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Murine Pharmacokinetics of 6-Aminonicotinamide (NSC 21206), a Novel Biochemical Modulating Agent

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ABSTRACT. The pyridine nucleotide 6-aminonicotinamide (6AN) was shown recently to sensitize a number of human tumor cell lines to cisplatin *in vitro*. The present studies were undertaken to compare the drug concentrations and length of exposure required for this sensitization *in vitro* with the drug exposure that could be achieved in mice *in vivo*. Human K562 leukemia cells and A549 lung cancer cells were incubated with 6AN for various lengths of time, exposed to cisplatin for 1–2 hr, and assayed for Pt-DNA adducts as well as the ability to form colonies. K562 cells displayed progressive increases in Pt-DNA adducts and cisplatin sensitivity during the first 10 hr of 6AN exposure. An 18-hr 6AN exposure was likewise more effective than a 6-hr 6AN exposure in sensitizing A549 cells to cisplatin. HPLC analysis of 6AN and its metabolite, 6-amino-NAD^+, permitted assessment of exposures achieved *in vivo* after i.v. administration of 10 mg/kg of 6AN to $CD2F_1$ mice. 6AN reached peak serum concentrations of 80–90 μM and was cleared rapidly, with $T_{1/2\alpha}$ and $T_{1/2\beta}$ values of 7.4 and 31.3 min, respectively. Bioavailability was 80–100% with identical plasma pharmacokinetics after i.p. administration. At least 25% of the 6AN was excreted unchanged in the urine. The metabolite 6-amino-NAD^+ was detected in perchloric acid extracts of brain, liver, kidney, and spleen, but not in serum. Efforts to prolong systemic 6AN exposure by administering multiple i.p. doses or using osmotic pumps resulted in lethal toxicity. These results demonstrated that 6AN exposures required to sensitize tumor cells to cisplatin *in vitro* are difficult to achieve *in vivo*. BIOCHEM PHARMACOL **58**;6:1057–1066, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. 6-aminonicotinamide; cisplatin; pharmacokinetics

The nicotinamide analog 6AN†† (NSC 21206) currently is undergoing preclinical investigation as an agent that modulates the cytotoxicity of antineoplastic treatments, particularly DNA-damaging chemotherapeutic agents. Originally synthesized as a niacin antagonist [1], 6AN has been reported to enhance sensitivity of mammalian tumor cells to bis(chloroethyl)nitrosourea [2–4] and ionizing radiation [5]. In combination with *N*-(phosphonacetyl)-L-aspartate and 6-methylmercaptopurine riboside, 6AN also has been observed to enhance the therapeutic efficacy of 5-fluorouracil, paclitaxel, and doxorubicin in animal tumor models [6–9]. None of these previous preclinical studies included any assessment of 6AN pharmacokinetics or metabolism.

Recent results have indicated that 6AN also sensitizes a number of human tumor cell lines to the cytotoxic effects of cisplatin *in vitro* [4, 10]. In a previous study from our laboratory, this increase in cisplatin sensitivity was explained by enhanced formation of Pt-DNA adducts and the subsequent induction of apoptosis [4]. These changes appear to result from 6AN-induced increases in cisplatin accumulation on a neutral amino acid transporter [4]. Our studies also indicated that 6AN is metabolized to 6-amino-NAD^+. Moreover, inhibition of 6-amino-NAD^+ formation prevented the effects of 6AN [4], suggesting that formation of 6-amino-NAD^+ is required for sensitization of cells to cisplatin.

Although the *in vitro* results suggested that 6AN might augment the effects of cisplatin, initial experiments per-

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†† *Abbreviations*: 6AN, 6-aminonicotinamide; 6AN-NO, the N-oxide of 6AN; 6-amino-NAD^+, the nicotinamide adenine dinucleotide analog containing 6AN in place of nicotinamide; amu, atomic mass unit; AUC, area under the curve; and 6MN, 6-methylnicotinamide.

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formed by the National Cancer Institute failed to demonstrate therapeutic synergy between cisplatin and 6AN in tumor-bearing mice.* These latter experiments employed a single bolus of 6AN at a wide range of concentrations 3 hr before cisplatin. The apparent failure to modulate the antitumor effects of cisplatin *in vivo* prompted us to compare conditions of 6AN exposure required to sensitize human tumor cell lines to cisplatin *in vitro* with exposure parameters that can be achieved *in vivo*. In these studies, we have examined the duration of 6AN exposure required to sensitize two different human tissue culture cell lines to 6AN *in vitro*. We have also characterized the pharmacokinetics and metabolism of 6AN administered to mice by two different routes and three different schedules. Comparison of the results of these two sets of experiments indicates that the 6AN exposures required to sensitize human tumor cell lines *in vitro* can be achieved in mice, but only with considerable toxicity.

MATERIALS AND METHODS

Materials

6AN was supplied by the Pharmaceutical Resources Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute. 6MN, nicotinamide, NAD^+, $NADP^+$, NADPH, *Bungarus fasciatus* venom NADase, and cisplatin were purchased from the Sigma Chemical Co. Ultrapure hydrochloric acid (Tracepure Plus, EM Sciences) was redistilled into clean Teflon containers. Platinum and rhodium standards from J. T. Baker were diluted serially in redistilled ultrapure hydrochloric acid to yield working standards from 0.2 to 20 ng/mL. All other chemicals were analytical grade; all solvents were HPLC grade.

Synthesis of 6AN-NO was achieved by reaction of 6AN (0.5 g, 3.65 mmol) with hydrogen peroxide (0.41 g, 3.65 mmol) in glacial acetic acid (10 mL) at 75° for 3 hr. The yellow residue obtained by removal of the solvent under reduced pressure was recrystallized from ethanol. The product was characterized by ^{1}H-NMR and electrospray ionization-mass spectroscopy.

