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Neurotoxicity and dermatotoxicity of cyanomorpholinyl adriamycin

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Summary. The highly lipophilic cyanomorpholinyl adriamycin (CMA) is the most potent antineoplastic anthracycline yet described. CNS distribution and toxicity were examined after i.v. administration of CMA to mice. At doses $\geqslant 0.1$ mg/kg, a neurotoxic syndrome including ataxia, hypokinesia, and tremors appeared. At doses of $\leqslant 0.05$ mg/kg, which have been reported to be antineoplastic, no neurotoxicity was observed. On histopathologic examination, no changes were observed in the brain, spinal cord, or dorsal root ganglia. Unlike adriamycin (ADR), which rapidly appears in the nuclei of several tissues, CMA showed no fluorescence, suggesting a different cellular microcompartmentalization. The i.d. injection of CMA disclosed a 200-fold increase in toxicity compared with that of adriamycin. In comparisons of CMA and ADR, neurotoxicity and cardiotoxicity occurred equally only at higher doses; however, the dermatotoxicity and antineoplastic activity of CMA were increased several hundred-fold.

Introduction

The delivery of antineoplastic drugs to brain tumors is problematic for two principal reasons. Barriers exist that deny access of many drugs to regions within and adjacent to brain neoplasms. Furthermore, tumors metastatic to the brain are often insensitive to agents effective against the same tumors at other sites [14]. The selection of drugs capable of gaining access to these regions must take into consideration lipophilicity, degree of ionization, molecular weight, protein binding, and a host of other pharmacokinetic factors [21].

Adriamycin (ADR), an amphipathic chemotherapeutic agent, is virtually excluded from the brain; it has been shown to be ineffective against malignancies in the CNS, although carcinomas, sarcomas, or hematologic malignancies respond systemically [6]. The cyanomorpholinyl analog of adriamycin (CMA) has recently been described; it has several hundred-fold the in vitro antineoplastic activity of ADR against a number of tumor cell lines [1–3, 16, 17, 29, 31, 32]. CMA lacks the ionizing side group of ADR and is 81 times more lipophilic [3], suggesting the possibility of either a neurotoxic response to systemic administration or, in the absence of such, an effective antineoplastic activity in the CNS. The present experiments in mice examined the brain distribution of CMA, clinical and CNS histopathologic responses to this drug, and its dermatotoxicity, which is an important source of morbidity in patients receiving ADR.

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Materials and methods

Neuropathology. CMA was dissolved in dimethyl sulfoxide (DMSO) (J. T. Baker Chemical Co., Phillipsburg, NJ) and diluted in phosphate-buffered saline (PBS; pH 7.2) such that no injection contained >30 µl DMSO. Swiss Webster mice (10 weeks old) weighing approximately 20 g were lightly anesthetized with Metafane. They were then injected either i.v. or i.d. with a fresh solution of CMA at doses ranging from 0.01 to 10 mg/kg body weight. Controls were injected with identical solutions of DMSO in PBS without any drug. Regular examinations of spontaneous activity, gait, coordination, and response to auditory threat were carried out. The brain and vertebral column with spinal cord were removed from i.v. injected animals when they were found dead or at preselected times. The tissues were fixed in Vaughn's phosphate-buffered paraformaldehyde for 4 h, spines were decalcified, and brains and spines were paraffin-embedded, H/E-stained, and examined under the microscope.

Tissue fluorescence. The detection of CMA and ADR with fluorescence microscopy was verified by dropping solutions of varying concentrations onto slides of freshly sliced brain tissue. Both drugs showed distinctive fluorescence in the nuclei of brain cells, with the limit of detection for ADR being at a somewhat lower concentration ($> = 0.1\ \mu M$) than that of CMA ($> = 1.0\ \mu M$). For in vivo studies, mice were anesthetized and injected i.v. with a 0.2-ml bolus of either CMA (3–10 mg/kg), ADR (3–6 mg/kg), or vehicle alone. After varying lengths of time (20 min – 36 h), animals were sacrificed and the brain, kidney, liver, spleen, small intestine, and heart were removed and fixed in Vaughn's phosphate-buffered paraformaldehyde for 48 h. Frozen 6-µm-thick sections were taken and examined by phase contrast and fluorescence microscopy using a Rhodamine filter.

