6-Aminonicotinamide Sensitizes Human Tumor Cell Lines to Cisplatin

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ABSTRACT

The nicotinamide analogue 6-aminonicotinamide (6AN) is presently undergoing evaluation as a potential modulator of the action of various antineoplastic treatments. Most previous studies of this agent have focused on a three-drug regimen of chemical modulators that includes 6AN. In the present study, the effect of single-agent 6AN on the efficacy of selected antineoplastic drugs was assessed in vitro. Colony-forming assays using human tumor cell lines demonstrated that pretreatment with 30–250 μM 6AN for 18 h resulted in increased sensitivity to the DNA cross-linking agent cisplatin, with 6-, 11-, and 17-fold decreases in the cisplatin dose that diminishes colony formation by 90% being observed in K562 leukemia cells, A549 non-small cell lung cancer cells, and T98G glioblastoma cells, respectively. Morphological examination revealed increased numbers of apoptotic cells after treatment with 6AN and cisplatin compared to cisplatin alone. 6AN also sensitized cells to melphalan and nitrogen mustard but not to chlorambucil, 4-hydroperoxycyclohexadiene alone. 6AN also sensitized cells to melphalan and nitroscyanide; DRB, 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole; FBS, heat-inactivated fetal bovine serum; LD₅₀, (LD₁₀₀), dose that abolishes the formation of 6ANAD⁺, the increase in Pt-DNA adducts, and the sensitizing effect of 6AN in clonogenic assays. These observations identify 6AN as a potential modulator of cisplatin sensitivity and suggest that the 6AN metabolite 6ANAD⁺ exerts this effect by increasing cisplatin accumulation and subsequent formation of Pt-DNA adducts.

INTRODUCTION

Cisplatin is a Pt(II) coordination compound that is widely used in the treatment of carcinomas of the ovary, lung, and upper aerodigestive tract as well as glioblastomas and germ cell testicular tumors. This agent enters cells by passive diffusion and by facilitated uptake on one or more transporters (reviewed in Ref. 1). Once inside the cell, cisplatin is converted to a hydrated Pt(II) coordination complex that forms DNA interstrand and intrastrand cross-links (2). Although the role of each type of cross-link in the effects of cisplatin remains a subject of debate, the Pt-DNA cross-links set into motion a series of events that result in G₂ arrest (3), followed in some cells by apoptosis (4, 5).

In tissue culture cell lines, several potential mechanisms of cisplatin resistance have been described (reviewed in Refs. 1, 2, and 6–8), including diminished drug uptake, increased detoxification by GSH and metallothionein, increased repair of Pt-DNA adducts by a process that appears to involve nucleotide excision repair proteins ERCC1 (9) and XPG (10, 11), and alterations in mismatch repair (12, 13). These observations have led to the investigation of agents that might modulate cisplatin resistance.
Fig. 1  Effect of 6AN on cisplatin sensitivity. A, K562 leukemia cells incubated for 18 h in medium A lacking (solid line) or containing (dotted line) 250 μM 6AN were treated for 1 h with various concentrations of cisplatin (CDDP), washed, and plated in agar to allow determination of survival by colony-forming assays. B, K562 cells were incubated with 0, 7.8, 15.6, 31.2, 62.5, 250, or 1000 μM 6AN for 18 h, exposed to various concentrations of cisplatin for 1 h, and plated in agar to determine clonogenic survival. C–F, effect of 6AN on cisplatin sensitivity of A549 (C), T98G (D), MCF-7 (E), and Ovcar-3 (F) cells. Cells were incubated for 18–24 h in tissue culture medium lacking (solid line) or containing (dotted line) 6AN at 500 μM (F), 250 μM (C), 125 μM (D), or 31 μM (E) and then treated with various concentrations of cisplatin for 2 h. Colony formation was determined as described in “Materials and Methods.” Bars, ±1 SD from quadruplicate (A) or triplicate (C–E) samples.

Another factor in cisplatin resistance might be PARP, a nuclear enzyme that has been implicated in the response to other types of DNA damage (15–19). PARP is a zinc finger protein that binds with high affinity to bent DNA (20). This DNA binding enhances the catalytic activity of the enzyme up to 100-fold (16, 21), resulting in transient accumulation of protein-linked polymers containing up to 200 ADP-ribose subunits within cells (22). Covalent attachment of these ADP-ribose polymers to topoisomerase I, topoisomerase II, and DNA polymerase α can inhibit these enzymes (reviewed in Ref. 23), contributing to diminished DNA and RNA synthesis in cells with damaged DNA. In addition, the highly charged ADP-ribose polymers can compete histones away from DNA (24) and enhance accessibility of damaged chromatin to repair enzymes (25).

Several lines of indirect evidence have implicated PARP in cisplatin resistance: (a) treatment of O-342 rat ovarian carcinoma cells with cisplatin results in increased synthesis of poly-(ADP-ribose) polymers (26); (b) a cisplatin-selected subline of 342 cells (O-342/CDDP) contains elevated PARP levels compared to parental cells (27); (c) O-342/CDDP cells are sensitized to cisplatin by the PARP inhibitor 3-aminobenzamide (28).

In the present study, we evaluated 6AN, a known PARP inhibitor (29), as a potential modulator of cisplatin cytotoxicity in vitro. Previous studies have shown that 6AN, when used in the context of a cocktail of modulators that includes N-(phosphonomethyl)-L-aspartate and 6-methylmercaptopurine riboside, contributes to sensitization of spontaneous autochthonous mu-
Fasciatus venom NADase, yeast GSH reductase, yeast phospho-
in this sensitization: (a) 6AN has been shown to inhibit PARP directly (29); (b) 6AN treatment is associated with inhibition of the NAD'-requiring enzyme 6-phosphogluconate dehydro-
rase, accumulation of its substrate 6-phosphogluconate, and depletion of products downstream from this enzyme, notably 5'-phosphoribosyl-1'-PP, (34–36), thereby decreasing 5'-phos-
phoribosyl-1'-PP-dependent biosynthesis of NAD' and ATP (37, 38). Because intracellular NAD' concentrations approxi-
mate the K_m of PARP for this substrate, NAD' depletion also inhibits formation of ADP-ribose polymers (39, 40).