Cell Culture

Human K562 leukemia cells and A549 lung non-small cell lung cancer cells (from American Type Tissue Culture) were cultured at 37° in a humidified atmosphere of 95% air:5% CO_2 using RPMI 1640 medium containing 5% heat-inactivated fetal bovine serum, 100 U/mL of penicillin G, 100 μg/mL of streptomycin, and 2 mM glutamine (medium A).

To perform colony forming assays with K562 cells, 1-mL aliquots containing 3–5 × 10^5 cells were incubated with 250 μM 6AN in medium A for 0–24 hr. A 1-μL aliquot of DMSO containing the indicated final concentration of cisplatin was added subsequently for 1 hr. After cells were washed, dilutions were plated in 0.3% agar in the medium of Pike and Robinson [11], incubated for 10–14 days at 37°, and examined on an inverted phase contrast microscope so that colonies containing ≥ 50 cells could be counted. Survival was expressed relative to control cells incubated with the corresponding concentration of 6AN in the absence of cisplatin treatment. Control experiments indicated that 6AN treatment for 24 hr diminished proliferation but did not affect the plating efficiency of K562 cells. Plates of untreated or 6AN-treated cells generally contained 300–500 colonies.

Clonogenic survival of A549 cells was determined as described previously [4]. In brief, aliquots containing 300–500 A549 cells were plated in 35-mm tissue culture plates containing 2 mL of medium A and incubated for 18 hr in the absence or presence of the indicated concentration of 6AN. Alternatively, cells were incubated for 14 hr in the absence of 6AN followed by 6 hr in the presence of the indicated concentration of 6AN. Then cisplatin was added at the indicated final concentration. A somewhat longer cisplatin exposure time (2 hr) was utilized to decrease any effect of small differences in exposure time that might result from the need to wash large numbers of plates of adherent cells in order to perform these experiments. After a 2-hr incubation, cells were washed twice with serum-free RPMI 1640 and incubated in drug-free medium A for 7 days. The resulting colonies were stained with Coomassie brilliant blue and counted visually. Control plates generally contained 100–200 colonies.

Detection of Pt-DNA Adducts

K562 cells treated with 250 μM 6AN for 0–24 hr were treated with 40 μM cisplatin as described previously [4]. At the completion of the cisplatin treatment, cells were sedimented at 200 *g* for 10 min, washed three times with ice-cold PBS, and lysed in 5 mL of TEN buffer [10 mM Tris–HCl (pH 7.4 at 21°), 10 mM EDTA, 150 mM NaCl] supplemented with 0.4% SDS and 1 mg/mL of proteinase K. After incubation at 50° for 16 hr, highly purified DNA samples were prepared by extraction with phenol/$CHCl_3$ and $CHCl_3$, ethanol precipitation, RNAse A treatment, phenol/$CHCl_3$ and $CHCl_3$ extraction, and *Hin*dIII digestion as previously described in detail [4, 12]. After aliquots (2 μg of DNA) were subjected to electrophoresis on agarose minigels to confirm complete removal of RNA and digestion of DNA, DNA was re-extracted with phenol/$CHCl_3$ and $CHCl_3$, ethanol precipitated, resuspended in 750 μL of 0.6 M HCl, and heated to 95° for 30 min. The DNA concentration was estimated by measuring absorbance at 260 nm; elemental Pt was assayed by inductively coupled plasma mass spectrometry using a modification of a procedure previously described [13–16]. In brief, a Gilson AS90 autosampler operating at a rate of 0.5 mL/min was used to infuse samples into a Perkin-Elmer Sciex Elan 6000 mass spectrometer operating at the following settings: Ar nebulizer flow rate, 0.9 L/min; inductively coupled plasma RF

* Sausville EA, personal communication. Cited with permission.

power, 1200 W; lens voltage, 8.0 V; analog stage voltage, -2100 V; pulse stage voltage, 1700 V; dwell time, 100 nsec/amu. Pt was expressed as the sum of Pt species detected at 194 and 195 amu using a program that sweeps from 1 to 263 amu 50 times per reading. Pt standards (0.2 to 20 ng/mL in 0.6 M HCl) were utilized to confirm the linearity of the assay ($R \geq 0.999$), and rhodium 103 served as an internal standard. Each unknown was determined in duplicate. Each experiment was performed a minimum of three times.

HPLC Analysis

Reverse-phase HPLC analysis was performed on a Hewlett-Packard 1090M tertiary gradient liquid chromatograph equipped with an SGE ODS-Inertsil column (100 mm × 4 mm i.d., 5 μm) and a Brownlee RP-18 (15 mm × 3.2 mm, 7 μm) guard column. 6MN served as the internal standard. To detect 6AN, proteins were precipitated from biological fluids (0.2 mL) by the addition of ice-cold methanol (0.9 mL). Following centrifugation, supernatants were evaporated by centrifugation under reduced pressure, and residues were reconstituted in 100 μL of 50 mM KH_2PO_4, pH 7 (Solvent A) prior to chromatography. Cell extracts prepared by extraction of 2–5 × 10^6 cells with 0.5 M $HClO_4$ were neutralized with KOH/potassium phosphate [17]. The separation of 6AN from its metabolites in biological fluids and extracts of tumor cells was accomplished by using a gradient from 100% Solvent A to 10:90 methanol:Solvent A (Solvent B). The elution profile was as follows: 0–4 min, Solvent A; 4–14 min, a linear gradient from Solvent A to Solvent B; 14–20 min, Solvent B; 20–25 min, Solvent A. The flow rate was 0.7 mL/min, and the eluate was monitored at 206 and 267 nm. Standard curves constructed by graphing the peak area ratio of 6AN/6MN versus the 6AN plasma concentration had a lower limit of sensitivity of 0.05 μg/mL; they were linear over the concentration range of 0.05 to 10.0 μg/mL and had coefficients of variation of the slope of 8.2%. Quantitation of 6-amino-NAD^+ was based on the number of moles of 6AN that were formed when the metabolite was hydrolyzed by NADase as previously described [4].

