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Effect of 6-Aminonicotinamide and Other Protein Synthesis Inhibitors on Formation of Platinum-DNA Adducts and Cisplatin Sensitivity

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ABSTRACT

The present study was undertaken to examine the mechanistic basis for the recent observation that the pyridine nucleotide derivative 6-aminonicotinamide (6AN, NSC 21206) enhances the accumulation and resulting cytotoxicity of cisplatin in a variety of tumor cell lines. When A549 lung cancer cells or K562 leukemia cells were treated with 62.5 μM 6AN for 21 h and then pulse-labeled with [^{35}S]methionine for 1 h, increased labeling of five polypeptides, one of which corresponded to a M_r ~78,000 glucose-regulated protein (GRP78), was observed. Two subsequent observations, however, suggested that up-regulation of these polypeptides was unlikely to explain the interaction between 6AN and cisplatin: 1) the concentration of 6AN required to induce GRP78 was 4-fold higher than the dose required to sensitize cells to cisplatin; and 2) simultaneous treatment of cells with 6AN and cycloheximide prevented the increase in GRP78 but not the sensitizing effect of 6AN. On the contrary, treatment with the protein synthesis inhibitors cycloheximide, anisomycin, or puromycin as well as prolonged exposure to the RNA synthesis inhibitor actinomycin D mimicked the biochemical modulating effects of 6AN on cisplatin action. Conversely, 6AN inhibited protein synthesis, whereas 18 6AN analogs that failed to enhance Pt-DNA adducts and cisplatin cytotoxicity failed to inhibit protein synthesis. These observations are consistent with a model in which 6AN and other inhibitors of protein synthesis act as modulating agents by increasing cisplatin accumulation, thereby enhancing the formation of Pt-DNA adducts and subsequent cisplatin-induced cell death.

The nicotinamide analog 6-aminonicotinamide (6AN) is undergoing preclinical evaluation as a potential agent for modulating the cytotoxicity of antineoplastic drugs. Studies performed by Berger and coworkers (1983, 1989) revealed that 6AN sensitized L1210 murine leukemia cells to bis(2-chloroethyl)nitrosourea in vitro and in a murine model. In subsequent studies, a mixture containing the modulators *N*-(phosphonacetyl)-L-aspartate, 6-methylmercaptopurine riboside, and 6AN has been reported to enhance the efficacy of ionizing radiation, 5-fluorouracil, doxorubicin, and paclitaxel against spontaneous autochthonous breast cancers in mice (reviewed in Martin and Schwartz, 1997). More recently, 6AN was observed to enhance the cytotoxicity of cisplatin in V79 Chinese hamster cells (Chatterjee et al., 1997) and a variety of human tumor cell lines in vitro (Budihardjo et al., 1998). Collectively, these studies raised the possibility that 6AN might enhance the effectiveness of various anticancer agents in vitro and in vivo.

Despite these promising results, studies performed to evaluate the feasibility of using 6AN to modulate the cytotoxicity of cisplatin in vivo have been disappointing (Walker et al., 1999). Optimal sensitization of A549 or K562 cells to cisplatin in vitro required pretreatment with 15 to 30 μM 6AN for at least 10 h. Pharmacokinetic studies, on the other hand, revealed that 6AN had a terminal serum half-life of only 20 to 30 min in mice (Walker et al., 1999). After bolus administration of 10 mg/kg 6AN (one-fourth the single-dose LD_{50}), serum 6AN levels peaked at ~90 μM but rapidly declined, preventing prolonged exposure to concentrations that sensitized cells to cisplatin in vitro. Conversely, a continuous

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ABBREVIATIONS: 6AN, 6-aminonicotinamide; 6ANAD$^+$, the 6AN-containing analog of nicotinamide adenine dinucleotide; DRB, 5,6-dichloro-1β-D-ribofuranosylbenzimidazole; GRP78, the M_r ~78,000 glucose-regulated protein; MeAIB, methylaminoisobutyrate; NEPHGE, nonequilibrium pH gradient electrophoresis; PBS, calcium/magnesium-free phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

infusion strategy was capable of maintaining 6AN plasma levels at 15 μM for 24 to 48 h but was uniformly lethal (Walker et al., 1999), most likely as a consequence of the well-documented neurotoxicity of 6AN (Sternberg and Philips, 1958; Herken et al., 1969). These observations indicate the need to identify a less toxic analog of 6AN, a process that would be facilitated by a better understanding of the mechanism by which 6AN modulates the action of other agents.

Our previous study (Budihardjo et al., 1998) demonstrated that 6AN enhanced cisplatin-induced apoptosis but did not affect the cytotoxicity of etoposide, topotecan, 4-hydroperoxycyclophosphamide, or chlorambucil, raising the possibility that 6AN might preferentially affect a drug-specific step in the cell death pathway. Consistent with this hypothesis, 6AN was observed to enhance cisplatin accumulation and the subsequent formation of Pt-DNA adducts (Budihardjo et al., 1998). Although the mechanisms responsible for cisplatin accumulation are not completely understood, a current model suggests that cisplatin uptake involves both passive diffusion and carrier-mediated transport (Gately and Howell, 1993). Accordingly, the observation that 6AN enhances intracellular cisplatin levels raises the possibility that 6AN alters the expression or function of polypeptide(s) involved in cisplatin transport across the plasma membrane.

A different view of 6AN action has emerged from other studies. In particular, the observation that 6AN enhances expression of the glucose-regulated protein GRP78 (Chatterjee et al., 1995, 1997) has raised the possibility that GRP78 might somehow regulate cisplatin action. GRP78, however, is a stress-inducible chaperone protein that resides in the endoplasmic reticulum, where it is hypothesized to function in the translocation of proteins from the cytosol and in the correct assembly of proteins during early protein processing (Lee, 1992; Little et al., 1994; reviewed in Brostrom et al., 1995). A mechanistic link between 6AN-induced GRP78 expression and altered cisplatin action has not been established.

In the present study, the cellular effects of 6AN were examined in greater detail. Labeling with [^{35}S]methionine, followed by two-dimensional nonequilibrium pH gradient electrophoresis (NEPHGE)/SDS-polyacrylamide gel electrophoresis (PAGE), was used to more completely define the effects of 6AN on protein expression. Protein synthesis inhibitors were employed to examine the role of 6AN-induced polypeptides on cisplatin accumulation and cytotoxicity. Assays of methionine uptake and incorporation were performed to assess the effect of 6AN on amino acid transport and protein synthesis. Finally, 18 structural analogs of 6AN were tested for their ability to inhibit protein synthesis, increase Pt-DNA adducts, and enhance cisplatin cytotoxicity. Results of these studies are consistent with a model in which 6AN-associated inhibition of protein synthesis prompts a compensatory increase in amino acid and cisplatin accumulation, with subsequent enhancement of Pt-DNA adducts and cisplatin-induced cytotoxicity.

Experimental Procedures

Materials. Reagents were purchased from the following suppliers: cisplatin, L-alanine, L-serine, 6AN, 6-aminonicotinic acid, nicotinamide, methylaminoisobutyrate (MeAIB), cycloheximide, anisomycin, puromycin, DRB, actinomycin D, and digitonin from Sigma (St. Louis, MO); *Hin*dIII from Life Technologies/BRL (Gaithersburg, MD); proteinase K from Boehringer-Mannheim (Indianapolis, IN); bicinchoninic acid from Pierce (Rockford, IL); enhanced chemiluminescence reagents (ECL) or Amplify (Amersham, Arlington Heights, IL); pH 3.5 to 10 ampholytes from Pharmacia/LKB (Piscataway, NJ); mouse monoclonal anti-GRP78 antibody from StressGen (Victoria, British Columbia); and platinum standard from J.T. Baker (Phillipsburg, 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. All chemicals for synthesis were of the highest available purity.

