarcoma tumor was injected s. c. into the left flank of male Wistar rats. With the exception of control animals, all received either CPA (from 2.8 to 23.4 mg/kg) or PM (from 2.1 to 20.7 mg/kg) i. p. once daily for 5 consecutive days beginning 36 h after tumor implantation. Drug activity against subcutaneous tumors was assessed by surgical excision of each tumor 8 days after implantation, followed by comparison of mean tumor wet weight with that of controls.

A minimum of eight animals were included in each control and treatment group. A two-tailed Student's *t*-test was performed to compare two means [36]; when more than two means were compared, one-way analysis of variance and the Bonferroni multiple *t*-test were used [36]. Statistical significance was taken at the level of $P<0.05$.

Results

Figure 2 shows plasma and brain concentrations of PM following its i. v. administration to rats. A peak level of 757 nmol/ml was determined at 5 min; no plasma samples were obtained prior to this time; concentrations then declined monophasically in plasma, with a disappearance half-life of 15.1 min. The volume of distribution and clearance of PM from adult rats were 339.3 ml/kg and 15.7 ml min^{-1} kg^{-1}, respectively. The areas under the time-dependent concentration profile of PM in plasma were 14,014 nmol min ml^{-1}, measured between 5 and 60 min, and 15,153 nmol min ml^{-1}, calculated between 5 min and

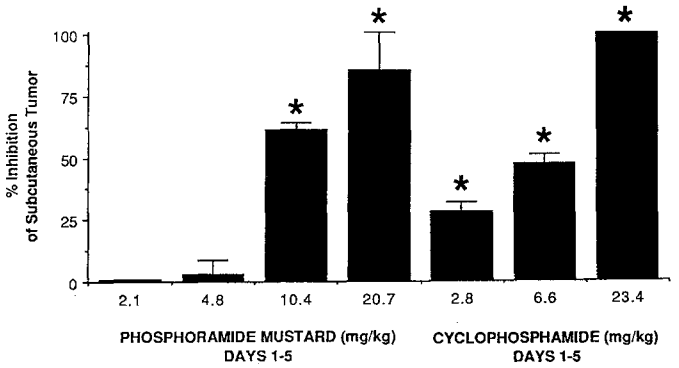


Fig. 4. Anticancer activities of increasing doses of PM (between 2.1 and 20.7 mg/kg PM free base) and CPA (between 2.8 and 23.4 mg/kg CPA free base), given i.p. once daily for 5 consecutive days, against subcutaneously implanted Walker 256 carcinosarcoma tumor in the rat. Each group contains a minimum 8 animals. Means ±SEM are shown. * $P < 0.05$ for the difference between treated and control animals

infinity. The plasma fraction of PM bound to protein varied between 20% and 60% at concentrations of 1,000 and 100 nmol/ml, respectively.

Substantially lower concentrations of PM were found in brain than in plasma. A peak level of 57.6 nmol/g was measured at 15 min; thereafter, brain levels rapidly declined and could not be reliably detected after 60 min. The AUC of PM in brain tissue amounted to 2,509 nmol min g^{-1}, calculated between 5 and 60 min.

Figure 3 shows the plasma and brain concentrations of total alkylating metabolites following i.v. administration of CPA, expressed as equivalents of PM. Peak levels of 296 nmol/ml were achieved in plasma between 15 and 30 min; thereafter, they declined monophasically, with a disappearance half-life of 63 min. Peak brain concentrations (69.8 nmol/g) were substantially lower than concomitant plasma levels and occurred at 60 min; thereafter, brain concentrations rapidly declined and could not be reliably detected after 240 min. The AUC of total alkylating metabolites derived from CPA in brain tissue was 7,895 nmol min g^{-1}, measured between 5 and 240 min, and that for plasma was 38,950 nmol min ml^{-1}, measured between 5 and 240 min, and 42,305 nmol min ml^{-1}, calculated between 5 min and infinity.