Murine Pharmacokinetic Studies

Non-tumored male $CD2F_1$ mice (20–30 g), supplied by the National Cancer Institute, were housed five per cage on commercially obtained pure wood shaving bedding in an on-site facility with light provided from 6:00 a.m. to 8:00 p.m. Food (Purina Rodent Chow) and tap water were provided *ad lib*. 6AN (1 mg/mL in 0.85% NaCl) at a dose of 10 mg/kg was administered i.v. or i.p. using tuberculin syringes fitted with 27-gauge needles. The i.v. dose was administered via the tail vein to mice restrained in standard Broome-type restraints. Blood samples (1 mouse per time point) were obtained by cardiac puncture from mice anesthetized under ether vapors during the 48-hr period following drug administration (10–15 time points). Specimens were drawn into citrated syringes (150 μL of citrate-phosphate-dextrose-adenine anticoagulant/mL of whole blood) and transferred to silanized microcentrifuge tubes. Plasma was separated by centrifugation (8300 *g* × 3 min) and stored frozen at −20° until analysis.

6AN Administration by Repeated i.p. Dosing or Osmotic Pump Infusion

To examine the effect of repeated 6AN administration, 20-g female athymic NCr-nu mice received 6AN at 10 mg/kg in 0.2 mL of 0.85% (w/v) NaCl–0.1% Tween 80 i.p. at 3-hr intervals for a total of three doses on days 1, 5, and 9. To examine the effect of continuous infusion, osmotic pumps (Alza) were implanted i.p. into athymic mice. Two solutions (9.5 and 14.3 mg/mL in 10^{-4} M HCl) were administered by 24-hr infusion at a rate of 8 μL/hr (3.8 and 5.7 mg/kg/hr, respectively). A single solution (76.1 mg/mL in 10^{-4} M HCl) was infused with a 7-day pump at a rate of 1.0 μL/hr (3.8 mg/kg/hr).

Analysis of 6AN Plasma Concentration–Time Data

Data were fitted by nonlinear least squares regression to a two-compartment open model using the program PCNONLIN. AUC values were determined by trapezoidal approximation.

RESULTS

6AN-Mediated Sensitization of K562 and A549 Cells to Cisplatin In Vitro

The present studies were undertaken to determine whether the 6AN exposures that sensitize human tumor cell lines to cisplatin *in vitro* could be achieved in mice. To facilitate this comparison, we initially examined the 6AN concentrations and durations of exposure required to sensitize cells to cisplatin *in vitro*.

As illustrated by the results in Fig. 1A, treatment of K562 human leukemia cells with 250 μM 6AN for 24 hr prior to a 1-hr exposure to cisplatin decreased the cisplatin LD_{90} in colony forming assays by a factor of 6.2 ± 1.4 (mean ± SD, N = 16). Additional experiments [4] have revealed that this 6AN treatment increases the number of cells undergoing cisplatin-induced apoptosis, confirming that the colony forming assays are accurately reflecting enhanced cytotoxicity of cisplatin in the presence of 6AN. When the 6AN pretreatment time is fixed at 18 hr and the 6AN concentration varied, sensitization has been observed with 6AN concentrations as low as 8 μM and is maximal at 31–1000 μM [4]. In the present study, the 6AN concentration was fixed at 250 μM and the effect of exposure duration was examined. Sensitization was almost maximal after a 10-hr 6AN pretreatment (Fig. 1A). In contrast, little sensitization was observed with a 1- or 3-hr 6AN pretreatment (Fig. 1A). Measurement of Pt in highly purified preparations of DNA