Despite the promising results obtained with the 6AN-con-
taining cocktail of modulating agents in vivo (30–33), there have been few studies describing the ability of single-agent 6AN to modulate the effects of DNA-damaging treatments (36, 41–43). In the present study, the effect of 6AN on the cytotoxicity of a number of different DNA-damaging antineoplastic agents was examined. At 6AN concentrations that are themselves non-
cytotoxic, 6AN was observed to relatively selectively increase the toxicity of cisplatin in a variety of tissue culture cell lines. Additional investigations revealed that this effect did not corre-
late with NAD' or ATP depletion, PARP inhibition, or altered Pt-DNA adduct removal. Instead, 6AN treatment resulted in increased cisplatin accumulation and Pt-DNA adduct formation.

Materials and Methods

Materials. Reagents were purchased from the following suppliers: cisplatin, melphalan, GSH, chlorambucil, 6AN, 3AP, 6PG, ATP, nicotinamide, NAD', NADP', NADPH, Bungarus fasciatus venom NADase, yeast GSH reductase, yeast phospho-
gluconate dehydrogenase, luciferin-luciferase reagent, and [14C-
carbonyl]nicotinamide (31.5 mCi/mol) from Sigma Chemical Co. (St. Louis, MO); 5,5'-dithiobis(2-nitrobenzoic acid) and DHQ from Aldrich (Milwaukee, WI); HindIII from Life Tech-
ologies, Inc. (Gaithersburg, MD); proteinase K from Boehringer-
Mannheim (Indianapolis, IN); bicinechonic acid from Pierce (Rockford, IL); platinum standard from J. T. Baker (Phillips-
burg, NJ); and BSO from Chemical Dynamics (South Plainfield, NJ). RNase A (RAF grade; Worthington, Freehold, NJ) was prepared as a 10 mg/ml solution in H_2O and boiled before storage at ~20°C. BCNU and 4HC were kindly provided by the Drug Synthesis Branch of the National Cancer Institute and Dr. O. Michael Colvin (Duke University Cancer Center, Durham, NC), respectively.

Cell Culture. Cell lines (from American Type Culture Collection, Rockville, MD, unless indicated) were cultured at 37°C in a humidified atmosphere of 95% air:5% CO_2 using the following media, all of which contained 100 units/ml penicillin G, 100 µg/ml streptomycin, and 2 mM glutamine: K562 myeloid leukemia cells (from Richard J. Jones, Johns Hopkins Oncology Center) and A549 non-small cell lung cancer cells in RPMI 1640 containing 5% FBS (medium A); T98G glioblastoma cells in Eagle’s minimal essential medium containing Earle’s balanced salt solution, nonessential amino acids, and 10% FBS supplemented with 1 mM sodium pyruvate (medium B); MCF-7 breast adenocarcinoma cells in medium B containing 10 µg/ml bovine insulin; and Ovcar-3 ovarian adenocarcinoma cells in RPMI 1640 containing 10 µg/ml bovine insulin and 20% FBS. To ensure logarithmic growth, cultures were maintained at densities below 1 x 10^6 cells/ml (K562) or 70–80% confluence.

Fig. 2 Effect of 6AN on cisplatin-induced apoptosis in K562 (A) and HL-60 (B) cells. A. K562 cells incubated with diluent or 250 µM 6AN for 18 h were treated for 1 h with diluent or 10 µM cisplatin. After washing, cells were incubated for 4 days in drug-free medium and stained with Hoechst 33342. The number of morphologically apoptotic cells (arrowhead in A') was determined by counting >600 cells exposed to the indicated treatment. B. The number of morphologically apoptotic cells observed on slides of HL-60 cells treated with diluent or 10 µM cisplatin after an 18-h incubation in the absence or presence of 62.5 µM 6AN. A' and B', cells treated with diluent for 18 h prior to a 1-h exposure to 10 µM cisplatin. A" and B", cells treated with 6AN for 18 h prior to a 1-h exposure to 10 µM cisplatin. Results are representative of three experiments with each cell line.
A549 cells were incubated for 18–24 h in tissue culture medium lacking (solid line) or containing (dotted line) 125–250 µM 6AN. Melphalan (A), nitrogen mustard (B), or 4HC (C) was then added for an additional 2 h. After the cells were washed, colony formation was determined as described in “Materials and Methods.” In D and E, K562 cells were incubated for 18–24 h in tissue culture medium lacking (solid line) or containing (dotted line) 250 µM 6AN and then treated for 1 h with the indicated concentration of chlorambucil (D) or etoposide (E). At the completion of the treatment, colony formation was determined as described in “Materials and Methods.” Bars, ±1 SD.

**Colony-forming Assays.** Aliquots containing 3–5 × 10⁵ log phase K562 cells in 1 ml of medium A were incubated with 0–1000 µM 6AN for 18 h as indicated. A 1-µl aliquot of DMSO containing the indicated final concentration of cisplatin was added for 1 h. Cells were then sedimented at 200 × g for 10 min, washed, and plated in 0.3% agar in the medium of Pike and Robinson (44). After a 10–14-day incubation at 37°C, colonies containing ≥50 cells were counted on an inverted phase contrast microscope. Survival was expressed relative to control cells incubated with the corresponding concentration of 6AN in the absence of cisplatin treatment. Control experiments revealed that 6AN treatment for 18 h did not affect the plating efficiency of K562 cells.

Aliquots containing 300–500 A549 cells were plated in 35-mm tissue culture plates containing 2 ml of medium A and incubated for 16–18 h in the absence or presence 125–250 µM 6AN. Cisplatin was then added at the indicated final concentration. After a 2-h 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 manually. Control plates generally contained 100–200 colonies. Experiments with T98G and MCF-7 cells were performed in an identical fashion, except that 6AN was decreased to 125 and 31 µM, respectively (concentrations chosen to minimize the effect of 6AN on colony formation in these cell lines); medium A was replaced with media described above; 750 MCF-7 cells were plated to insure 100–200 colonies in control plates; and MCF-7 cells were incubated for 14 days to allow visible colonies to grow.