Figure 4 illustrates the inhibitory effects of PM and CPA administration on the subcutaneous growth of Walker 256 carcinosarcoma tumor in rats. Both agents were injected i.p. at daily doses ranging between 2.1 and 20.7 mg/kg for PM and between 2.8 and 23.4 mg/kg for CPA on 5 consecutive days starting 36 h after tumor implantation. Whereas 50% inhibition of subcutaneous tumor growth was produced by a daily dose of 6.6 mg/kg CPA, an equivalent dose of PM caused only 3% inhibition. A daily dose of 12 mg/kg PM was required to produce 50% inhibition of subcutaneous tumor growth.

Five consecutive daily doses of up to 40 mg/kg PM and of CPA were given to rats bearing intracerebral implants of Walker 256 carcinosarcoma tumor. Neither PM nor CPA significantly extended the survival of these animals compared with that of untreated controls.

Discussion

In our pharmacokinetic studies of CPA and PM, quantitation of active drug was based on the measurement of a product formed by reaction of an alkylating species with NBP. For quantitation of alkylating agents in biological samples, particularly in brain tissue, our measurement of reaction product by fluorescence proved to be more sensitive and reliable than colorimetric analysis by spectrophotometer at wavelengths between 540 and 575 nm. Whereas quantitation of active metabolites derived from CPA by the NBP technique is simple, rapid, and inexpensive as compared with more sophisticated and sensitive gas chromatography (GC) assays, with and without mass spectrometry (MS) [7, 14, 33], the NBP method measures total alkylating agents at pH 4.6 and cannot discriminate tumor-active metabolites specifically [62]. Although PM, 4-HC, and AP express significant alkylating activity at pH 4.6 and readily react with NBP, tumor-inactive CPA metabolites such as carboxyphosphoramide, which, unlike PM, is neither cytotoxic nor an alkylating agent at physiological pH [56], may slightly contribute to formation of the NBP reaction product at pH 4.6. Despite this, variations of the NBP technique have proved to be valuable in determining the pharmacokinetics of CPA and analogues such as ifosfamide in both rodent and clinical studies [15, 31, 41, 61]; moreover, results of these studies compare favorably with those of other investigations using more sophisticated analytical techniques [32, 33, 56].

Furthermore, in the present studies CPA pharmacokinetic data were expressed as equivalents of PM for three reasons. First, extensive studies suggest that PM is the ultimate active cytotoxic species of CPA and that nornitrogen mustard, used by some for the quantitation of CPA [31, 32], is minimally cytotoxic at physiological pH, with its presence in plasma now being considered as largely artifactual [30]. Second, whereas 4-HC and AP represent important principles in the pharmacokinetics and pharmacological actions of CPA, both agents, unlike PM, undergo rapid biotransformation in vivo and are relatively unstable in vitro, undergoing general base- [35] as well as (3'-5'-exonuclease) enzyme-catalyzed [3, 57] hydrolysis to PM and acrolein. This limits their use as standards for analytical procedures. Finally, the expression of CPA pharmacokinetics in PM equivalents enables their direct comparison with our PM pharmacokinetic studies.

Following the i.v. administration of CPA or equimolar PM to rats, significant concentrations of alkylating metabolites were present in plasma. The peak concentration after PM administration was greater than that achieved after CPA administration (757 and 296 nmol/ml, respectively) and occurred earlier. However, disappearance of PM from plasma was 4-fold that of total alkylating metabolites derived from CPA, with half-lives of 15.1 and 63 min, respectively. As a consequence, the concentration versus time integral of CPA-derived alkylating metabolites was 3-fold that of PM (42,305 and 15,153 nmol min ml^{-1}, respectively). The clearance of PM (15.7 ml min^{-1} kg^{-1}) and its volume of distribution (339.3 ml/kg) were consistent with those of an ionized water-soluble agent. The composite volume of distribution and clearance of CPA-