Dermatotoxicity. Mice were shaven and a depilatory agent (Nare) was applied 2–3 times, with warm-water rinses between applications. After 2–7 days, each mouse received

Table 1. Neurotoxicity in CMA-treated mice

Dose mg/kg	*n*	Method of injection	Neurotoxic status	Number of days surviving
10.0	2*	i.v.	+++	1
3.0	2	i.d.	+++	1
1.0	3	i.v.	+++	1–1.5
0.5	3	i.d.	+++	2–4
0.1	1*	i.v.	+++	2
0.05	2*	i.v.	N	14(s), 28(s)
0.015	3	i.d.	N	7(s)
0.0	1*	i.v.	N	14(s)

Mice were injected with CMA and observed. Animals with a status of +++ were observed to have neurotoxicity including ataxia, hypoactivity, and tremor at rest. A status of N indicates no evidence of neurotoxicity; * indicates that the animal's brain, spinal cord, and dorsal root ganglia were observed microscopically – no differences from the vehicle-treated control were seen; (s) indicates that the animal was sacrificed

five to seven 0.05-ml i.d. injections: one of vehicle alone, two or three of CMA (.0025–50 μg), and two or three of ADR (.0025–50 μg). To verify that the vehicle had no influence on neuro- or dermatotoxicity, two mice were given six i.d. injections of each vehicle; no sequelae were observed in these controls. Injection sites were examined daily for 7 days for induration, erythema, and ulceration. Additionally, mice receiving CMA were observed for evidence of behavioral neurotoxicity.

Results

Neuropathology

Results of the observations of mice injected with CMA are presented in Table 1. With increasing dose of CMA, survival times decreased; survival was prolonged with the i.d. route of injection, compared with the i.v. route. A threshold was observed for neurotoxicity: at doses of CMA $\geqslant 0.1$ mg/kg, all mice were seen to be hypothermic, cyanotic, severely ataxic, hyporesponsive to auditory threat, and hypoactive, with tremor at rest, within 6–12 h of injection. On microscopic examination, neurons, glia, white matter, and dorsal root ganglia at multiple levels were not found to be significantly different from those of the control brain or spinal cord; mild autolytic changes were seen in most specimens. None of the animals injected with ADR for the fluorescence studies displayed evidence of neurotoxicity.

Fluorescence

Table 2 shows the results of fluorescence studies of ADR and CMA in different tissues. The nuclei in CMA and control tissue specimens showed no fluorescence; exceptions were the liver and spleen, which exhibited the autofluorescent greenish hue also seen in specimens treated with ADR. The background fluorescence interfered with the localization of trace amounts of anthracyclines in these tissues. In the brain, a very faint staining only in the plasma membranes of neurons and of the choroid plexus was seen 40 min after i.v. injection of 6.5 mg/kg CMA. A similar membrane-staining pattern was seen in the kidney; this membranous staining pattern could not be photographically reproduced. Brain parenchyma and endothelia did not fluoresce after i.v. injection of either drug. ADR fluoresced in all kidney tubules, hearts, and small intestines, as well as in the choroid plexus in 6/6 animals examined. Positive ADR fluorescence was distinctly orange, nuclear in location, and consistently well above the lower levels of detection. Since no such nuclear staining was observed with CMA after in vivo administration, even at a dose of 10 mg/kg, it appears that this hydrophobic anthracycline analog, unlike ADR, does not accumulate in the cell nucleus. Figures 1 and 2 are photographs of the choroid plexus of a mouse that was sacrificed 20 min after an i.v. injection of 10 mg/kg ADR.

Dermatopathology

The results of i.d. injections of CMA and ADR are listed in Table 3. These mice survived 7 days and exhibited no neurotoxic symptoms. Three other animals receiving 1, 10, and 50 μg of ADR on the left side and identical doses of CMA on the right side displayed erythema and induration when they succumbed to neurotoxicity at 1–4 days after i.d. injection (data not shown).

Table 2. Fluorescence microscopic detection of CMA and ADR

Drug	Dose mg/kg	Time before sacrifice	Brain			Kidney
			Endothelia	Parenchyma	Choroid plexus	
CMA	3.0	30 min	–	–	–	–
	6.4	40 min	–	–	+/–	+/–
	10.0	36 h	–	–	–	–
ADR	6.0	40 min	–	–	+	+
	7.5	30 min	–	–	+	+
	10.0	15 min	–	–	+	N
	10.0	20 min	–	–	+	N
	10.0	30 min	N	N	N	+
	10.0	2 h	–	–	+	+
	10.0	6 h	–	–	+	+