When cisplatin was replaced by other drugs, experiments were performed identically. Melphalan, BCNU, and chlorambucil prepared as 10 mM stocks in 25 mM HCl (melphalan) or 95% ethanol (BCNU, chlorambucil) were stored at −20°C. Stocks of 4HC (10 mM in H₂O), nitrogen mustard (10 mM in 25
mm HCl), etoposide, and daunorubicin (17 mm and 1 mm, respectively, in DMSO) were prepared fresh. Concentrated (×1000) working dilutions of these agents were prepared in the same diluents immediately before the addition to the cells.

**Morphological Assessment of Apoptosis.** Aliquots containing 5 × 10⁶ K562 cells in 1 ml of medium A were incubated with or without 250 μM 6AN for 18 h and then treated with 1 μl of DMSO containing the indicated final concentration of cisplatin. After a 1-h incubation, the supernatant was removed, and the cells were resuspended in 1 ml of medium A. One h later, the supernatant was again replaced with fresh medium A. Cells were diluted 1:3 (cisplatin-treated) or 1:10 (DMSO-treated) in medium A and incubated for 4 days at 37°C in an atmosphere containing 5% (v/v) CO₂. HL-60 cells were treated identically, except that the 6AN concentration was decreased to 62.5 μM and the incubation time after cisplatin treatment was shortened to 2 days. Cells were then sedimented at 200 × g, fixed in 3:1 (v/v) methanol:acetic acid, deposited on glass slides, stained with 1 μg/ml Hoechst 33342, and examined under epi-illumination using a Zeiss Axiosplan microscope equipped with a N.A. 1.40 × 63 objective, a 365-nm excitation filter, and a 420-nm emission filter. A minimum of 600 cells/sample were scored for apoptotic changes as described previously (45, 46).

**Measurement of Pt-DNA Adducts.** For each data point, 10⁸ log phase K562 cells or 10 subconfluent 100-mm tissue culture plates (∼10⁷ adherent cells/plate) were incubated with 40 μM cisplatin in the media described above for 1 h (K562 cells) or 2 h (adherent cells). At the completion of the incubation, adherent cells were washed once with ice-cold PBS and released by brief trypsinization. All cells were collected by centrifugation 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°C), 10 mM EDTA, and 150 mM NaCl) supplemented with 0.4% SDS and 1 mg/ml proteinase K. After incubation at 50°C for 16 h, highly purified DNA samples were prepared by extraction with phenol/CHCl₃ and CHCl₃, ethanol precipitation, RNase A treatment, phenol/CHCl₃ and CHCl₃ extraction, and HindIII digestion as described previously in detail (47, 48). After aliquots (2 μg DNA) were subjected to electrophoresis on agarose minigels to confirm complete removal of RNA and digestion of DNA, DNA was then reextracted with phenol/CHCl₃ and CHCl₃, ethanol precipitated, resuspended in 750 μl of 0.6 M HCl, and heated to 95°C for 30 min. The DNA concentration was estimated using an enzyme cycling assay described previously (45, 46).

**Whole-Cell Cisplatin Accumulation.** Duplicate 100 mm plates of subconfluent A549 cells were incubated in medium A in the absence or presence of 250 μM 6AN for 24 h. Freshly prepared cisplatin was added to a final concentration of 40 μM from a ×1000 concentrated stock. After a 2-h incubation at 37°C, cells were washed once with ice-cold PBS, briefly trypsinized at 37°C, sedimented at 200 × g for 10 min, washed three times with ice-cold PBS, reacted overnight at 21°C with 0.5 ml concentrated nitric acid, heated to 100°C for 5 min, diluted with 0.5 ml 30% (w/w) H₂O₂, and heated again to 100°C for 5 min (47). Elemental platinum was determined as described for platinum-DNA adducts. Protein content was measured by the bicinchoninic acid method (49).

**Estimation of GSH, NAD, ATP, and 6PG.** Samples containing 2–5 × 10⁶ cells were extracted with 0.5 M HClO₄ and neutralized with KOH/potassium phosphate as described previously (50). GSH was assayed by a modification of the method of Eyer and Podhradsky (51). Freshly prepared cell extracts or reduced GSH standards (0–1500 pmol) in 40 μl of neutralized HClO₄ were mixed in the wells of a 96-well microtiter plate with 140 μl of reaction mixture containing 0.1 M 4-morpholineethanesulfonic acid, 50 μM phosphoric acid, 1 μM viologen, 5,5'-dithiobis(2-nitrobenzoic acid), 100 μM NADPH, and 0.02% (w/v) BSA. After GSH reductase (100 ng in 20 μl of 50 mM sodium phosphate-2 mM EDTA, pH 7.0) was added to each well, plates were incubated at 37°C for 45 min. Absorbance at 405 nm was measured using a microtiter plate reader.

Aliquots of neutralized perchloric acid extracts were stored at −70°C until NAD⁺, ATP and 6PG were assayed. NAD⁺ was estimated using an enzyme cycling assay described previously (52). Purified NAD⁺ (0–20 pmol) provided a standard curve. ATP content of the same neutralized perchloric acid extracts was estimated using a luciferin/luciferase luminescent assay according to instructions provided by the supplier. ATP (0.025–250 pmol) provided a standard curve. 6PG was assayed by spectrophotometrically measuring the production of NADPH from 6PG and NADP⁺ in the presence of 6-phosphogluconate.