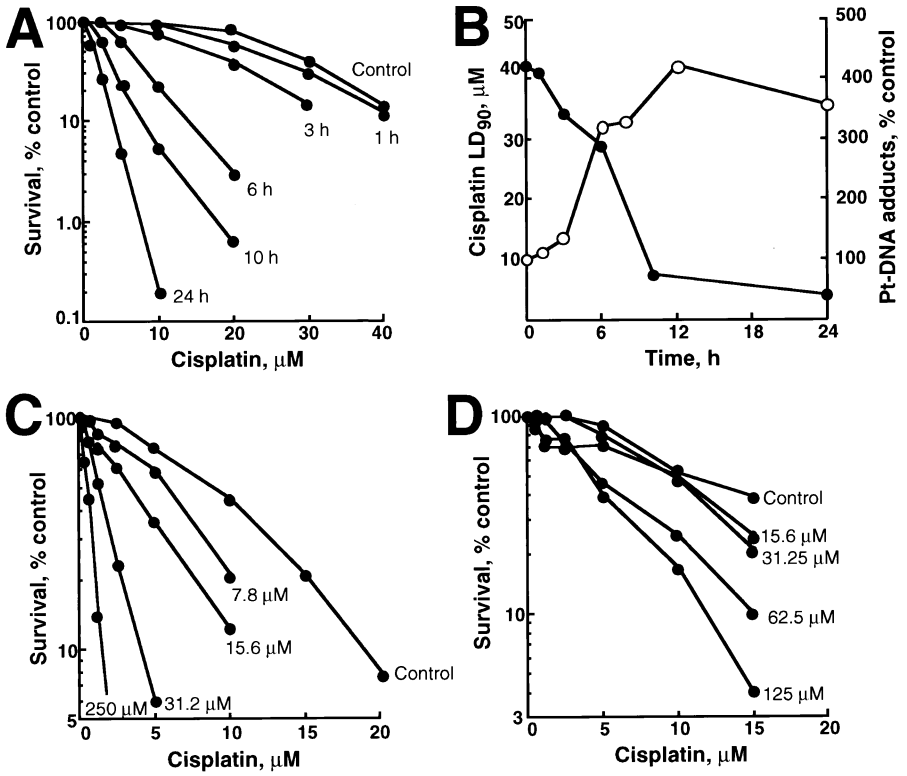

FIG. 1. Effect of 6AN treatment on K562 cells and A549 cells *in vitro*. (A) K562 cells were incubated with 250 μM 6AN for 0–24 hr. The indicated concentration of cisplatin was then added for 1 hr. Cells were washed and plated in drug-free 0.3% agar to allow colonies to form. (B) Data from panel A plotted as LD_{90} vs duration of 6AN pretreatment (●). Also shown are relative levels of Pt-DNA adducts formed in K562 cells after various durations of pretreatment with 250 μM 6AN (○). (C and D) A549 cells were treated with the indicated concentration of 6AN for 18 (C) or 6 hr (D) prior to the addition of cisplatin for an additional 2 hr. At the completion of the incubation, cells were incubated in drug-free medium for 8 days to allow colonies to form. All results are representative of at least three experiments. Control samples in panels A, C, and D contained 330 ± 20, 130 ± 10, and 160 ±10 colonies, respectively.

revealed that the 6AN pretreatment enhanced the formation of Pt-DNA adducts over the same time course that it enhanced the cytotoxicity of cisplatin (Fig. 1B), providing further support for the view that these two changes are related. In additional experiments, we observed that a 1-hr exposure to 250 μM 6AN followed by a 5-hr drug-free interval was no more effective than a 1-hr exposure to 6AN (data not shown).

To confirm that the requirement for relatively prolonged 6AN exposure was not unique to the K562 cell line, A549 cells were incubated with various concentrations of 6AN for 6 hr or 18 hr prior to cisplatin exposure. Results of these studies indicated that an 18-hr 6AN exposure sensitized cells to cisplatin (Fig. 1C). This effect was readily detectable at 16 μM 6AN and resulted in an 8-fold decrease in LD_{50} (from 10 to 1.25 μM) at 31 μM 6AN. In contrast, when the 6AN preincubation was shortened to 6 hr, the maximal decrease in LD_{50} was 3-fold (Fig. 1D); high concentrations of 6AN were required for this sensitization. Identical results also were obtained with HT29 human colon carcinoma cells (data not shown). These observations provided the impetus for measuring 6AN pharmacokinetics in mice to ascertain whether concentrations of 15–30 μM for 24 hr would be potentially attainable.

Murine Pharmacokinetic Studies After Single Dose Administration

Reverse-phase HPLC was utilized to analyze 6AN pharmacokinetics in mice. This analysis method permitted separation and quantitation of 6AN, 6MN (internal standard), nicotinamide, 6-aminonicotinic acid, nicotinic acid, and 6-amino-NAD^+ [Fig. 2A and Ref. 4]. Endogenous nicotinamide was detected routinely in plasma, but was fully separated from 6AN, internal standard, and metabolites under the HPLC conditions utilized (Fig. 2, B and C).

Elimination of 6AN initially was characterized in mice following i.v. administration of 10 mg/kg. This dose, which has been utilized extensively in previous studies employing 6AN *in vivo* [6–9], is one-fourth the reported single-dose LD_{50} in mice [1]. Plasma 6AN concentrations peaked at 80–90 μM and declined rapidly to undetectable values (<1 μM) within 3 hr of drug administration (Fig. 3). Analysis of distribution and plasma elimination revealed $T_{1/2\beta}$, total