6AN analogs C, D, F through J, L through P, and R were provided by the Pharmaceutical Resources Branch of the National Cancer Institute (Rockville, MD). Compound E was synthesized by reacting 1.82 mmol of 6AN with 18.2 mmol of acetic anhydride in 5 ml of acetic acid on a steam bath for 30 min under N_2 atmosphere. The resulting precipitate was filtered, washed with acetic acid, and recrystallized from ethanol. Compound K was synthesized by reacting 3.65 mmol of 6AN in acetic acid with 1 equivalent of hydrogen peroxide (added as a 30% solution) at 75°C for 3 h. After the solvent was evaporated under reduced pressure, the yellow residue was recrystallized from ethanol. Compound Q was synthesized by reacting 1.46 mmol of 6AN with 1.54 mmol of benzoic anhydride in 0.2 ml of triethylamine on a steam bath overnight under N_2 atmosphere. The resulting solid was filtered, rinsed with acetone, and recrystallized from ethanol. 6AN and analogs B, C, D, F, H, J, L, M, O, and R were prepared as 2.5 mM stocks in medium A. The remaining analogs were prepared as 100 mM stocks in dimethyl sulfoxide with (E, K, Q) or without (G, I, N, P) 1% acetic acid.

Cell Culture. A549 nonsmall cell lung cancer cells and K562 chronic myelogenous leukemia cells from American Type Culture Collection (Manassas, VA) were cultured at 37°C in a humidified atmosphere of 95% air, 5% CO_2 in RPMI 1640 medium containing 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine (medium A). To ensure logarithmic growth, cultures were maintained at densities below 1×10^6 cells/ml (K562) or 70 to 80% confluence (A549). Cells were fed on the day before the start of each experiment.

Colony Forming Assays. Aliquots containing 3 to 5×10^5 log phase K562 cells in 1 ml of medium A were incubated with diluent or a concentration (250 μM) of the various 6AN analogs for 18 h. A 1-μl aliquot of dimethyl sulfoxide containing the indicated final concentration of cisplatin was added for 1 h. Cells were then sedimented at 200*g* for 10 min, washed, diluted 1:500, and plated in 0.3% agar as described (Budihardjo et al., 1998; Walker et al., 1999). After a 10- to 14-day incubation at 37°C, colonies containing $\geq$50 cells were counted using an inverted phase-contrast microscope. Survival was expressed relative to control cells incubated with the corresponding concentration of 6AN analog in the absence of cisplatin treatment. Control plates typically contained 200 to 400 colonies.

Aliquots containing 300 to 500 A549 cells were plated in 35-mm tissue culture plates containing 2 ml of medium A and allowed to adhere for 12 to 14 h. After cells were treated with the indicated concentration of 6AN or cycloheximide for 6 h, cisplatin was added to the indicated final concentration. Following 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 to 200 colonies.

Measurement of Whole Cell Cisplatin Accumulation and Pt-DNA Adducts. For assessment of cellular cisplatin accumulation, duplicate 100-mm plates of A549 cells grown to 70 to 80% confluence were incubated in medium A in the absence or presence of 6AN, cycloheximide, puromycin, anisomycin, DRB, or actinomycin D for 6 to 24 h as indicated. Freshly prepared cisplatin was then added to a final concentration of 40 μM from a 1000$\times$ concentrated stock. After a 2-h incubation at 37°C, cells were washed once with ice-cold PBS, briefly trypsinized, sedimented at 200*g* for 10 min, and washed

three times with ice-cold PBS. At the last wash step, cells were resuspended in a total volume of 1 ml. A 100-μl aliquot of cells was removed, solubilized in 100 μl of 1 M NaOH at 21°C overnight, neutralized with 100 μl of 1 M HCl, and assayed for protein by the bicinchoninic acid method (Smith et al., 1985). The remainder of the cells were sedimented, reacted overnight at 21°C with 0.5 ml of concentrated nitric acid, heated to 100°C for 5 min, diluted with 0.5 ml of 30% (w/w) H_2O_2, and heated again to 100°C for 5 min. Elemental platinum in the lysates was assayed by the Metals Laboratory of the Mayo Clinic using inductively coupled plasma mass spectroscopy as recently described (Walker et al., 1999).

To measure Pt-DNA adducts, plates of 50 to 70% confluent A549 cells were incubated with 6AN, cycloheximide, or actinomycin D as indicated. Cisplatin was then added to a final concentration of 40 μM. Two h after addition of cisplatin, cells were washed with ice-cold PBS, briefly trypsinized, 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°C), 10 mM EDTA, 150 mM NaCl] supplemented with 0.4% SDS and 1 mg/ml proteinase K. The lysates were incubated at 50°C for 16 h. Highly purified DNA samples were then prepared by phenol/$CHCl_3$ and $CHCl_3$ extraction, ethanol precipitation, RNase A treatment, phenol/$CHCl_3$ and $CHCl_3$ extraction, and *Hin*dIII digestion as previously described in detail (Budihardjo et al., 1998). 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°C for 30 min. The DNA concentration was estimated by measuring absorbance at 260 nm, and elemental platinum was assayed as described above. Similar techniques were used to measure Pt-DNA adducts in K562 cells treated with 6AN or compounds B through R.

Methionine Labeling, Protein Electrophoresis, and Immunoblotting. A549 cells were incubated in the absence or presence of 62.5 μM 6AN in medium A for 18 to 24 h. After culture medium was removed, cells were briefly trypsinized and washed twice with RPMI 1640 medium lacking methionine. Cells were resuspended in 1 ml of methionine-free RPMI 1640 medium supplemented with 5% dialyzed fetal bovine serum and 50 μCi/ml [^{35}S]methionine in the continued absence or presence of 62.5 μM 6AN and incubated for 1 h at 37°C. After labeling, the cells were sedimented at 800*g* for 10 min at 4°C; washed twice with ice-cold PBS containing 5 mM unlabeled methionine; sonicated in SDS sample buffer consisting of 4 M urea, 2% (w/v) SDS, 62.5 mM Tris-HCl (pH 6.8), and 5% (v/v) β-mercaptoethanol; and heated to 70°C for 30 min. Aliquots containing equal amounts of trichloroacetic acid-perceptible radiolabel (500,000 cpm) were mixed with four volumes of IEF buffer [9.5 M urea, 8% (w/v) Nonidet P-40, 2% (w/v) pH 3.5 to 10 ampholytes, and 5% (w/v) β-mercaptoethanol] and subjected to NEPHGE in the first dimension followed by SDS-PAGE in the second dimension as previously described (O'Farrell et al., 1977; Kaufmann and Shaper, 1984). After the second dimension, gels were impregnated with Amplify and exposed to Kodak X-Omat AR film for 4 days at −70°C in the presence of intensifying screens as specified by the supplier.

To perform immunoblotting, subconfluent A549 cells were treated with increasing 6AN concentrations for 18 h at 37°C, washed twice with ice-cold PBS, released by brief trypsinization, diluted with medium A, sedimented at 200*g*, washed twice with ice-cold PBS, lysed in 6 M guanidine hydrochloride under reducing conditions, and prepared for SDS-PAGE as previously described (Kaufmann, 1989). Aliquots containing 50 μg of protein, as determined by the bicinchoninic acid assay (Smith et al., 1985), were heated to 65°C for 20 min, subjected to SDS-PAGE on gels with 5 to 15% (w/v) acrylamide gradients, and transferred to nitrocellulose. Blots were probed using murine monoclonal anti-GRP78 or chicken anti-lamin B_1 (Kaufmann, 1989) as primary antibodies.

Methionine Uptake and Incorporation. To assess methionine uptake, A549 cells in 24-well plates were treated with 0 to 1000 μM 6AN for 18 to 24 h. Cells were then treated with 1 μCi/ml [^{3}H]methionine in medium A for 5 min, washed five times with ice-cold PBS, and lysed in 0.5 ml of 0.5% SDS. Aliquots were subjected to scintillation counting and protein measurement (Smith et al., 1985). To correct for nonspecific trapping of label, replicate aliquots were treated with [^{3}H]methionine for <10 s and handled in an identical fashion.