**Table 1** Effect of 6AN on sensitivity of human cancer cell lines to various antineoplastic agents

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Drug</th>
<th>Dose-modifying factor (n)</th>
</tr>
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<tbody>
<tr>
<td>K562</td>
<td>Cisplatin</td>
<td>6.2 ± 1.4 (16)</td>
</tr>
<tr>
<td></td>
<td>Melphalan</td>
<td>1.9 ± 0.6 (5)</td>
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<tr>
<td></td>
<td>Chlorambucil</td>
<td>0.9 ± 0.2 (3)</td>
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<tr>
<td></td>
<td>Daunorubicin</td>
<td>1.1 ± 0.1 (3)</td>
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<tr>
<td></td>
<td>Etoposide</td>
<td>1.5 ± 0.2 (3)</td>
</tr>
<tr>
<td>A549</td>
<td>Cisplatin</td>
<td>11.4 ± 3.4 (5)</td>
</tr>
<tr>
<td></td>
<td>Melphalan</td>
<td>8.0 ± 2.9 (4)</td>
</tr>
<tr>
<td></td>
<td>Nitrogen mustard</td>
<td>3.3 ± 1.2 (4)</td>
</tr>
<tr>
<td></td>
<td>4HC</td>
<td>1.3 ± 0.3 (4)</td>
</tr>
<tr>
<td>T98</td>
<td>Cisplatin</td>
<td>17 ± 5 (3)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Cisplatin</td>
<td>9 ± 5 (4)</td>
</tr>
<tr>
<td></td>
<td>4HC</td>
<td>0.8 ± 0.2 (2)</td>
</tr>
<tr>
<td>Ovcar-3</td>
<td>Cisplatin</td>
<td>2.2 ± 0.2 (3)</td>
</tr>
</tbody>
</table>

*Cells were incubated in the absence and presence of 6AN for 18–24 h and then exposed to the indicated agent for 1 h (K562 cells) or 2 h (adherent cell lines) in the continued presence of 6AN. After removal of 6AN, colony formation was determined as depicted in Figs. 1 and 3. The effect of 6AN on colony formation was quantitated by determining the dose-modifying factor, i.e., the LD₉₀ with 6AN + LD₉₀ without 6AN. The number of replicate experiments is indicated in parentheses.

Because Ovcar-3 cells do not form colonies, experiments were performed by incubating 20,000 cells in 60-mm tissue culture plates in medium without or with 500 μM 6AN for 18 h, exposing cells to cisplatin for 2 h, washing the cells, incubating for 5 days in drug-free medium, and counting surviving cells in a hemocytometer after release by trypsinization.
dehydrogenase as described (53). 6PG (2–500 pmol) provided a standard curve.

Effect of 6AN on NAD\(^+\) Synthesis. Log phase K562 cells in medium A were incubated with 5 \(\mu\)M \[^{14}\text{C}\]nicotinamide in the absence or presence of 6AN for up to 24 h. At the end of the incubation, cells were washed twice with ice-cold PBS and lysed in 70% ethanol (54). After macromolecules were removed by sedimentation at 16,000 \(\times\) g for 5 min, supernatants were dried under vacuum; solubilized in ethanol; supplemented with unlabeled pyridine nucleotides including nicotinamide, NMN, NAD\(^+\), and NADP\(^+\) to permit visualization under UV light; and subjected to TLC on polyethyleneimine cellulose plates (55). After two ascents in 1:3 (v/v) 2 M NH\(_4\)Cl in 0.12 M sodium citrate (pH 5.3); 95% ethanol, labeled NAD\(^+\) was scraped from the appropriate spot \((R_f \approx 0.15)\) and quantitated by scintillation counting.

Detection and Quantitation of 6AN and 6ANAD\(^+\). Reverse-phase HPLC was performed on a Hewlett-Packard 1090M ternary gradient liquid chromatograph equipped with an SGE ODS-Inertsil column (100 mm \(\times\) 4 mm internal diameter; 5 \(\mu\)) and a Brownlee RP-18 (15 mm \(\times\) 3.2 mm; 7 \(\mu\)) guard column. Fifty-\(\mu\)l aliquots of neutralized HClO\(_4\) extracts (see above) were applied to the system and eluted at a flow rate of 0.7 ml/min using a gradient from 50 mM KH\(_2\)PO\(_4\), pH 7 (solvent A) to 10:90 methanol:solvent A (solvent B) according to the following program: 0–4 min, solvent A; 4–14 min, a linear gradient from solvent A to solvent B; 14–20 min, solvent B; and 20–25 min, solvent A. The eluate was monitored at 267 nm. Authentic 6AN, nicotinamide, and NAD\(^+\) dissolved in neutralized perchloric acid were used as standards.

The identity of the 6AN metabolite as 6ANAD\(^+\) was based on electrospray ionization mass spectroscopy as well as confirmation that NADase hydrolyzed the metabolite to the expected products of 6AN and ADP-ribose. For mass spectroscopy, 6AN metabolite eluting at 14 min was isolated from 6AN-treated K562 cells using the HPLC procedure outlined above. To desalt the metabolite, pooled fractions were lyophilized, reconstituted in H\(_2\)O, injected onto the chromatograph a second time, and eluted with water replacing solvent A. Pooled metabolite was concentrated by lyophilization, resuspended in 1:1 methanol: H\(_2\)O, and subjected to electrospray ionization mass spectroscopy in the negative mode. This analysis revealed a parent ion \(m/e = 667\), the expected mass of 6ANAD\(^+\). To confirm this identification, the isolated metabolite was also dissolved in 50 mM KH\(_2\)PO\(_4\), 1 mM EDTA (pH 7.5), incubated for 2 h at 37°C with NADase [isolated from Bungarus fasciatus venom as reported by Anderson and Anderson (56)], and subjected to HPLC as described above using authentic 6AN and ADP-ribose as standards. Comparison to standard curves indicated that equimolar amounts of these compounds had been generated upon hydrolysis of the 6AN metabolite with NADase. Quantitation of the 6ANAD\(^+\) peak in subsequent experiments was based on the number of moles of 6AN that were formed when the metabolite peak was hydrolyzed by NADase.