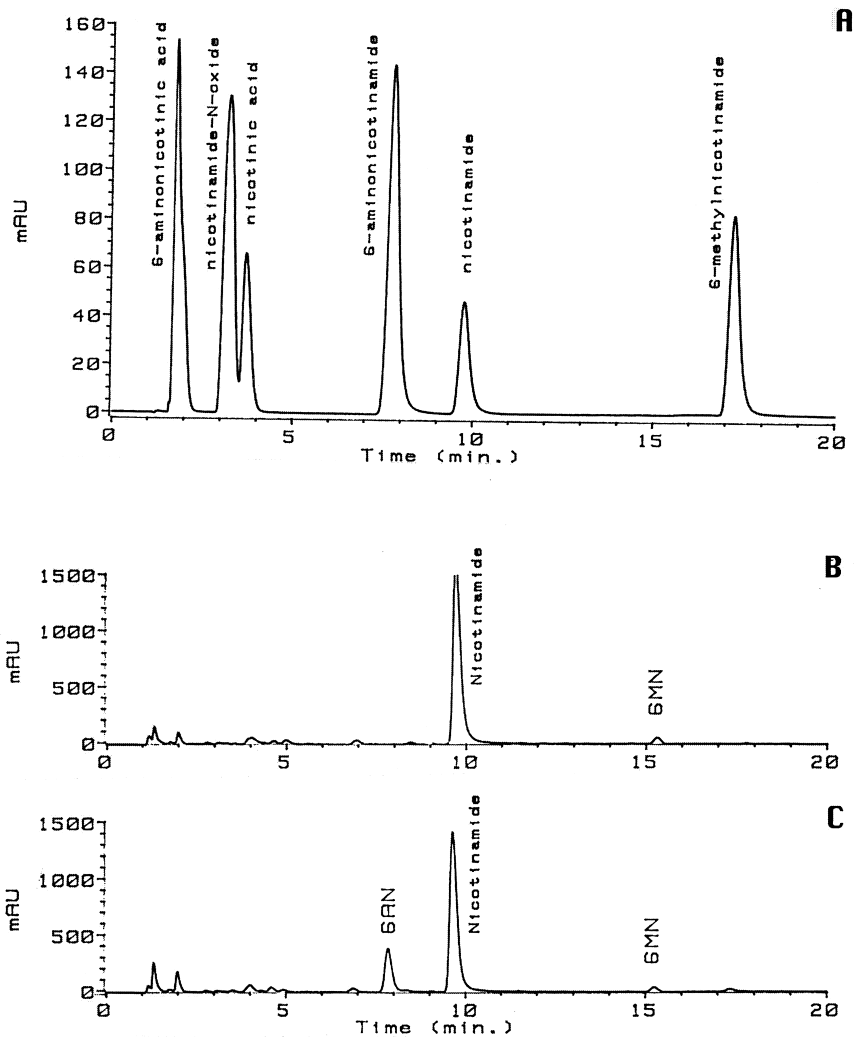

FIG. 2. HPLC chromatograms of 6AN and related analogs (10 μg/mL) prepared in mobile phase (A) and blank mouse plasma (B) and mouse plasma collected 3 min following i.v. administration of 6AN (C).

body clearance (Cl_{TB}), and steady-state volume of distribution (V_{ss}) values (mean ± SD) of 35.6 ± 13.6 min, 31 ± 2 mL/min/kg, and 315 ± 21 mL/kg, respectively.

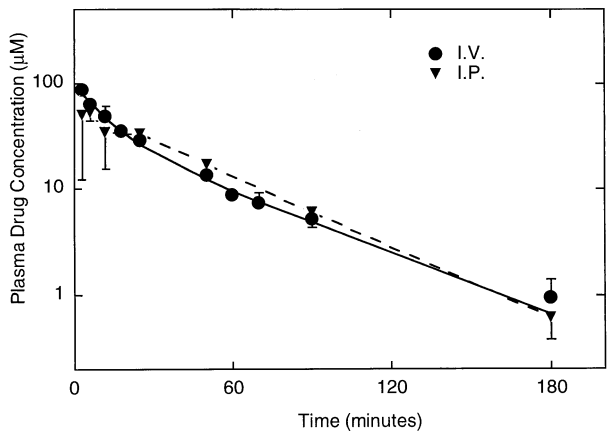

FIG. 3. Plasma profiles of 6AN following i.v. and i.p. administration of 10 mg/kg to male $CD2F_1$ mice. Symbols represent the means ± SD of three mice at each time point.

Urinary recovery of parent drug was 25–35% over 48 hr. We could not detect the presence of 6AN metabolites in urine, although the presence of many interfering materials in HPLC eluates limits the ability to detect metabolites. Based on previous reports of metabolism of nicotinamide to nicotinamide N-oxide [18, 19], we anticipated that 6AN-NO might be formed from 6AN. 6AN-NO was not detected in serum (Fig. 2C) or urine.

Because many of the preclinical studies examining the potential role of 6AN as a sensitizing agent *in vivo* (see introduction) have utilized an i.p. route for this agent, 6AN pharmacokinetics also were examined after i.p. administration. Plasma drug concentrations were variable for the first 3–12 min following i.p. administration but thereafter were similar to those detected after i.v. administration (Fig. 3). The total bioavailability of 6AN following i.p. administration was 80–100% of that following i.v. administration. Systemic exposure to 6AN following i.p. administration, therefore, was similar to that achieved following i.v. administration.

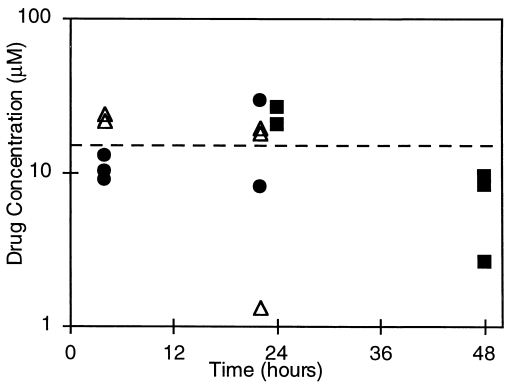

FIG. 4. Plasma concentrations of 6AN following 24-hr osmotic pump infusion of 9.5 mg/mL (low dose) (●), 14.3 mg/mL (high dose) (Δ), or 48-hr infusion with a 7-day pump at a concentration of 76.1 mg/mL (■) (equivalent to low dose in the 24-hr pump group). The target plasma concentration was 15 μM (—).

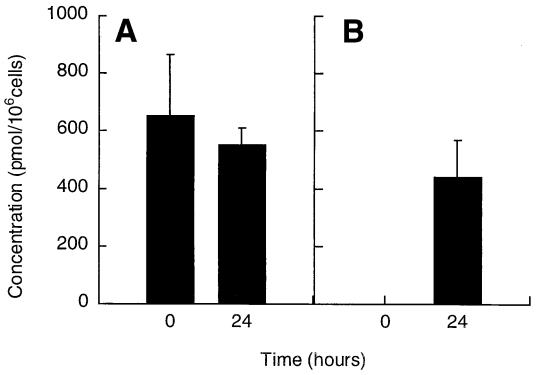

FIG. 5. Levels (pmol/10^6 cells) of NAD (A) and 6-amino-NAD^+ (B) in extracts of K562 cells treated with 250 μM 6AN for 24 hr. Values are means ± SD, N ≥ 3.