To measure methionine incorporation into macromolecules, A549 cells were treated with 6AN, cycloheximide, puromycin, anisomycin, or actinomycin D for the indicated length of time. Radiolabeled methionine was added at a final concentration of 1 μCi/ml. After a 60-min incubation, the medium was removed and cells were washed once with ice-cold PBS. All further steps were performed at 4°C.

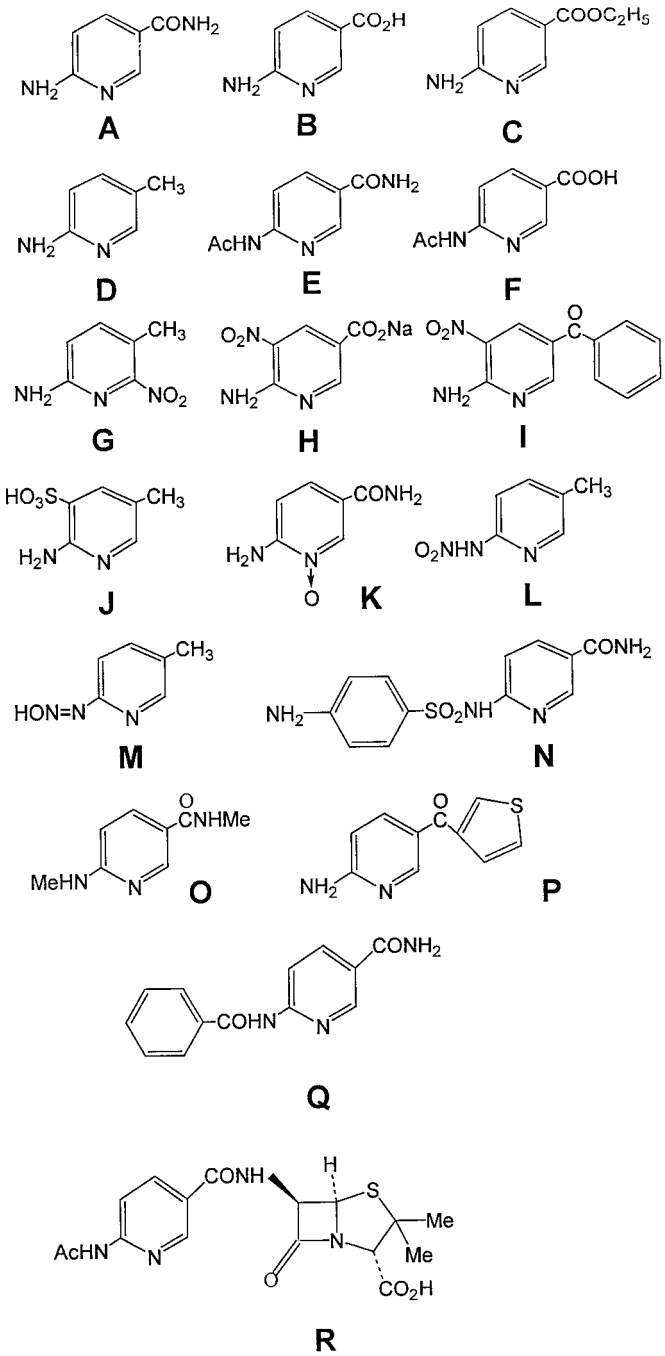

Fig. 1. Structures of 6AN analogs used in this study. 6AN is depicted as compound A.

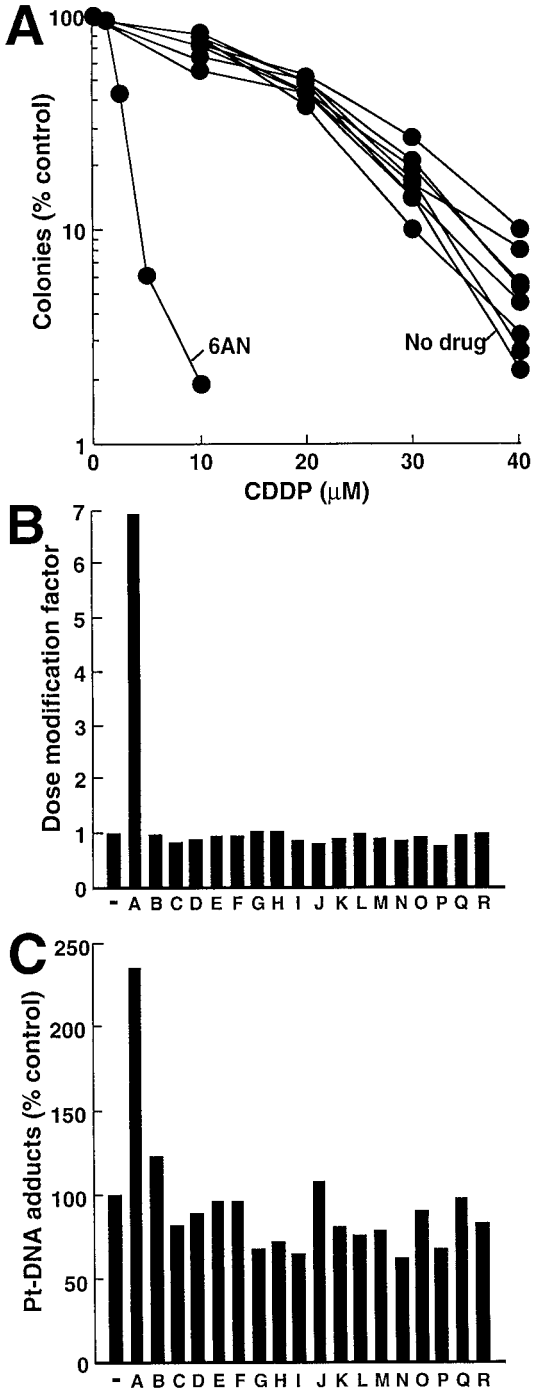

Fig. 2. Effects of 6AN and its analogs. A, K562 cells were incubated for 21 h with 250 μM 6AN or 250 μM analogs D, F, H, L, M, and R. Cisplatin was then added at the indicated final concentration. After a 1-h incubation with cisplatin, cells were washed and plated in drug-free agar to allow colonies to form. Data are expressed as the ratio of the number of colonies in samples treated with the 6AN analog + cisplatin to the number of colonies formed in samples treated with the 6AN analog alone. B, summary of the effects of all 18 analogs on cisplatin sensitivity in clonogenic assays performed as indicated in A. Results are expressed as the ratio of the LD_{90} in absence of the analog to the LD_{90} in the presence of the analog. Results are the means of two to six experiments with analogs B through R. C, summary of the effects of all 18 analogs on formation of Pt-DNA adducts. Data are expressed as the ratio of Pt-DNA adducts in the presence of the analog to Pt-DNA adducts in the absence of any analog. Results are the means of 17 experiments with 6AN (compound A, included as a control in each experiment) and two to four experiments with analogs B through R.

Cells were scraped into 10% (w/v) trichloroacetic acid (TCA). After the lysates were sedimented at 12,000*g* for 5 min, the pellets were washed once with 10% TCA and three times with acetone. The pellets were then solubilized in 0.5 M NaOH, heated to 70°C for 30 min, and neutralized with 0.5 M HCl. After an aliquot was removed for determination of protein (Smith et al., 1985), the remainder was subjected to scintillation counting. Similar techniques were applied to K562 cells except that analogs B through R were substituted for 6AN and radiolabeled cells were collected by sedimentation at 3200*g* for 1 min before lysis in 10% TCA. Similar results were obtained using either ^{35}S-labeled or *methyl*-^{3}H-labeled methionine.