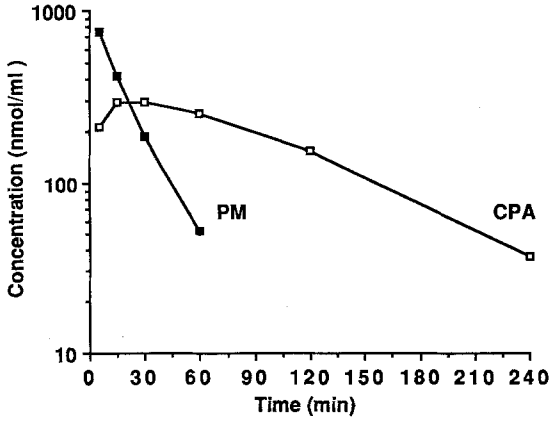


Fig. 5. Comparative time-dependent plasma concentration profiles of total alkylating metabolites generated by equimolar i. v. administration of PM and CPA at 59.4 and 70 mg/kg (free base), respectively, to rats

derived alkylating metabolites were 519 ml/min and 5.7 ml min^{-1} kg^{-1}, respectively. The interpretive value of these composite parameters is limited, as the individual metabolites differ widely in their pharmacokinetic properties and profiles [62]. Nevertheless, the cytotoxic effects of CPA are directly proportional to the summed AUC values of 4-HC, AP, and PM, its alkylating metabolites.

Our pharmacokinetic data (Fig. 5) compare favorably with those of Sladek [56] and Sladek et al. [57], who reported a half-life and volume of distribution for PM of 14 min and 440 ml/kg, respectively, following i.v. administration of PM cyclohexylamine salt (50 mg/kg) to rats. The combined composite half-life and volume of distribution of 4-HC and AP, generated by i.v. administration of 4-hydroperoxycyclophosphamide and ASTA Z 7557 and quantitated by the fluorometric measurement of released acrolein, were determined to be 14.2 min and 994 ml/min, respectively [57]. Furthermore, Sladek [56] and Sladek et al. [57] reported the actual half-life of CPA to be 29 min. As in our study, these actual half-lives are dramatically shorter than the composite plasma half-life obtained after administration of CPA in our study (63 min) and in that of Sladek et al. (55 min) [57]. The greater AUC and longer composite half-life of total alkylating metabolites derived from CPA administration, as compared with equimolar administration of PM, support the contention of Sladek [56] and Juma et al. [32] that a major determinant in the in vivo activity of CPA is the rate of formation of the intermediates 4-HC and AP. The subsequent distributions of 4-HC and AP, which are widely considered to represent the transport forms of CPA [56], and their final metabolism to PM account for the lower and later peak plasma levels and the larger AUC of alkylating metabolites generated after CPA administration as compared with those obtained after equimolar PM.

As reviewed by Sladek [56], most human and rodent tumor cells are more sensitive to 4-HC and AP than to PM. This has been explained by the finding that although PM represents the final cytotoxic species of CPA, its cellular uptake is restricted by its ionization at physiological pH [16]; however, 4-HC and AP are predicted to enter tumor cells more readily [34], where they are subsequently hydrolyzed to PM. The larger AUC of alkylating metabolites generated after CPA administration and the predicted, higher intracellular uptake of 4-HC and AP as compared with PM probably account for the greater anticancer activity of CPA as compared with equimolar PM against subcutaneous Walker 256 carcinosarcoma in our studies; we achieved equal anticancer activity at a CPA dose 50% lower than that of PM.

There appears to be some controversy concerning the amount of CPA that enters the brain after its systemic administration. Duncan et al. [14] measured the concentration of parent drug and metabolites by gas chromatography/mass spectrometry (GC/MS) and found only a minute quantity in cerebrospinal fluid. However, various studies using [^{3}H]-CPA have suggested that it penetrates the blood-brain barrier with ease. Graul et al. [23] and Simon et al. [54] measured the distribution of tritium-labeled drug in blood, cerebrospinal fluid, normal brain tissue, and brain tumor tissue following its i.v. administration to nine patients. After finding this drug in each tissue at concentrations similar to that in blood, these authors concluded that it freely enters the brain. Neuwelt et al. [38] measured levels of [^{3}H]-CPA in blood, extracerebral tissues, avian sarcoma-induced brain tumor, and surrounding brain in rats after its i. v. administration. This brain tumor possesses a highly leaky blood-tumor barrier [25], and drug levels in brain tumor were similar to those in extracerebral tumor.