Effect of Repeated or Prolonged 6AN Administration

When considered in the context of the *in vitro* sensitization data described in Fig. 1, the 6AN pharmacokinetic data suggested that i.v. or i.p. bolus administration of 6AN to mice might not produce the concentration and duration of systemic exposure required to enhance cytotoxicity of cisplatin. Observation of animals treated with single boluses of 6AN revealed anorexia and decreased activity levels 6–36 hr after the administration of a single 6AN dose of 10 mg/kg, but no lethality. In an attempt to prolong the systemic exposure to 6AN, mice were given multiple i.p. injections of 10 mg/kg of 6AN at 3-hr intervals for three doses. Observation of these animals revealed a 10% weight loss 4 days after the injection. When the series of 6AN injections was performed according to this q3 hr × 3 schedule on days 1, 5, and 9 (a commonly utilized cisplatin schedule in xenograft work), animals displayed a 20% weight loss by day 9 and were uniformly dead by day 12 even in the absence of cisplatin administration. Similar toxicity was observed with 6AN doses as low as 6.7 mg/kg/dose on this schedule.

In an effort to evaluate an alternative dosing strategy, 6AN was administered continuously by implanted osmotic pumps with infusions planned for 24 hr and for 7 days. Doses and delivery rates of drug were selected based on the i.v. pharmacokinetic data, with the intent of achieving plasma concentrations of 15–30 μM for periods of ≥ 24 hr. Plasma concentration–time data for 24-hr infusions of two 6AN doses (3.8 and 5.7 mg/kg/hr) and for 48 hr of a planned 7-day (3.8 mg/kg/hr) infusion of 6AN to mice are shown in Fig. 4. The desired 24-hr plasma concentration of ~15 μM was achieved for the higher 24-hr dose rate and was nearly achieved at the lower 24-hr dose rate. As expected, when the 76 μg/hr delivery rate was employed in the 7-day infusion study, similar concentrations were achieved for 24 and 48 hr, after which the 7-day infusion was halted. Animals receiving the continuous infusion of 6AN demonstrated profound toxicity. Several days after completion of the 24-hr infusion schedule, and by 48 hr of the planned 7-day infusions, mice receiving 6AN by continuous osmotic infusion displayed decreased motor activity, depression of body temperature, body weight loss, and a general irreversible course of toxicity that required the killing of the animals and the termination of the experiments. Although pharmacokinetic analyses suggested that continuous infusion schedules in mice could yield the desired 6AN steady-state plasma concentrations and exposure times, toxicity of these treatments precluded further testing for *in vivo* efficacy in tumor-bearing mice.

Tissue Levels of 6AN and 6-Amino-NAD^+

Based on previous results suggesting that metabolism of 6AN to 6-amino-NAD^+ might be required for the 6AN-mediated sensitization of cells to cisplatin *in vitro* [4] and on earlier metabolic studies indicating that 6AN might be converted *in vivo* to 6-amino analogs of nicotinamide mononucleotide and $NADP^+$ as well [20, 21], we examined the formation of 6AN-derived pyridine dinucleotides *in vitro* and *in vivo*. When K562 cells were treated with 250 μM 6AN for 24 hr *in vitro*, parent drug (173 ± 51 pmol/10^6 cells) and 6-amino-NAD^+ (450 ± 50 pmol/10^6 cells) were readily detected (Fig. 5).*

Following administration of 6AN to mice for pharmacokinetic studies, plasma, urine, and selected tissues were assayed likewise for the presence of 6AN and 6-amino-NAD^+. Brain, liver, kidney, and spleen were removed from mice at various time points and subjected to perchloric acid extraction. HPLC of these perchloric acid extracts revealed that 6AN was readily detected in these tissues (Fig. 6B). Concentrations of parent drug initially were highest in liver, kidney, and spleen. As was observed in plasma, 6AN was eliminated rapidly from all tissues, with half-life values ranging from 19–74 min (Fig. 7A).

Although 6-amino-NAD^+ was not detectable in plasma

* For purposes of comparison with levels measured in murine tissues after administration of 6AN *in vivo*, these levels in K562 cells correspond to ~1000 pmol 6-amino-NAD^+/mg protein.

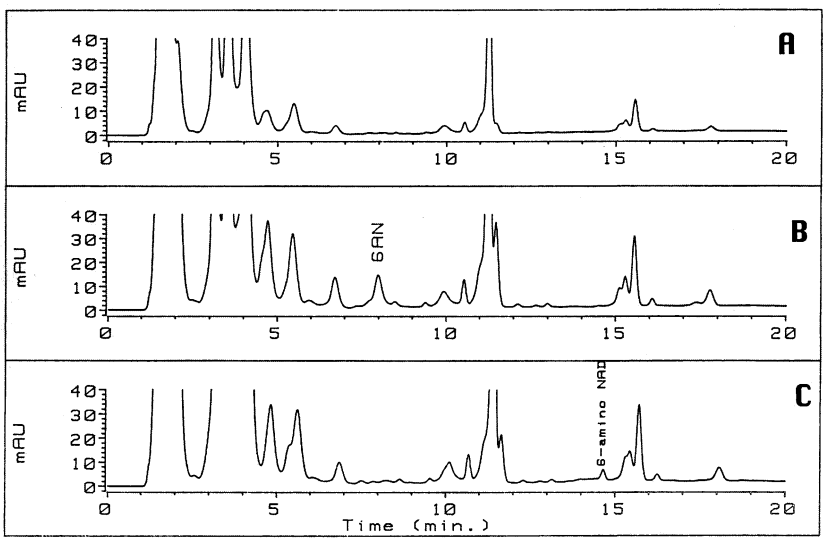

FIG. 6. HPLC chromatograms of blank mouse liver extract (A) and extracts of mouse liver collected 3 min (B) and 6 hr (C) following i.v. administration of 6AN to mice.