RESULTS

6AN Sensitizes Human Cancer Cells to Cisplatin. Recent reports implicating PARP in the response to platinating agents (see "Introduction") prompted us to examine the effect of 6AN on cisplatin sensitivity. Treatment of K562 leukemia cells with 250 \(\mu\)M 6AN for 18 h prior to cisplatin exposure decreased the cisplatin LD\(_{50}\) by a factor of 6.2 \(\pm\) 1.4 (mean \(\pm\) SD, \(n = 16\); Fig. 1A). Dose-response curves revealed that the effect of 6AN was evident at doses as low as 8 \(\mu\)M and was essentially maximal at 32 \(\mu\)M (Fig. 1B). Increased cisplatin sensitivity was
Sequential examination of K562 cells treated for 1 h with 10-40 μM 6AN indicated that cisplatin sets into motion a series of biochemical changes that culminate in programmed cell death. A previous observation from this laboratory (4) and others have suggested that DNA repair or PARP inhibition is not involved in the sensitization to cisplatin induced by 6AN. The degree of sensitization varied from much as 9-fold after cisplatin treatment (Fig. 2B). Additional studies were performed to confirm that the results were not an artifact of the colony-forming assays. Previous observations from this laboratory (4) and others (5, 57) have indicated that cisplatin sets into motion a series of biochemical changes that culminate in programmed cell death. Sequential examination of K562 cells treated for 1 h with 10-40 μM cisplatin revealed that the cells stopped proliferating, maintained a normal morphology for 3–4 days, and then began to take up the vital dye trypan blue over the ensuing 4–5 days (data not shown). These changes are presumably responsible for the inhibition of colony formation. Examination of K562 cells 4 days after a 1-h treatment with 10 μM cisplatin revealed that a small number displayed apoptotic morphological features (Fig. 2A, A'). Although 6AN itself had no effect, treatment with 250 μM 6AN for 24 h prior to cisplatin exposure increased the number of apoptotic cells an average of 3.4-fold (± 1.1-fold, n = 3) relative to cisplatin alone (Fig. 2A, A'). In a similar fashion, 62.5 μM 6AN enhanced the number of apoptotic HL-60 cells as much as 9-fold after cisplatin treatment (Fig. 2B, B' and B″).

To determine whether the effects of 6AN were unique to cisplatin, 6AN-treated cells were also exposed to melphalan, chlorambucil, nitrogen mustard, 4HC, daunorubicin, or etoposide. Results of these experiments are illustrated in Fig. 3 and summarized in Table 1. In A549 cells, for example, 6AN treatment was associated with an 11 ± 3-fold decrease in cisplatin LD90 (n = 5), an 8 ± 3-fold decrease in the melphalan LD90 (n = 4), and a 3.3 ± 1.2-fold decrease in nitrogen mustard LD90 (n = 4). In contrast, there was minimal effect on 4HC sensitivity.

Sensitization to Cisplatin Is Not Due to Altered DNA Repair or PARP Inhibition. Additional studies were performed in an attempt to elucidate the mechanism of the effects observed in Figs. 1–3. Based on observations summarized in the “Introduction,” we initially postulated that 6AN treatment resulted in depletion of NAD⁺, diminished ability of the cells to...
Fig. 6 Relationship between Pt-DNA adducts and enhanced CDDP sensitivity. A, relationship between 6AN concentration, Pt-DNA adduct formation, and cisplatin sensitivity in K562 cells. Cells were incubated with the indicated concentration of 6AN for 18 h and then treated with 40 μM cisplatin for 1 h, washed, and assayed for Pt covalently bound to DNA (-) or clonogenic survival as indicated in Fig. 1B. cisplatin LD₉₀ from the clonogenic assays. B, effect of 6AN on Pt-DNA adduct formation in additional cell lines. Cells were treated for 18 h in the absence (-) or presence (+) of 6AN at the concentrations listed in the legend to Fig. 1, incubated with 40 μM cisplatin for 1 h (K562) or 2 h (other cell lines), and assayed for DNA-bound Pt. C-E, effect of 6AN and other agents on total cellular cisplatin accumulation. In C, A549 cells were incubated for 24 h in the absence or presence of 250 μM 6AN. Digitonin (10 μM) was added simultaneously with cisplatin (40 μM), and the incubation was continued for an additional 2 h. In D, cells were incubated for 60 min in the presence of diluent, 90 μM DRB, or 100 μM cordycepin. 6AN was then added to a final concentration of 250 μM in the continued presence of diluent, DRB, or cordycepin. Twelve h after the addition of 6AN, cisplatin was added to a final...
form poly(ADP-ribose) polymer in response to DNA damage, and consequent inhibition of DNA repair. If this model were correct, one would predict that: (a) 6AN pretreatment would inhibit removal of Pt-DNA adducts; (b) other PARP inhibitors would have an effect similar to 6AN; and (c) the modulatory effect would occur only at 6AN concentrations that deplete NAD+. Experiments were performed to test each of these predictions.

The effect of 6AN on repair of cisplatin-induced DNA damage was evaluated by treating K562 cells with 0 or 250 μM 6AN for 18 h, adding 40 μM cisplatin for 1 h, and then examining the amount of Pt covalently bound to DNA 0–8 h after removal of cisplatin from the medium. This approach has been used previously to detect alterations in repair of Pt-DNA adducts associated with changes in expression of the helicase ERCC1 (58) or in cisplatin-resistant cells (47, 59–61). Previous studies from our laboratory indicate that this approach can detect the inhibitory effect of the intercalating topoisomerase inhibitor pyrazoloacridine on repair of Pt-DNA adducts (48).

In the present study, removal of Pt-DNA adducts from cisplatin-treated K562 cells approximated a first-order process (Fig. 4A), with a 43 ± 3% (n = 3) decrease in the number of adducts during the 8 h after cisplatin removal. 6AN did not inhibit this repair process.

To examine the possibility that the effect of 6AN resulted from inhibition of some other process requiring (ADP-ribose) polymers, 6AN was replaced with other PARP inhibitors. Immunoblotting with the anti-polymy antibody 10H performed as described by Shah et al. (62) confirmed that the potent PARP inhibitor DHQ (63) inhibited formation of polymer in K562 cells exposed to DNA-damaging agents (data not shown). Cisplatin sensitivity of K562 cells, however, was not enhanced by DHQ (Fig. 4B). Another PARP inhibitor, 3-aminobenzamide, likewise failed to sensitize K562 cells to cisplatin. The failure of these inhibitors to reproduce the effect of 6AN suggests that 6AN is modulating cisplatin sensitivity by altering some process other than poly(ADP-ribose) synthesis.