Mice were injected i.v. with ADR, CMA, or with drug-free solvents as controls. The heart and small intestine were positive in 3/3 animals injected with ADR (not shown). The liver and spleen exhibited nonspecific background fluorescence in all animals. PBS-injected controls were uniformly negative in all tissues examined. No fluorescence is indicated by –; positive fluorescence, by +. Occasional, faintly positive cells in a tissue are designated as +/–. Samples that were not examined are marked by N

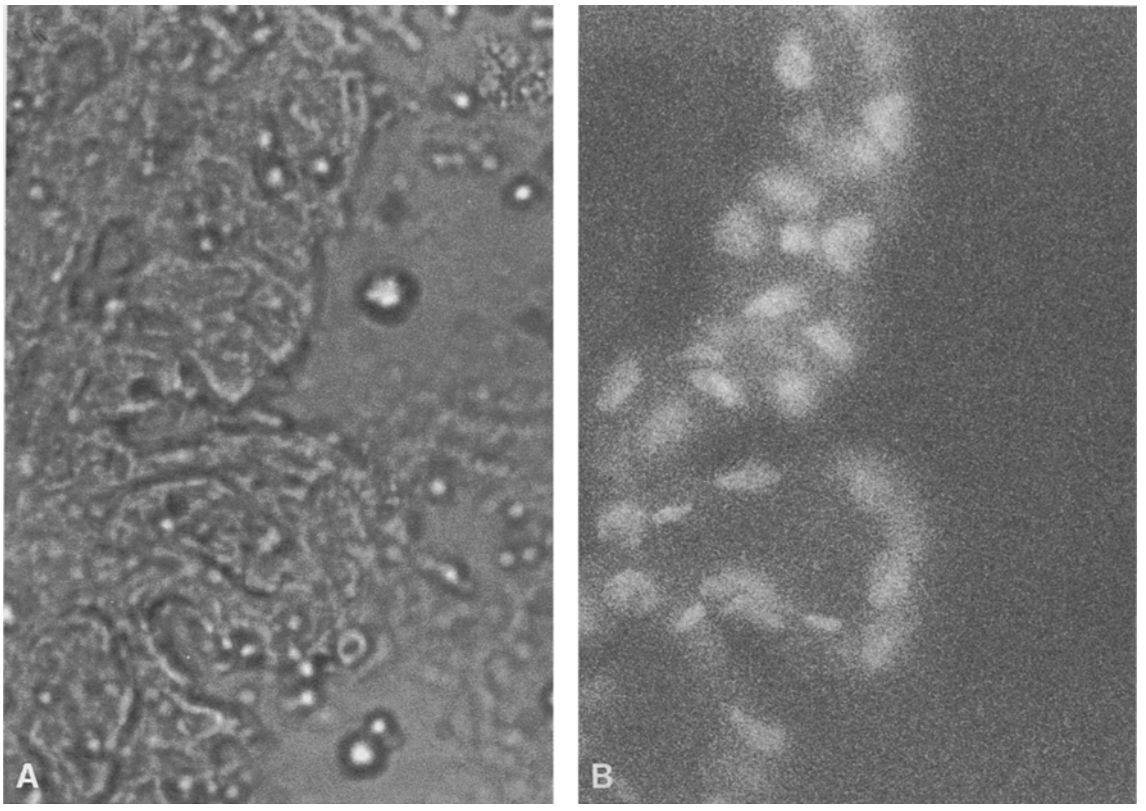

Fig. 1. Coronal section (6 μm) through the mid-cerebrum of the brain of a mouse sacrificed 20 min after i.v. injection of 10 mg/kg ADR. **A,** light microscopy, × 400. **B,** fluorescence microscopy of the same section using a Rhodamine filter. Using identical concentrations of CMA, no nuclear accumulation of the drug was detectable