(Fig. 2C and data not shown), it became detectable in tissue 12 min (kidney, liver, and spleen) to 90 min (brain) following i.v. administration of 6AN. Results obtained in liver at 3 min and 6 hr following 6AN administration are shown in panels B and C of Fig. 6. The identity of the indicated metabolite as 6-amino-NAD^+ was confirmed by its conversion to 6AN and ADP-ribose in the presence of NADase as well as its comigration with authentic 6-amino-NAD^+. In contrast to 6AN, which was eliminated rapidly from tissue, 6-amino-NAD^+ concentrations increased over the first several hours (Fig. 7B). Although 6-amino-NAD^+ levels in spleen declined dramatically by 24 hr, 6-amino-NAD^+ concentrations of 3–30 pmol/mg tissue were observed in the other three tissues 48 hr following administration of a bolus of 6AN (Fig. 7B).

DISCUSSION

The present results represent the first reported pharmacokinetic analysis of 6AN. This agent was originally described as a niacin antagonist [1] and was found subsequently to have antineoplastic activity in animal studies [22–24]. Phase I trials conducted over 30 years ago suggested that 6AN also might have antineoplastic activity in the clinical setting [25, 26], but development of this agent was abandoned after it failed to show clinical activity in a phase II trial in renal cell carcinoma [27]. All of these studies were performed before pharmacokinetic analyses were a routine component of anticancer drug development and before HPLC was developed.

Interest in 6AN has been rekindled by a series of reports indicating that 6AN, alone or in combination with *N*-(phosphonacetyl)-L-aspartate and 6-methylmercaptopurine riboside, enhances the therapeutic efficacy of bis(chloroethyl)nitrosourea, 5-fluorouracil, paclitaxel, doxorubicin and ionizing radiation in animal tumor models [2, 5–9]. More recently, 6AN has been reported to sensitize a number of human tumor cell lines to cisplatin *in vitro* [4, 28]. Because the studies conducted by the National Cancer Institute did not suggest therapeutic synergism between a single i.v. dose of 6AN and cisplatin *in vivo*, we compared conditions of 6AN exposure required for sensitization of human tumor cell lines to cisplatin *in vitro* with *in vivo* 6AN exposure. Given the strong evidence that 6AN metabolites are ultimately responsible for the enhanced cytotoxicity of cisplatin [4], we also investigated 6AN metabolism. The results of these studies have implications for future efforts to develop 6AN as a potential modulating agent.

Studies with multiple tumor cell lines revealed that prolonged (10–24 hr) exposures to 6AN were required to effectively sensitize tumor cell lines to cisplatin *in vitro* (Fig. 1). In contrast, we found that 6AN was eliminated from murine plasma after i.v. or i.p. injection with a terminal half-life of ~30 min (Fig. 3). Even though peak plasma concentrations at ~1/4 the murine LD_{50} were 80–90 μM (Fig. 3), well above *in vitro* concentrations required for the enhanced cytotoxicity of cisplatin, rapid clearance of 6AN quickly reduced the levels to < 1 μM. In short, our data suggest that the lack of synergy when 6AN and cisplatin are administered to tumor-bearing animals as single boluses 3 hr apart is explained by the fact that the duration of 6AN exposure achieved after a single bolus *in vivo* (Fig. 3) was much shorter than the duration of exposure required to sensitize cells to cisplatin (Fig. 1).

In view of the short half-life of 6AN in the bloodstream (Fig. 3), coupled with rapid clearance of this water-soluble agent from peripheral tissues (Fig. 7), it did not appear that increasing the amount administered as a single bolus would achieve the exposure required for sensitization of tumor cells to cisplatin. Anticancer drugs are frequently administered by continuous infusion, particularly when mechanistic considerations such as cell cycle dependence or phar-