Results

Evaluation of the Effects of 6AN Analogs. Previous experiments demonstrated that the niacin derivative 6AN enhances cisplatin-induced Pt-DNA adducts and cytotoxicity in a variety of human tumor cell lines (Budihardjo et al., 1998). To search for structural analogs that might have similar effects, 18 6AN derivatives (Fig. 1) were evaluated for their ability to increase Pt-DNA adducts and cisplatin toxicity in K562 cells. Results of these experiments are summarized in Fig. 2.

In the absence of cisplatin, 6AN was cytostatic but not cytotoxic during the 24-h exposure period. In contrast, the analogs were neither cytostatic nor cytotoxic by themselves. When cisplatin was added, 6AN was found to be relatively unique among these agents in its ability to enhance the cytotoxicity of cisplatin (Fig. 2, A and B). In particular, modification of the side chain amide (compound A) to a carboxylic acid (compound B), ethyl ester (compound C), ketone (compound P), or methyl group (compound D) completely abolished the activity of 6AN. Likewise, oxidation of the pyridine nitrogen (compound K) abolished activity. Finally, most modifications of the amino group at the 6-position, including methylation (compound O) and amidation with acetic or benzoic acid (compounds E and Q, respectively), abolished activity. The inability of these analogs to enhance the cytotoxicity of cisplatin (Fig. 2, A and B) paralleled their inability to enhance formation of Pt-DNA adducts (Fig. 2C). Further studies were undertaken in an attempt to better understand the relatively unique effects of 6AN on cisplatin action.

6AN Selectively Increases the Expression of Five Polypeptides. A549 cells were used for most of the subsequent studies because the effects of 6AN on cisplatin sensitivity were somewhat greater in this cell line (Budihardjo et al., 1998). All major findings presented below, however, were also replicated in K562 cells to confirm that the results were not unique to one line.

The previous observation that the effect of 6AN on formation of Pt-DNA adducts requires exposure of cells for 6 to 10 h (Walker et al., 1999) raised the possibility that the effect of 6AN might reflect the synthesis of one or more polypeptides. To examine this possibility, A549 cells were incubated in the absence or presence of 62.5 μM 6AN for 21 h, then pulse-labeled with [^{35}S]methionine for 1 h. Compared with cells treated with diluent alone, 6AN-treated cells displayed increased incorporation of [^{35}S]methionine into a small subset of polypeptides (cf. Fig. 3, A and B), including species at M_r ~45,000 (pI ~9.1), M_r ~66,000 (pI ~8.9), M_r ~78,000 (pI ~5.3), and M_r ~90,000 (pI ~7.1). The mobility of the M_r ~78,000 (pI ~5.3) polypeptide was consistent with the reported mobility of GRP78 (Patierno et al., 1987), a polypep-

tide previously shown to be up-regulated in 6AN-treated hamster cells (Chatterjee et al., 1995, 1997). Immunoblotting with an anti-GRP78 antibody confirmed that GRP78 polypeptide content increased 3- to 4-fold in 6AN-treated A549 cells (Fig. 4B, inset lanes 5 to 7). The M_r ~45,000 (pI ~9.1) polypeptide comigrated with a major Coomassie Brilliant Blue-stained polypeptide that was identified as the translation elongation factor EF-1α by microsequencing. The identity of the other three 6AN-induced polypeptides is currently unknown.

To examine the potential relationship between increased GRP78 and enhanced cisplatin sensitivity, the effects of various 6AN concentrations on these processes were compared. Interestingly, the 7.8 to 31.2 μM concentrations of 6AN that reproducibly sensitized A549 cells to cisplatin (Fig. 4A) caused only small changes in GRP78 levels (Fig. 4B, inset lanes 3 and 4) relative to those seen at higher 6AN concentrations, raising the possibility that up-regulation of GRP78 and enhancement of cisplatin sensitivity might be two independent 6AN-induced processes.

Protein Synthesis Inhibitors Mimic the Effects of 6AN on Formation of Pt-DNA Adducts and Cisplatin Sensitivity. To further examine the potential role of the 6AN-induced polypeptides in sensitizing cells to cisplatin, A549 cells were treated with 6AN or diluent in the presence of 107 μM cycloheximide, a concentration that inhibited protein synthesis in A549 cells by >90%, before cisplatin exposure. Treatment with 250 μM 6AN for 6 h resulted in a 4.2- $\pm$ 1.5-fold (n = 3) increase in Pt-DNA adducts (Fig. 5A). Addition of cycloheximide 30 min before 6AN did not prevent this increase. On the contrary, treatment with cycloheximide alone for 6.5 h enhanced the formation of Pt-DNA adducts 2.9- $\pm$ 0.9-fold (n = 3, Fig. 5A). Consistent with these results, pretreatment with cycloheximide also failed to diminish the effects of 6AN on cisplatin sensitivity in colony forming assays (Fig. 5B). A 6-h pretreatment with 6AN decreased the cisplatin LD_{90} by 3.5- $\pm$ 1.5-fold (n = 6). This effect was not prevented by adding cycloheximide 30 min before 6AN. Instead, treatment with cycloheximide alone resulted in a 2.3- $\pm$ 0.2-fold decrease in the LD_{90}, and the combination of cycloheximide plus 6AN resulted in a 5.2- $\pm$ 0.4-fold decrease in the LD_{90} (n = 3). In other words, cycloheximide mimicked the effects of 6AN when applied alone and augmented the effects of 6AN when combined with this agent. Interestingly, all the changes observed after the 6-h incubation occurred without any change in GRP78 (Fig. 5B, inset), providing additional support for the view that induction of GRP78 and effects of 6AN on cisplatin action can be dissociated.

These effects were not limited to cycloheximide. Treatment of cells for 24 h with actinomycin D, a classical inhibitor of RNA synthesis, also enhanced the formation of Pt-DNA adducts (Fig. 5C). This effect occurred at the same actinomycin D concentrations that caused inhibition of protein synthesis as assessed by a 1-h methionine pulse at the end of the