Sensitization to Cisplatin Does Not Correlate with Depletion of NAD+ or ATP. To determine whether the modulatory effects of 6AN reflected the ability of this compound to deplete NAD+, the effects of various concentrations of 6AN on NAD+ biosynthesis and cellular NAD+ levels were examined under the same conditions where 6AN was observed to sensitize the cells. When K562 cells were incubated with [14C]nicotinamide, 70% of the cellular radiolabel was converted to 14C-labeled NAD+. Incubation with 6AN inhibited NAD+ synthesis in a dose-dependent fashion (Fig. 5A), as reported previously (37, 38).

Consistent with these results, an 18-h 6AN treatment resulted in decreased cellular NAD+ content (Fig. 5B). However, two observations indicate that this NAD+ depletion was not the cause of the increased cisplatin sensitivity: (a) comparison of Figs. 1B and 5B indicates that 6AN sensitizes cells to cisplatin at concentrations that are 10-fold lower than the 6AN concentrations required to appreciably diminish NAD+ levels; and (b) an 18-h treatment of K562 cells with 3AP, another nicotinamide analogue that affects pyridine nucleotide metabolism (64), resulted in an equivalent or greater depletion of NAD+ (Fig. 5C) without sensitization to cisplatin (Fig. 5D). Collectively, these results argue against the possibility that the modulatory effect of 6AN involves NAD+ depletion.

It has also been suggested that 6AN might exert some of its effects by depleting intracellular ATP (37). To examine the potential role of this metabolic alteration, ATP levels were measured 18 h after treatment with various concentrations of 6AN. Results of these assays (Fig. 5E) indicated minimal changes in ATP concentrations at the 31–62.5 μM 6AN concentrations that resulted in maximal cisplatin sensitization. Even after treatment with 250 μM 6AN, ATP concentrations were 81 ± 11% (n = 5) of control levels. These results indicate that 6AN causes little depletion of ATP at concentrations that enhance cisplatin sensitivity.

Despite the limited effect of 6AN on NAD+ and ATP levels, 6AN treatment definitely affected other aspects of intermediary metabolism at concentrations that sensitized cells to cisplatin. In agreement with previous studies (30, 34, 36, 42, 65), the pentose phosphate shunt intermediate 6PG increased 43 ± 2-fold (n = 4) after treatment of K562 cells with 250 μM 6AN for 18 h. Moreover, dose-response curves (Fig. 5F) demonstrated a progressive increase in 6PG levels over the 8–31 μM 6AN concentration range that resulted in progressive cisplatin sensitization (Figs. 1B and 6A).

Increased Pt-DNA Adducts in 6AN-treated Cells. Further investigation revealed that Pt-DNA adducts at the end of a 1-h incubation with 40 μM cisplatin were 100 ± 60 pg Pt/μg DNA in 6AN-treated K562 cells compared with 39 ± 17 pg Pt/μg DNA in control cells (n = 10), raising the possibility that 6AN might be sensitizing cells by increasing the formation of Pt-DNA adducts. To explore this hypothesis further, the relationship between 6AN concentration and Pt-DNA adduct formation was examined (Fig. 6A). In K562 cells, the number of Pt-DNA adducts increased 2.3-fold after an 18-h pretreatment with 16 μM 6AN and reached a maximum after treatment with 31 μM 6AN. This dose-response curve is the mirror image of the effect of 6AN on cisplatin sensitivity in the same cells (Fig. 6A), reflecting a strong negative correlation (r = −0.94, P = 0.002) between Pt-DNA adduct formation and cisplatin LD₅₀ after 6AN pretreatment.

Measurement of Pt-DNA adducts in additional cell lines concentration of 40 μM. In E, cells were incubated for 24 h in the absence or presence of 250 μM 6AN. MeAIB (20 mM) was added 30 min prior to 40 μM cisplatin. After the completion of the cisplatin treatment, cells were washed and assayed for total cellular platinum accumulation as described in "Materials and Methods." F, effect of neutral amino acids on formation of Pt-DNA adducts. A549 cells were incubated in the absence or presence of 250 μM 6AN for 18 h. MeAIB, alanine, or serine was added to a final concentration of 20 mM. After an additional 30 min incubation, cisplatin (40 μM) was added for 2 h. Cells were then washed and assayed for Pt-DNA adducts.
provided additional support for the relationship between increased Pt-DNA adduct formation and sensitization to cisplatin. 6AN pretreatment was associated with a 7.4 ± 2-fold (n = 15) increase in Pt-DNA adducts in A549 cells and a 5.8 ± 0.8-fold (n = 3) increase in T98G cells, but only a 1.8 ± 0.5-fold (n = 3) increase in Ovcar-3 cells (Fig. 6B). In other words, the degree of sensitization roughly paralleled the increase in Pt-DNA adducts.

To determine whether the 6AN-induced increase of Pt-DNA adducts (Fig. 6, A and B) reflected increased cisplatin accumulation or diminished cisplatin detoxification, both processes were examined. For these and several subsequent experiments, A549 cells were used because they were easier to manipulate and the 6AN-induced increase in Pt-DNA adducts was larger. As illustrated in Fig. 6C, pretreatment of A549 cells with 250 µM 6AN for 18 h increased total cell-associated Pt by a factor of 3.2 ± 0.5-fold (n = 14). Because <10% of the cell-associated Pt could be accounted for as Pt-DNA complexes, these results suggested that total cellular cisplatin accumulation had been enhanced by 6AN. When digitonin was used to introduce small holes in the plasma membrane during the cisplatin incubation, total cellular cisplatin accumulation increased, and the effect of 6AN was abolished (Fig. 6C), as would be expected if transport of cisplatin across the plasma membrane were rate-limiting (1) and 6AN were affecting that process.