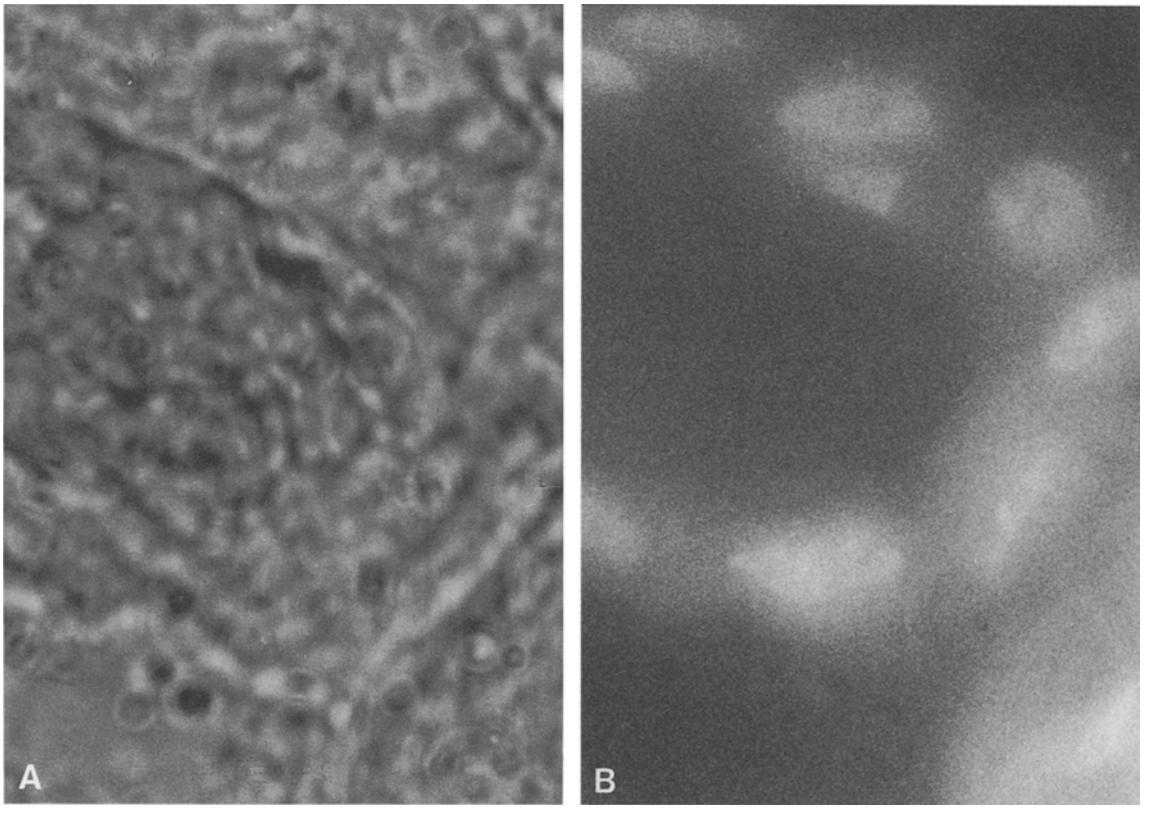

Fig. 2. Coronal section (6 μm) from the brain of the same mouse as in Fig. 1. **A,** light microscopy, × 1,000. **B,** fluorescence microscopy of the same section using a Rhodamine filter

Table 3. Dermatotoxicity of CMA

Dose (μg/kg)	CMA	ADR
12.5	4-mm² ulcer with erythema	0
12.5	4-mm² ulcer with erythema	0
12.5	8-mm² ulcer with erythema	0
1.25	2-mm² ulcer with erythema	0
1.25	1-mm² ulcer with erythema	0
0.125	0	0
0.125	0	0
0.125	0	0

Mice received multiple 0.05-ml i.d. injections; the local skin response to each is described for mice surviving 7 days. Doses are in the μg/kg injection site. Vehicle-injected control sites were normal in all cases.

Discussion

The nuclei of choroid epithelium were found to fluoresce brilliantly in 6/6 mice 15 min after i.v. injection of 6–10 mg/kg ADR; this particular finding has not previously been described. ADR has been reported to have negligible distribution in the brain, although choroid epithelium has not been considered independently [4, 5, 20, 28, 30, 36]. It is not detectable in human cerebrospinal fluid (CSF) 1–18 h after i.v. administration [6]. Merker et al. [22] found that monkeys receiving lower concentrations (3–10 μ*M*) of CSF-perfused ADR had occasions of increased rates of CSF formation. Although these increases may have been due to leaks caused by ADR-related damage to the choroid epithelium, an ADR-induced increase in Na^+/K^+ ATPase activity may be causative. Ouabain inhibition of ATPase activity is known to be biphasic in a number of tissues, including choroid epithelium; an increase in CSF production occurs at lower ouabain concentrations [26]. At concentrations of 0.1 m*M*, ADR inhibits Na^+/K^+ ATPase in kidney slices, although at lower concentrations (0.05 m*M*) it may actually stimulate the enzyme [13]. As with ouabain, low concentrations of ADR may increase CSF production rates, a possibility that requires further investigation.