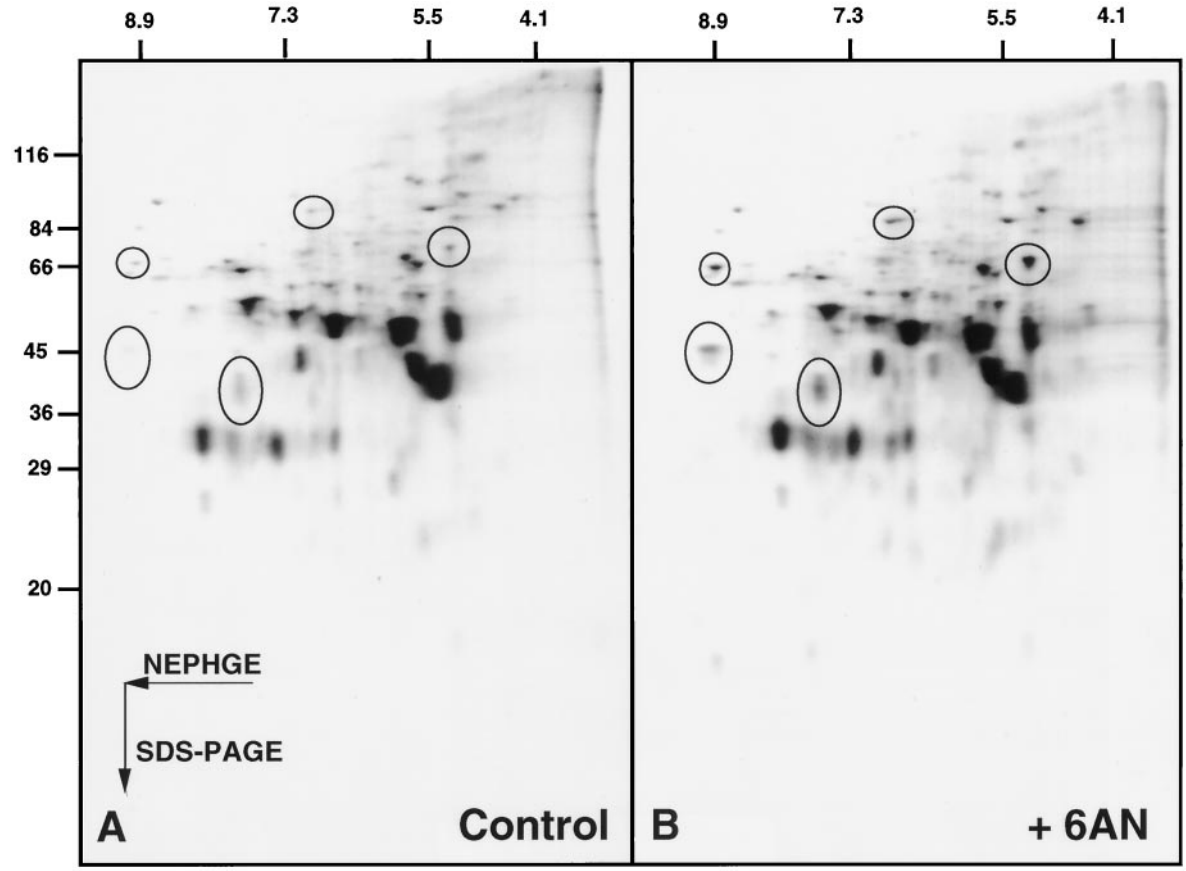

Fig. 3. 6AN treatment is accompanied by enhanced expression of multiple cellular polypeptides. Two-dimensional gels of A549 cells were treated with buffer (A) or 62.5 μM 6AN (B) for 21 h then pulsed with [^{35}S]methionine for 1 h before lysis. NEPHGE (right to left) was followed by SDS-PAGE (top to bottom). Numbers at left are molecular weights of standard proteins (run on the edge of the gel) $\times$ 10^3. Numbers at top are pH values of corresponding sections of blank NEPHGE gel run in parallel. Circled areas are species that increase in 6AN-treated cells. Results are representative of three experiments.

pretreatment (Fig. 5C). In additional experiments, a 24-h DRB exposure likewise inhibited protein synthesis and increased formation of Pt-DNA adducts.

Protein Synthesis Inhibitors Increase Cisplatin Accumulation. Previous observations suggested that the effect of 6AN on Pt-DNA adducts is mediated by a 6AN-induced increase in cisplatin accumulation that is partially inhibited by the nonmetabolizable amino acid MeAIB (Budihardjo et al., 1998). To determine whether protein synthesis inhibitors might be modulating the action of cisplatin by a similar mechanism, A549 cells were incubated for 24 h with 107 μM cycloheximide, 100 μM puromycin, or 38 μM anisomycin. Even though each of these agents inhibited protein synthesis by >90%, cell viability as determined by trypan blue exclusion remained ≥98% after 24 h. When these cells were assayed for cisplatin accumulation, treatment with each of the protein synthesis inhibitors was accompanied by increased accumulation of cisplatin (Fig. 6A). In other experiments, a 20- to 24-h exposure to actinomycin D or DRB had the same effect. Addition of 20 mM MeAIB at the same time as cisplatin partially reversed the effect of the protein synthesis inhibitors just as it partially reversed the effect of 6AN (Fig. 6B and data not shown). In contrast, serine had no effect on cisplatin accumulation (data not shown). Taken together, these data indicate that 6AN and protein synthesis inhibitors increase cisplatin accumulation, possibly through a mechanism that involves a neutral amino acid transporter.

6AN Inhibits Protein Synthesis at the Concentrations That Increase Cellular Cisplatin Accumulation. Because cycloheximide, puromycin, and anisomycin produced effects similar to those of 6AN, we next investigated the possibility that 6AN might be capable of inhibiting protein synthesis. When A549 cells were incubated with increasing concentrations of 6AN for 18 h, a dose-dependent decrease in incorporation of radiolabeled methionine into TCA-precipitable macromolecules was observed (Fig. 7A). Decreased methionine incorporation was evident at 8 μM 6AN and reached a maximum of 70 to 80% inhibition at 62.5 μM. To rule out the possibility that these results reflect decreased methionine uptake rather than diminished protein synthesis, uptake of radiolabeled methionine into cells was measured at time points ranging from 30 s to 5 min. In 10 separate experiments, 6AN enhanced methionine uptake by a factor of 1.8- ± 0.5-fold (e.g., inset, Fig. 7A). These results argue against the possibility that 6AN is inhibiting methionine uptake and instead suggest that 6AN is inhibiting protein synthesis. Furthermore, the effects of 6AN on protein synthesis (Fig. 7A), cellular cisplatin accumulation (Budihardjo et al., 1998), and cisplatin cytotoxicity (Fig. 4) were observed at similar concentrations (half-maximal effects at 16 μM 6AN in all cases), suggesting that these changes were related.

To confirm that these results were not unique to the A549 cell line, K562 cells were treated with varying concentrations of 6AN before measurement of methionine incorporation. Once again, the dose-response curves for the effect of 6AN on protein synthesis and cisplatin LD_{90} were essentially superimposable (data not shown). Additional experiments revealed that 6AN was unique among the 18 6AN analogs in its ability to inhibit protein synthesis. Although analog G inhibited protein synthesis modestly and irreproducibly, 6AN was the only niacin derivative that inhibited protein synthesis by 70 to 80% (Fig. 7B), providing further support for the relationship between inhibition of protein synthesis and enhancement of cisplatin action.

In a final series of experiments, cells were incubated with 6AN in the presence of a 10-fold excess of nicotinamide. Previous observations indicated that excess nicotinamide inhibits conversion of 6AN to its active metabolite $6ANAD^+$ and abolishes the effect of 6AN on cisplatin sensitivity (Budihardjo et al., 1998). As shown in Fig. 7C, excess nicotinamide abrogated the effects of 6AN on both protein synthesis (Fig. 7C, left) and formation of Pt-DNA adducts (Fig. 7C, right). These results not only suggest that 6AN must be metabolized to $6ANAD^+$ to inhibit protein synthesis but also provide additional support for the relationship between inhibition of protein synthesis and enhanced cisplatin action.