Unfortunately, the radioactive label alone provides no information concerning the structure of the compound in these studies. Bearing in mind the number of active and inactive metabolites of CPA, exactly what was being measured remains unclear. Yamada et al. [63] discriminated between active and inactive metabolites derived from CPA metabolism by measuring their alkylating activity and reported their plasma and cerebrospinal fluid pharmacokinetics in dogs. Calculated from the data of these authors, the cerebrospinal fluid/plasma time-dependent concentration integral ratios were 0.45 for total drug and metabolites, 0.29 for active drug alone in cerebrospinal fluid and plasma, and 0.17 for active drug in cerebrospinal fluid versus total drug and metabolites in plasma. Using a salmonella/mutagenesis assay in mice, Sulings et al. [60] confirmed the low accumulation of active drug in cerebrospinal fluid. Our studies confirm that only low concentrations of active metabolites derived from CPA enter brain tissue following systemic CPA administration; moreover our findings demonstrate that the brain uptake of PM, the active end product of CPA metabolism, is also low.

We previously reported that a number of factors combine to determine the concentration of a drug that is achieved and maintained in brain after its parenteral administration [24, 26]. First the permeability of the agent at the blood-brain barrier, which is related to its lipophilicity; second, the time-dependent plasma concentration profile of the agent, related to its distribution volume, metabolism, and elimination [24]; third, the binding of the drug to plasma and tissue constituents, its binding off-rates [49], and the question as to whether this binding is restrictive or nonrestrictive for brain uptake of drug [26], as only unbound agents are available to enter the brain. The final

factor is local cerebral blood flow, which is relatively unimportant for water-soluble drugs [26].

The cerebrovascular permeabilities of active metabolites derived from CPA have not been reported. As a consequence of the short half-lives of these metabolites, measurement of both their permeability at the blood-brain barrier and their binding to plasma constituents would be difficult, excepting those for PM. We determined the cerebrovascular permeability-surface area product (PA) of PM to be 7.5×10^{-5} s^{-1}. This value is similar to that of the water-soluable anticancer agent methotrexate (PA, 3.3×10^{-5} s^{-1} [45]), whose brain uptake is similarly restricted by its low cerebrovascular permeability. The binding of PM to plasma proteins rose from 20% to 60% at concentrations between 1,000 and 100 nmol/ml. As a significant fraction of drug remains unbound at concentrations achieved in vivo, the brain uptake of PM is restricted more by its low PA than by its binding to plasma constituents. Although significant concentrations of PM are present in plasma following its i.v. administration, as calculated from peak concentrations, it possesses a brain/plasma ratio of 0.08. As PM is eliminated more quickly from plasma as compared with the limited amount that enters brain tissue and becomes sequestered there (half-lives, 15.1 and 75 min, respectively), the brain/plasma concentration integral ratio of PM is 0.18 (calculated between 5 and 60 min).

Despite the maintenance of significant levels of alkylating metabolites in plasma after CPA administration, concomitant levels in the brain are relatively low. As calculated from the peak concentrations and concentration integrals, CPA-generated alkylating metabolites possess a brain/plasma ratio of 0.23 and 0.20, respectively. These compare favorably with the cerebrospinal fluid/plasma ratio of 0.20 that was previously reported by Egorin et al. [15] in ten patients as well as with that of 0.17, which was reported by Arndt et al. [2] in the rhesus monkey. Whereas the cerebrovascular permeability of 4-HC and AP would be expected to be greater than that of PM, the binding of the former to plasma constituents may also be greater than that of PM, which may limit their delivery to the brain and cerebrospinal fluid. CPA and 4-HC can react reversibly with various sulfhydryls to form thiol adducts [13, 56], and the brain uptake of these would certainly be restricted. It is likely that various individual combinations showing restrictive binding to plasma constituents and low cerebrovascular permeability limit the brain uptake of alkylating metabolites derived from CPA administration.

In our study, as in others [18, 52, 53], the activity of CPA against intracerebral tumor implants after its systemic administration was negligible, even at doses dramatically higher than those that completely eradicated systemic tumor. This confirms that the delivery of alkylating metabolites of CPA into brain tissue is limited and most often results in the achievement of subtherapeutic concentrations. Although CPA has proved to be of some value in the treatment of malignant brain tumors at high doses, we consider that the difficulty of maintaining therapeutic levels in the outermost shell of tumor and adjacent brain tissue [24, 50], which possess a relatively normal blood-brain barrier [25], probably accounts for the relatively short duration of responses.

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