Recent studies have indicated that 6AN can induce synthesis of the glucose-regulated protein GRP78 in hamster cells (66). To evaluate the possibility that enhanced cisplatin accumulation in the human tumor cell lines might reflect induction of one or more genes, A549 cells were incubated with 90 µM DRB (a concentration that inhibits [3H]uridine incorporation into RNA by 85% in these cells; Ref. 67) or 100 µM cordycepin during the 6AN treatment. These agents did not affect the ability of 6AN to enhance cisplatin accumulation (Fig. 6D), suggesting that the effect of 6AN on cisplatin accumulation is independent of any effect on the expression of GRP78 or other transcripts.

Although the transporter responsible for cisplatin accumulation remains to be definitively identified, it has been suggested that cisplatin uptake depends in part on one or more neutral amino acid transporters (68, 69). To assess the possibility that one or more of these might mediate the effect of 6AN, we examined the ability of various amino acids to affect cisplatin accumulation and Pt-DNA adduct formation. MeAIB, a nonmetabolizable neutral amino acid that is widely used in amino acid transport studies, diminished the 6AN-induced increase in cisplatin accumulation (Fig. 6E) and the subsequent Pt-DNA adduct formation (Fig. 6F) by 62 ± 4% (n = 4). The neutral amino acid alanine had a similar effect (Fig. 6F). In contrast, the amino acids serine, glutamine, and glycine had no effect (Fig. 6F and data not shown). These results raise the possibility that the effects of 6AN might be mediated through increased cisplatin accumulation on an amino acid transporter that binds MeAIB and alanine but not serine, glutamine, or glycine.

The effect of 6AN on cellular Pt accumulation was somewhat smaller than the effect on Pt-DNA adduct formation (Fig. 6, compare E and F). Although this might reflect the loss of free cisplatin from 6AN-treated cells during the incubation at 37°C after removal of cisplatin from the cells (see "Materials and Methods"), the possible role of GSH depletion in chemosensitization by 6AN was also examined. We focused on GSH because pyridine nucleotides (particularly NADPH) are involved in maintenance of GSH levels (reviewed in Ref. 36) and GSH has been postulated to play a role in resistance to cisplatin and alkylating agents (reviewed in Refs. 2 and 6–8). Treatment with 250 µM 6AN concomitantly decreased GSH levels to 56 ± 11% (n = 3) of control (Fig. 7A) and increased Pt-DNA adduct formation as much as 10-fold (Fig. 7B). In the same experiments, an 18-h pretreatment with 1 mM BSO decreased GSH levels to 8 ± 3% (n = 3) of control but did not increase Pt-DNA adducts, suggesting that GSH depletion does not play an appreciable role in the 6AN-induced increase in Pt-DNA adducts.

**6AN Metabolism Is Required for Chemosensitization**

In a final series of experiments, we attempted to determine whether sensitization was induced by 6AN itself or a metabolite of 6AN. HPLC analysis of neutralized HClO4 extracts from 6AN-treated K562 cells revealed peaks with retention times of 3.2, 7.5, 10.8, and 14 min (Fig. 8A). The first three of these peaks were identified as nicotinamide, 6AN, and NAD⁺, respectively, based on comigration with authentic standards and on absorbance spectra. The last peak was identified as 6ANAD⁺ based on the molecular weight (667 amu) determined by electrospray ionization mass spectroscopy and on the observation that treatment with NADase cleaved this compound to yield equimolar quantities of 6AN and ADP-ribose (Fig. 8B).

It was suggested previously that 6AN-containing dinucleotides are formed through an NADase-catalyzed reaction that exchanges 6AN for nicotinamide on NAD⁺ (70). Consistent with this hypothesis, we observed that incubation with a 10-fold excess of nicotinamide inhibited formation of 6ANAD⁺ but did not appreciably alter intracellular 6AN levels (Fig. 8C). Interestingly, nicotinamide also abolished the effect of 6AN on Pt-DNA adduct formation (Fig. 8D) and cisplatin sensitivity in clonogenic assays (Fig. 8E). These observations suggest that the effects of 6AN are due to formation of 6ANAD⁺ or a downstream metabolite rather than a direct action of 6AN itself.
DISCUSSION

6AN is currently undergoing preclinical evaluation as a potential biochemical modulating agent (30–33, 41, 43). The present report represents the first study of the effect of combining single-agent 6AN with a wide range of currently available antineoplastic agents. This study has demonstrated that pretreatment with 6AN sensitizes a variety of human cancer cell lines to the cytotoxic effects of cisplatin. Further investigation revealed that this effect required conversion of 6AN to 6ANAD and suggested that increased cisplatin accumulation and increased formation of Pt-DNA adducts were largely responsible for this effect. Each of these points is discussed in greater detail below.

Previous studies evaluating 6AN were generally performed using a three-drug cocktail of modulating agents that included 6-methylmercaptopurine riboside and N-(phosphonacetyl)-L-aspartate in addition to 6AN. This three-drug regimen sensitized CD8 murine breast carcinoma cells in vivo to a variety of treatments, including 5-fluorouracil, paclitaxel, doxorubicin, and γ-irradiation (30–33). Treatment with the three-drug regimen also induced a dramatic decrease of ATP levels in tumors (30, 71) and an increase in doxorubicin-induced tumor cell apoptosis compared with animals treated in the absence of the modulators (32). These results have led to the suggestion that the mixture of modulators sensitizes tumor cells to the cytotoxic effects of a variety of treatments by down-regulating ATP levels and altering the apoptotic threshold (72).