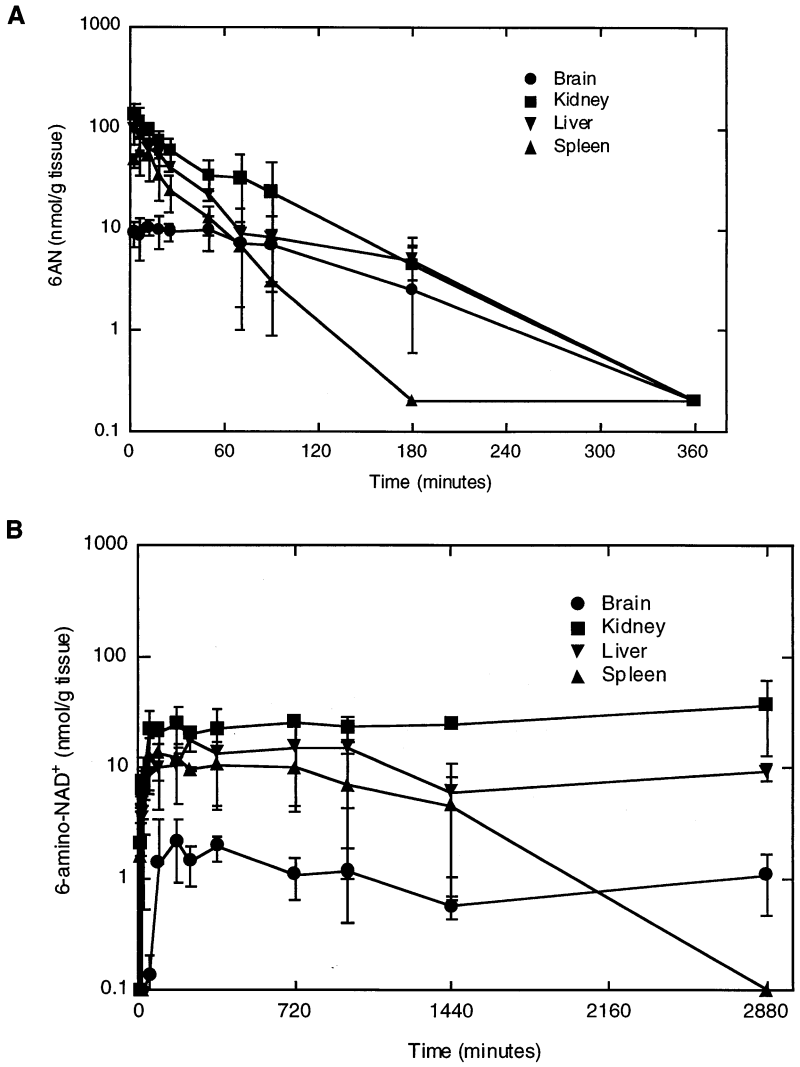

FIG. 7. Concentration of 6AN (nmol/g tissue) (A) and 6-amino-NAD$^+$ (nmol/g tissue) (B) in tissue collected from male CD2F$_1$ mice following i.v. administration of 6AN (10 mg/kg). Values are means ± SD, N = 3.

macokinetic considerations such as rapid elimination dictate such an approach. Based on the pharmacokinetic parameters derived from the bolus studies, 6AN was delivered by continuous infusions from osmotic pumps at rates calculated to yield plasma 6AN concentrations and durations of exposure required for sensitization to cisplatin *in vitro* (Fig. 1). Dose rates of 76 and 114 μg/hr for 24 or 48 hr of a planned 7-day infusion did yield plasma concentrations in the 15 μM range for ≥ 24 hr (Fig. 4). However, toxicities observed following completion of the 24-hr infusion, or at 48 hr into the planned 7-day infusion, appeared to preclude the prolonged continuous infusion schedules in mice. Likewise, repeated administration of the 10 mg/kg dose at 3-hr intervals for three doses was associated with extreme toxicity. Even though repeated bolus drug infusions and prolonged continuous infusions both provide exposures to 6AN concentrations that would be expected to produce enhanced sensitivity to cisplatin, neither of these approaches offers a practical 6AN treatment modality tolerated by mice.

Based on the previous observation that conversion of 6AN to 6-amino-NAD$^+$ was required for the sensitization of cells to cisplatin [4], 6-amino-NAD$^+$ was assayed in tissues following 6AN administration. The intracellular levels of 1–30 pmol 6-amino-NAD$^+$/mg protein that were formed in normal tissues (Fig. 7B) were far below those that formed in K562 cells sensitized to cisplatin (Fig. 5).* Nonetheless, these concentrations were sufficient to induce toxicity.

Early studies demonstrated that 6AN can cause profound neurological toxicity as a consequence of neuronal cell death [29]. Although the levels of 6AN and 6-amino-NAD$^+$ were not particularly high in the central nervous system (Fig. 7), the measured levels represent an average of concentrations in various cell types and might not accurately reflect neuronal levels of 6AN and 6-amino-NAD$^+$.

* For purposes of comparison with levels measured in murine tissues after administration of 6AN *in vivo*, these levels in K562 cells correspond to ~1000 pmol 6-amino-NAD$^+$/mg protein.

Results presented in Fig. 7B indicate that 6-amino-NAD^+ had a long half-life, possibly explaining why toxicity was cumulative when 6AN was administered continuously or by repeated bolus injection every 4 days. Although 6-amino-NAD^+ had an equally long half-life in some other tissues (Fig. 7B), two other factors might contribute to the profound neurotoxicity of 6AN. First, metabolites of 6AN have been shown to inhibit 6-phosphogluconate dehydrogenase, a key enzyme in the pentose phosphate shunt [21, 30, 31]. Given the strong reliance of neurons on this metabolic pathway [reviewed in Refs. 21 and 30], it is possible that inhibition of this pathway by 6AN is particularly toxic to neuronal cells. Second, Snyder and coworkers have demonstrated recently that consumption of NAD^+ facilitates neuronal cell death [32] and inhibition of NAD^+ consumption protects against neuronal cell death [32, 33]. It is possible that 6AN lowers NAD^+ more in neurons than in other tissues, thereby contributing to neuronal cell death. Further experiments are required to assess these possibilities.

Even though the pharmacological and toxicological features described above might make it difficult to utilize 6AN successfully to modulate cisplatin sensitivity *in vivo*, its chemosensitizing properties *in vitro* are striking [Fig. 1 and Ref. 4]. The observation that 6-amino-NAD^+ is formed *in vivo* raises the possibility that other precursors of this metabolite might have more favorable properties that could allow testing of this class of compound as a potential biochemical modulating agent *in vivo*. Alternatively, further investigations into the mechanism of action of 6AN might permit the identification of other agents that exert a similar effect on cisplatin accumulation and formation of Pt-DNA adducts without the neurotoxicity of 6AN. These possibilities appear to warrant further investigation.

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