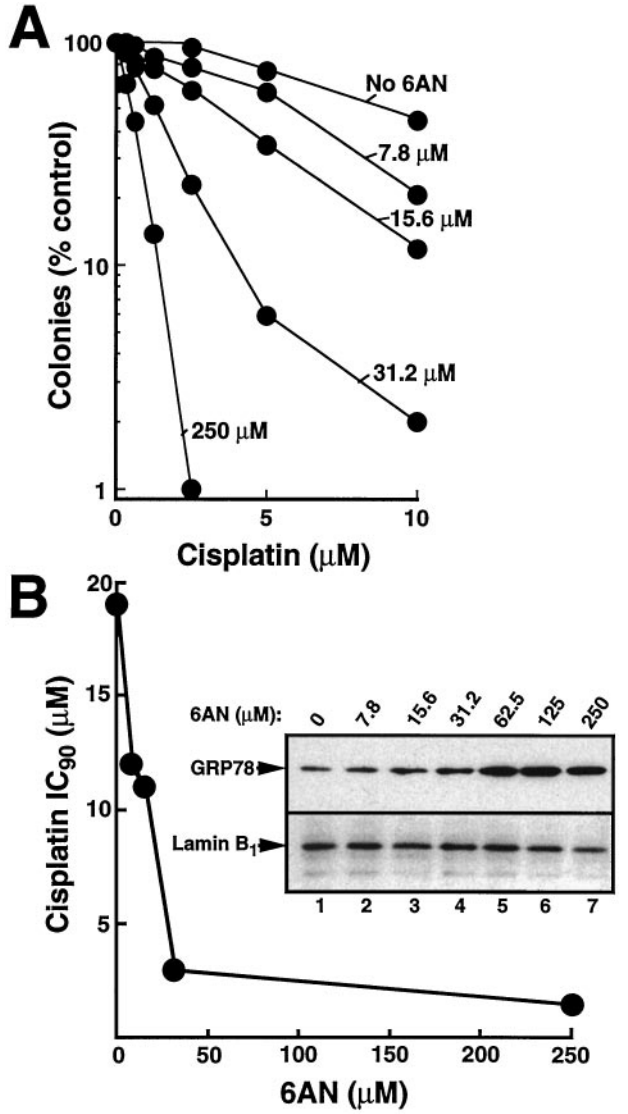

Fig. 4. Effect of 6AN on GRP78 expression and cisplatin cytotoxicity in A549 cells. A, A549 cells were treated with the indicated concentration of 6AN for 18 h. The indicated concentration of cisplatin was then added for 2 h. At the completion of the incubation, cells were washed and incubated for 7 days to allow colonies to form. B, from the data in A, the cisplatin LD_{90} was calculated at each 6AN concentration. Inset, immunoblotting for GRP78 and lamin B_1. A549 cells were incubated for 18 h with the indicated concentration of 6AN, harvested for SDS-PAGE, and probed with monoclonal anti-GRP78 (top). Lamin B_1 (bottom) served as a loading control. All results are representative of at least three experiments.

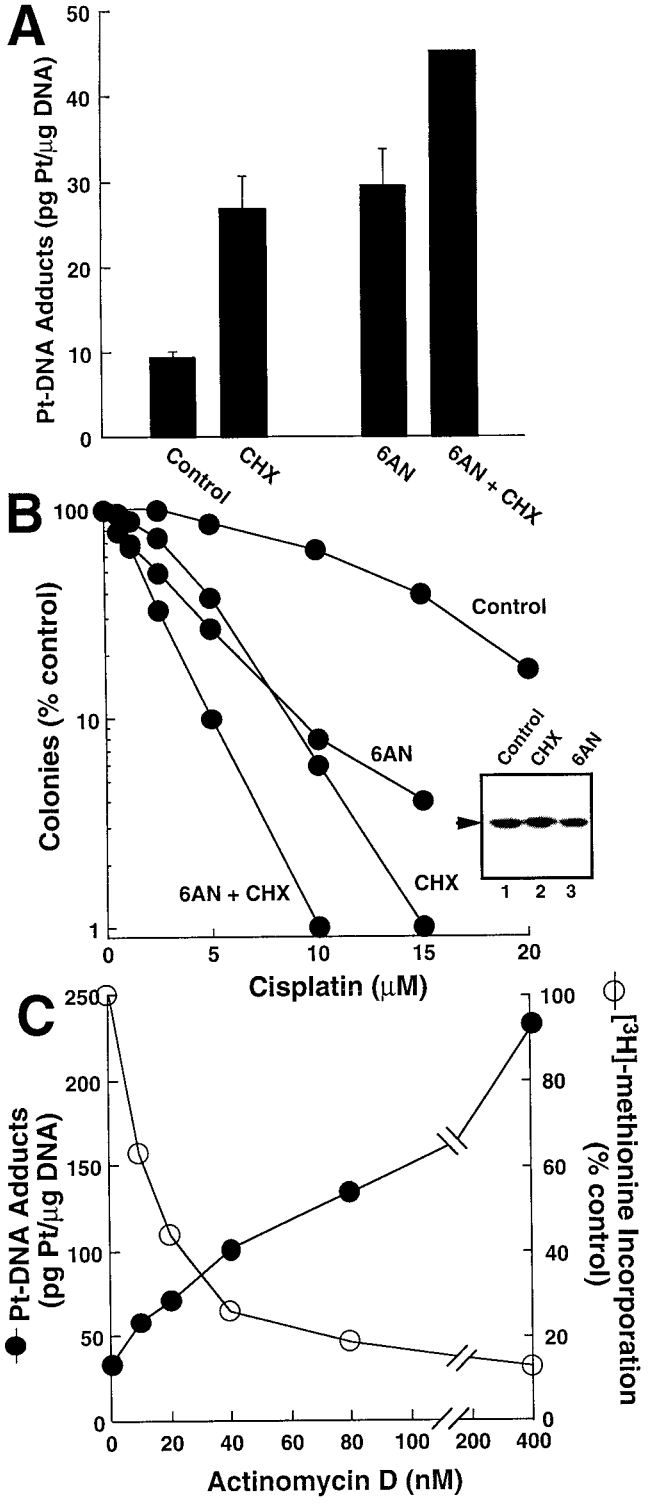

Fig. 5. Effect of cycloheximide on platinum-DNA adduct formation and cisplatin cytotoxicity. A, A549 cells were incubated for 6 h with 250 μM 6AN or 6.5 h with 107 μM cycloheximide. Cisplatin (40 μM) was then added, and the incubation was continued for an additional 2 h before cells were harvested for measurement of Pt-DNA adducts. Error bars, range of duplicate determinations in this experiment. The text summarizes results of multiple independent experiments. B, effect of 6AN and cycloheximide on A549 colony formation. A549 cells were incubated for 6 h with 250 μM 6AN or 6.5 h with 107 μM cycloheximide (CHX) before the addition of the indicated concentration of cisplatin. After a 2-h incubation with cisplatin, cells were washed and allowed to form colonies in drug-free medium for 7 days. Inset, in a parallel experiment, A549 cells treated with 250 μM 6AN or 107 μM cycloheximide for 6 or 6.5 h, respectively, were harvested for immunoblotting with monoclonal anti-GRP78 antibody. Both 6AN and cycloheximide enhance Pt-DNA adducts (A) and cytotoxicity of cisplatin (B) without increasing GRP78 (inset) over the time course of this exposure. C, effect of actinomycin D on cisplatin accumulation and protein synthesis. A549 cells were incubated for 22 h with the indicated concentration of actinomycin D. Cisplatin (40 μM) was then added, and the incubation was continued for an additional 2 h before cells were harvested for measurement of Pt-DNA adducts (●). Alternatively, incorporation of radiolabeled methionine into TCA-precipitable material was determined (○). Results in each panel present an experiment that is representative of three to six separate experiments.