The results of the present study do not conflict with these previous results. In the present study, single-agent 6AN was observed to enhance the cytotoxicity of cisplatin as measured by colony-forming assays (Fig. 1 and Table 1). Analysis of two different cell lines indicated that cisplatin-induced apoptosis was increased by 6AN treatment (Fig. 2). Additional studies indicated that 6AN (in contrast to the triple-drug regimen) is relatively selective, sensitizing cells to some agents and not others (Fig. 3 and Table 1). Because all of the chemotherapeutic agents listed in Table 1 have been shown to induce apoptosis in susceptible cells (73), a modulator that lowered the threshold for activating the apoptotic machinery would be expected to sensitize cells to all of these agents, much as the three-drug mixture of modulators (72) or the proapoptotic protein Bax does (74). The observation that 6AN selectively sensitizes cells to only certain agents (Table 1) argued against the possibility that 6AN was directly altering the apoptotic machinery and instead prompted us to examine drug-specific mechanisms that 6AN might affect.

Although we initially hypothesized that 6AN sensitized cells to cisplatin by depleting NAD+, inhibiting the formation of poly(ADP-ribose) polymer, and thereby diminishing the repair of Pt-DNA adducts, a number of observations ruled out this proposed mechanism: (a) 6AN treatment was not associated
with any demonstrable effect on the rate of removal of Pt-DNA adducts (Fig. 4A); (b) the effect of 6AN was not observed with other PARP inhibitors (Fig. 4B); and (c) sensitization to cisplatin was demonstrated at 6AN concentrations that are too low to appreciably diminish NAD⁺ levels (Figs. 1B and 5B), whereas other treatments that diminished NAD⁺ levels did not increase cisplatin sensitivity (Fig. 5, C and D). Although the depletion of NAD⁺ levels or inhibition of PARP might explain the effect of 6AN on the efficacy of other treatments, these effects do not appear to be the major factors responsible for the effect of 6AN on cisplatin sensitivity. Likewise, the present data do not support the hypothesis that depletion of ATP is responsible for the effects observed in Figs. 1–3. Single-agent 6AN was shown to have only a modest effect on cellular ATP levels (Fig. 6), in agreement with previous experiments performed both in vitro (75) and in vivo (30).

Although the present observations cannot rule out a small effect of 6AN on DNA repair, the predominant effect of 6AN appears to be enhanced formation of Pt-DNA adducts (Fig. 6). This increase in Pt-DNA adducts accompanied increased cellular accumulation of cisplatin (Fig. 6, C–E). Additional experiments indicated that the 6AN-induced increment in cisplatin accumulation and Pt-DNA adduct formation was greater in cell lines that were sensitized to a greater degree (Fig. 6B). Further studies revealed that the 6AN-induced increase in cisplatin accumulation and Pt-DNA adducts was inhibited by adding certain neutral amino acids (e.g., MeAIB) but not others (e.g., glycine) at the time of cisplatin treatment (Fig. 6, E and F). These results, combined with the previous suggestion that cisplatin might enter cells on a neutral amino acid transporter (68, 69), are consistent with the hypothesis that 6AN is enhancing the formation of Pt-DNA adducts by modulating cisplatin accumulation, possibly on a neutral amino acid transporter.

Results summarized in Fig. 3 and Table 1 indicate that 6AN also sensitizes cells to melphalan and nitrogen mustard. After the completion of the present studies, similar results were reported in abstract form by Belfi et al. (76). These effects are smaller than the sensitization observed with cisplatin and have not been investigated in detail. It is certainly possible that depletion of NAD⁺ or inhibition of PARP contributed to these effects (see “Introduction”). On the other hand, melphalan and chlorambucil differ from each other only by the presence or absence of a single amino group. The ability of 6AN to sensitize cells to melphalan, which is known to be transported into cells on a neutral amino acid transporter, but not chlorambucil, which enters cells by passive diffusion (reviewed in Refs. 77 and 78), is consistent with the hypothesis that 6AN up-regulates one or more neutral amino acid transporters. Likewise, the observation that 6AN enhances sensitivity to nitrogen mustard, an agent that enters cells on yet another transporter (68, 79), raises the possibility that 6AN might affect the activity of multiple drug uptake systems. The observation that 6AN can exert its effects in the presence of RNA synthesis inhibitors (Fig. 6D) suggests that expression of new transcripts is probably not required for enhanced cisplatin accumulation. Interestingly, stress-induced up-regulation of amino acid transport has also been shown to occur in the absence of protein synthesis (80).

Additional experiments suggest that 6ANAD⁺, rather than 6AN itself, is responsible for the modulation of cisplatin sensitivity. Results presented in Fig. 8 and by Saunders et al. (81) identify 6ANAD⁺ as the major intracellular metabolite of 6AN. We observed that treatment with a large molar excess of nicotinamide simultaneously abolished the formation of 6ANAD⁺ (Fig. 8C), the increase in Pt-DNA adducts (Fig. 8D), and the effect of 6AN on cisplatin sensitivity (Fig. 8E), with only a minimal effect on cellular 6AN accumulation (Fig. 8C), indicating that formation of 6ANAD⁺ is required for these effects. The observed correlation between 6PG elevation and cisplatin sensitization (Figs. 5F and 6A) suggests that a downstream metabolic alteration induced by 6ANAD⁺ might be responsible for the effects described in the present report. Although this correlation provides a potentially useful marker for the sensitizing effect of 6AN (43), the present studies do not establish whether 6PG elevation or some other effect of 6ANAD⁺ is actually responsible for cisplatin sensitization. Nonetheless, the demonstration that 6ANAD⁺ must be formed to modulate cisplatin sensitivity is potentially important because it raises the possibility that other precursors of 6ANAD⁺ might have similar effects. Although 6AN has been successfully used as part of a multiagent regimen that enhances the efficacy of a variety of treatments (30–33, 82), preclinical (83) and clinical studies (84, 85) have demonstrated that 6AN can also cause neurotoxicity under certain conditions. The demonstration that 6ANAD⁺ is the active compound raises the possibility that a more polar precursor of 6ANAD⁺ that does not cross the blood-brain barrier might also modulate cisplatin sensitivity. Additional preclinical studies are required to determine whether these observations can be used to increase the therapeutic efficacy of cisplatin in vivo.

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REFERENCES


6-Aminonicotinamide sensitizes human tumor cell lines to cisplatin.

I I Budihardjo, D L Walker, P A Svingen, et al.


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