CMA nuclear fluorescence was not detected in the CNS or any other tissue examined. ADR is concentrated in one cellular compartment, allowing for easy detection; however, CMA may be more diffusely distributed, leading to greater difficulty in detection. The lipophilic ADR analog AD-32 is taken up extensively by the liver [15]. No CMA-related hepatic fluorescence was seen, although the background fluorescence may have interfered with its detection. In the preliminary in vitro studies with tissue sections, CMA showed detectable nuclear fluorescence, which indicates that if it were concentrated in the nucleus in vivo, it would have been detectable. Because no substantial concentrations of CMA were detected in the kidney or liver between 0.5 and 36 h after injection, an altered metabolic fate is improbable, and changes at the tissue microcompartmental level are more likely.

None of the brains or spinal cords examined showed evidence of significant neuropathologic changes, although the time between CMA exposure and death was never longer than 2 days in this group of mice. However, CMA entrance into the CNS is suggested by the immediate appearance of ataxia, hypoactivity, tremors, and other neurotoxic symptoms in all 11 animals receiving doses ≥ 0.1 mg/kg. ADR is not normally associated with toxicity of the nervous system [7, 8, 19, 27, 34]. However, this neurotoxic syndrome has previously been seen with perfusion of ADR through the monkey CSF space. ADR (0.03–0.5 mg/kg) was delivered in 1–5 perfusions of 190 min duration. Of 12 animals, 5 died, 3 had an angiopathy, and 8 displayed hypokinesia, tremors, and/or ataxia soon after dosing [22]. This technique represents a relatively mild brain exposure to ADR. From the data of Blasberg and co-workers [9, 10], the expected depth of penetration for ADR from any brain-CSF interface was <3 mm. Neuwelt et al. [24, 25] directly exposed dog brains to ADR via intracarotid bolus after osmotic blood-brain barrier (BBB) modification. Most animals displayed seizures or coma and, on histopathologic examination, were found to have areas of necrosis and/or hemorrhage as early as 2.5 h after ADR administration. The CMA central neurotoxicity seen in mice (Table 1) is identical to that described by Merker et al. [22] in monkeys, although comparison is difficult due to the different routes of drug administration. CMA given i.v. or i.d. can therefore gain access to the CNS, although in a manner different from that with intracarotid bolus injected directly into brain tissue. We demonstrated a threshold of 0.1 mg/kg for the expression of CMA central neurotoxicity.

Evidence is lacking for CMA entrance into the CNS at subtoxic doses, which may represent a limitation of either drug access or neurotoxicity. In either case, CNS toxicity need not be considered in the use of CMA for non-CNS neoplasms, as effective systemic antineoplastic doses [22] appear to be below the threshold for CMA neurotoxicity. There is some evidence that lower concentrations of ADR, regardless of the route of delivery, can exist in the brain without causing toxicity [22, 24]. If the same holds true for CMA, ingress of the drug into the CNS will occur during systemic administration, but without consequence. Therefore, i.v. administration of CMA for CNS neoplasms might be a possibility.

Peripheral ganglion cell necrosis has been noted in the rat after high doses [11] and 2 days after low doses of ADR [18]. In the present study, no changes were noted in the dorsal root ganglion neurons after CMA administration in mice. The i.d. injection of ADR in the mouse has been validated as a model for toxicity due to ADR extravasation in humans [12]. In that study, 50 μg ADR produced ulcerations of approximately the same size as those seen with 0.25 μg CMA in the present study, indicating a 200-fold increase in dermatotoxicity. The i.v. infusion of CMA must be done at sufficiently dilute concentrations and with sufficient care to avoid this effect.

CMA is highly liphophilic and contains no ionizable size groups, retaining no charge with wide changes in pH [3]. Equilibration across the BBB is therefore not unexpected, since charged molecules are generally excluded from entry into the brain. Neurotoxicity does not occur at minute doses of CMA, which may correspond to effective antineoplastic doses, since CMA is the most potent anthracycline yet synthesized [34]. Sikic et al. [29] have previously shown that CMA cardiotoxicity is related to the dose of the anthracycline molecule, not to its relative antineoplastic potency. Free-radical production secondary to redox cycling is thought to be responsible for the cardiotoxicity

[23] and may underlie the neurotoxicity as well. The extraordinary antineoplastic effect of CMA may be derived from iminium ion production from the cyanomorpholinyl group [1] or from secondary events that induce DNA damage [35]. Either mechanism might also contribute to the observed 200-fold increase in CMA dermatotoxicity. In comparisons of CMA and ADR neurotoxicity and cardiotoxicity occur equally on a molar basis; however, the dermatotoxicity and antineoplastic activity of CMA are increased several hundred-fold.

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