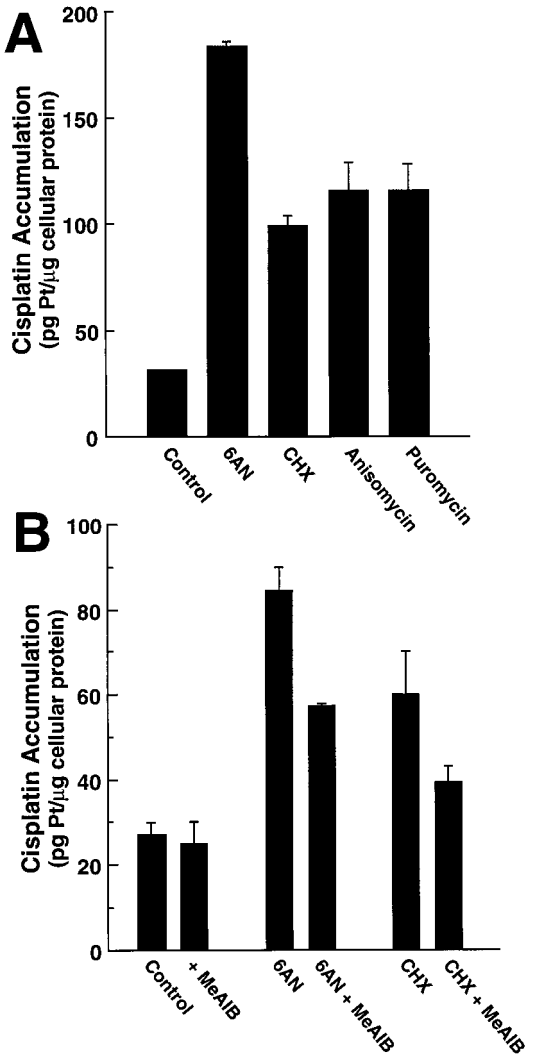

Fig. 6. Effect of protein synthesis inhibitors on cisplatin accumulation. A, effect of a 24-h treatment with 250 μM 6AN, 107 μM cycloheximide, 100 μM puromycin, or 38 μM anisomycin on cellular accumulation of cisplatin by A549 cells. Error bars, range of duplicate samples in this experiment. In multiple experiments, cisplatin accumulation was enhanced 2.6- ± 0.7-fold (mean ± S.D.) by cycloheximide, 3.6- ± 0.6-fold by puromycin, 2.8- ± 0.8-fold by anisomycin, and 3.8- ± 0.3-fold by actinomycin D. B, effect of MeAIB on the 6AN- and cycloheximide-induced increase in cisplatin accumulation. A549 cells were treated for 24 h with 250 μM 6AN or 107 μM cycloheximide. MeAIB was then added to a concentration of 20 mM, followed 30 min later by addition of 40 μM cisplatin. After a 2-h incubation with cisplatin, samples were harvested for determination of cell-associated platinum. Error bars, range of duplicate samples in this experiment. In three independent experiments, MeAIB diminished the effect of 6AN by 48 ± 8% and the effect of cycloheximide by 81 ± 18%.

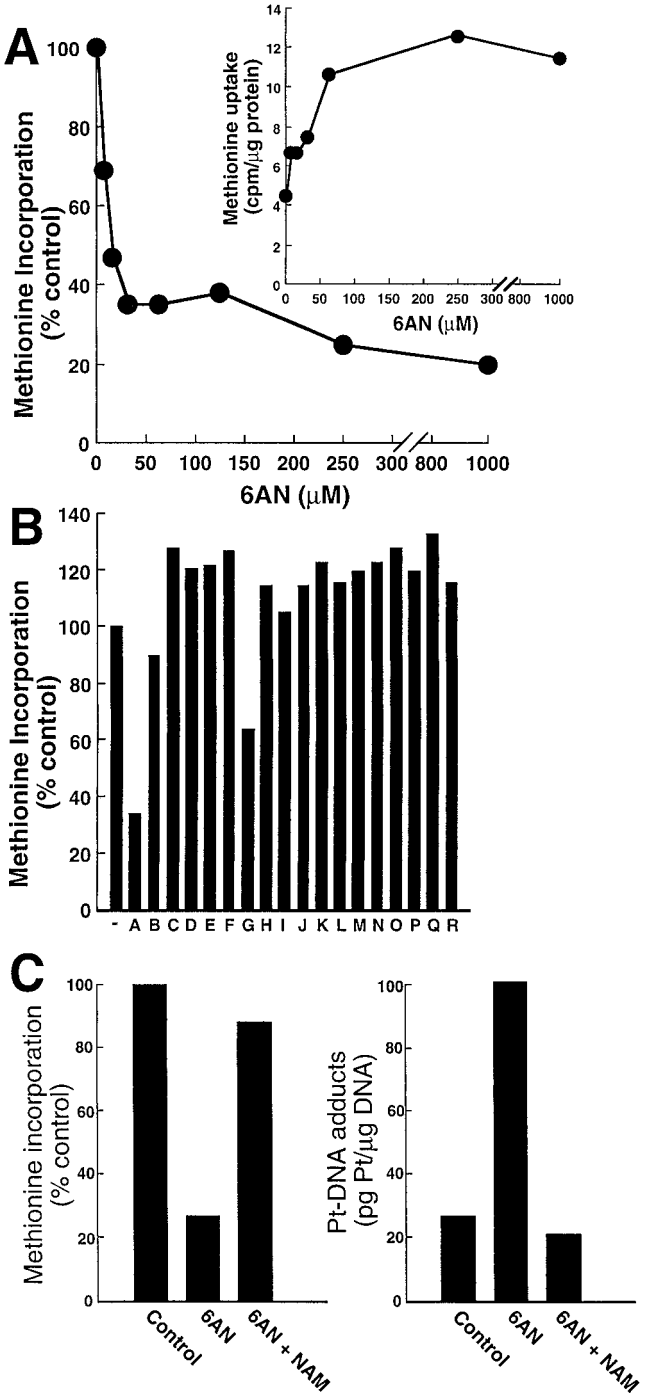

Fig. 7. Effect of 6AN and 6AN analogs on protein synthesis in A549 and K562 cells. A, effect of 6AN on methionine incorporation into TCA-precipitable material in A549 cells. Cells were incubated for 18 h with increasing concentrations of 6AN, then pulsed with radiolabeled methionine for 1 h before lysis in 10% TCA. Inset, effect of 6AN on radiolabeled methionine uptake in A549 cells. Cells were treated with the indicated concentration of 6AN for 22 h, then exposed to radiolabeled methionine for 5 min. B, summary of the effects of 18 6AN analogs on methionine incorporation into TCA-precipitable material in K562 cells. Cells were incubated with the indicated 6AN analog (see Fig. 1 for key) at 250 μM for 21 h. Radiolabeled methionine was then added for 1 h. At the completion of the incubation, TCA precipitates were collected and counted. Results of a single experiment in which all 18 analogs were tested simultaneously are presented. Similar results were obtained in two to three independent experiments. C, effect of nicotinamide on 6AN-inhibited protein synthesis and formation of Pt-DNA adducts. A549 cells were incubated for 21 h with 250 μM 6AN in the absence or presence of 2.5 mM nicotinamide. Aliquots were then pulsed for 1 h with radiolabeled methionine or treated with 40 μM cisplatin for assessment of Pt-DNA adducts. In three separate experiments, nicotinamide restored protein synthesis to 86 ± 17% of control levels and decreased Pt-DNA adducts from 560 ± 180% of control (6AN alone) to 90 ± 10% of control (6AN + nicotinamide).

Discussion

Recent studies have demonstrated that 6AN sensitizes cells to cisplatin in vitro (Chatterjee et al., 1997; Budihardjo et al., 1998). Because 6AN demonstrated neurotoxicity in animals in vivo (Sternberg and Philips, 1958; Walker et al., 1999) as well as unfavorable pharmacokinetics (Walker et al., 1999), studies were initiated to investigate the mechanism by which 6AN modulates the action of cisplatin and to search for analogs that might have similar effects but lack the neurotoxicity of 6AN. These experiments have led to a series of novel observations.

Previous studies have indicated that 6AN causes a variety of biochemical changes, including inhibition of 6-phosphogluconate dehydrogenase and depletion of products downstream from this enzyme, notably 5′-phosphoribosyl-1′-pyrophosphate and NADPH, in intact cells (Herken et al., 1969; Keller et al., 1976; Varnes, 1988; Street et al., 1996). Depletion of 5′-phosphoribosyl-1′-pyrophosphate in turn leads to the decreased synthesis of NAD^+ observed in 6AN-treated cells (Hunting et al., 1985; Berger et al., 1987; Martin and Schwartz, 1997), whereas depletion of NADPH contributes to glutathione depletion (Varnes, 1988; Budihardjo et al., 1998). In addition, 6AN has been shown to directly inhibit poly(ADP-ribose) polymerase (Sims et al., 1982), an NAD^+-consuming nuclear enzyme implicated in DNA repair (reviewed in de Murcia et al., 1997). Our earlier study not only confirmed that 6AN had these effects but also ruled out these biochemical changes as potential explanations for 6AN-induced enhancement of cisplatin toxicity (Budihardjo et al., 1998). In particular, 6AN enhanced cisplatin accumulation and formation of Pt-DNA adducts, whereas other agents that depleted glutathione, diminished NAD^+ levels, or inhibited poly(ADP-ribose) polymerase did not.

Experiments performed in the present study examined the possibility that effects on formation of Pt-DNA adducts might be due to 6AN-induced changes in expression of one or more polypeptides. After 6AN treatment for 16 to 24 h, increased labeling of at least five polypeptides was observed (Fig. 3). One of these comigrated with GRP78, a glucose-regulated heat shock family member that is localized in the lumen of the endoplasmic reticulum (Lee, 1992). While this work was in progress, Berger and coworkers reported that two different GRP78 inducers, 6AN and 2-deoxyglucose, each enhanced cisplatin sensitivity (Chatterjee et al., 1997). These data prompted the suggestion that GRP78 levels might affect cisplatin-induced DNA crosslinks and cytotoxicity (Chatterjee et al., 1997). Although it is conceivable that enhanced GRP78 expression and increased cisplatin sensitivity might be mechanistically linked in some cell lines, several observations suggest that this is not the case in A549 cells. First, dose-response curves indicate that cells are sensitized to cisplatin at 6AN concentrations that have small effects on GRP78 levels (Fig. 4). Second, treatment of cells with high 6AN concentrations (e.g., 250 μM) results in increased cisplatin accumulation and cytotoxicity beginning in ~6 h (Fig. 5) even though GRP78 is not elevated at this time (Fig. 5, inset).

Finally, cycloheximide prevents 6AN-induced increases in expression of GRP78 (I.I.B. and S.H.K., unpublished observations) but does not prevent the effect of 6AN on formation of Pt-DNA complexes (Fig. 5A) or cytotoxicity (Fig. 5B).

The observations with cycloheximide and other protein synthesis inhibitors are difficult to reconcile with the hypothesis that any of the 6AN-induced polypeptides (Fig. 3) play a critical role in altering the accumulation and cytotoxicity of cisplatin in A549 cells. Instead, the present results are consistent with a model (Fig. 8) in which 6AN treatment results in inhibition of protein synthesis; inhibition of protein synthesis causes enhanced uptake of a variety of compounds, including cisplatin; and enhanced accumulation of cisplatin results in increased formation of Pt-DNA adducts, which in turn results in increased cytotoxicity. This model is supported by a variety of observations.

First, we observed that 6AN inhibits protein synthesis by 70 to 80% (Fig. 7A) at concentrations that sensitize cells to cisplatin (Fig. 4). In contrast, 18 inactive 6AN analogs failed to inhibit protein synthesis by more than 30% (Fig. 7B). The effects of 6AN on protein synthesis, like the effects on Pt-DNA adducts, were antagonized by nicotinamide (Fig. 7C). All of these observations provide support for a link between inhibition of protein synthesis and modulation of cisplatin action. Although the suggestion that 6AN inhibits protein synthesis (Fig. 7A) appears to conflict with the enhanced expression of a subset of cellular polypeptides (Fig. 3), there is ample precedent for this observation. Studies examining stress-induced proteins have indicated that a number of cellular stresses (e.g., heat shock) simultaneously up-regulate expression of certain genes (e.g., heat shock protein genes) and inhibit protein synthesis (Lindquist and Craig, 1988). The net effect on cellular levels of these polypeptides reflects the increased abundance of message encoding these polypeptides vis á vis the diminished overall rate of translation. 6AN appears to exert a similar effect, increasing the expression of genes such as GRP78 (Chatterjee et al., 1995) while simultaneously decreasing overall protein synthesis (Fig. 7A).

Second, our studies establish a correlation between inhibition of protein synthesis and increased Pt-DNA adducts. In addition to 6AN, four different protein synthesis inhibitors had an effect similar to that of 6AN (Figs. 5 and 6). Actinomycin D inhibits protein synthesis by inhibiting transcription; anisomycin inhibits the transpeptidation step; cycloheximide inhibits the peptidyl transferase reaction on ribosomes; and puromycin causes premature chain termination (Moldave, 1985; Pain, 1986). The demonstration that all four of these compounds exert similar effects suggests that inhibition of protein synthesis is the common feature of their action. Additional experiments (IIB, unpublished observations) have demonstrated that a 22-h treatment of A549 cells with 10 mM 2-deoxyglucose also causes decreased protein synthesis and increased formation of Pt-DNA adducts, providing a means of reconciling the results of Chatterjee et al. (1997) with the present model. The observation that other protein synthesis inhibitors, notably sparsomycin, cinnamaldehyde, anguidine, and L-histidinol, also enhance the cytotoxicity of cisplatin (Hromas and Yung, 1986; Dornish et al., 1989; Hofs et al., 1995; Warrington et al., 1996) is likewise consistent with the model presented in Fig. 8, although the effects of the latter agents on Pt-DNA adducts remain to be evaluated.

Third, as indicated in the inset to Fig. 7A, we observed that 6AN treatment resulted in increased uptake of methionine (which is imported by a neutral amino acid transporter) as well as enhanced cisplatin accumulation. The possibility that protein synthesis inhibitors can alter membrane transport processes is well established. Treatment of 3T3-L1 adipocytes with cycloheximide causes a 7-fold increase in 2-deoxyglucose transport without any alteration in the plasma membrane levels of the glucose transporter (Clancy et al., 1991; Harrison et al., 1992). Likewise, cycloheximide rapidly stimulates leucine uptake on the system L amino acid transporter in cultured vascular smooth muscle cells (Low et al., 1994). Because the polypeptide corresponding to the system L amino acid transporter has not been identified, it is not known whether levels of this polypeptide are altered by cycloheximide. Nonetheless, these examples provide precedent for alterations in transport as a consequence of treatment that inhibits protein synthesis. In both cases, the authors postulated that cycloheximide inhibited the synthesis of a short-lived polypeptide that suppressed the activity of the transporters under basal conditions (Clancy et al., 1991; Harrison et al., 1992; Low et al., 1994).

In view of previous claims that diminished accumulation is a potentially important cause of cisplatin resistance (Gately and Howell, 1993), it is tempting to speculate that the addition of protein synthesis inhibitors to platinating agents might provide one means of overcoming cisplatin resistance. Of the anticancer drugs that are currently in clinical use, actinomycin D and L-asparaginase are capable of inhibiting protein synthesis. Each of these agents, however, causes a variety of toxicities in vivo (Chabner and Loo, 1996); and neither is routinely combined with cisplatin. Accordingly, further preclinical studies are required to determine whether the mechanism of biochemical modulation outlined above can be developed into a regimen suitable for testing in the clinical setting.

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This paper is dedicated to the memory of Mette Strand, who directed the Graduate Program in Pharmacology at the Johns Hopkins University School of Medicine during the period of this thesis project. We gratefully acknowledge the kind gift of 6AN analogs from

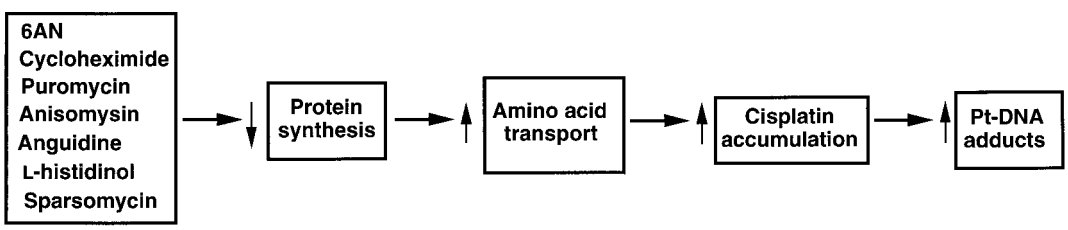

Fig. 8. Proposed mechanism by which 6AN enhances cisplatin cytotoxicity.

the National Cancer Institute, advice of Alex Adjei regarding the methionine transport assay, provocative discussions with Daniel S. Martin and Nathan Berger, secretarial help of Deb Strauss, and assistance of the Metals Laboratory of the Mayo Clinic (Thomas P. Moyer, Director) with the platinum assays.

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