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Reversal of acquired cisplatin resistance by nicotinamide in vitro and in vivo

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Abstract. At a concentration of 2.5 m*M*, nicotinamide (NA), an inhibitor of poly(ADP-ribose) polymerase (PARP), significantly potentiated the cytotoxicity of cisplatin (DDP) in a DDP-resistant rat ovarian tumor cell line (O-342/DDP) in vitro, whereas the same treatment had no substantial effect on DDP's cytotoxic activity against the DDP-sensitive parental line (O-342). Furthermore, in a nude mouse model where the O-342/DDP tumor grew intraperitoneally, whereas DDP given alone at 1 mg/kg $\times 3$ exhibited no antitumor activity as compared with control values due to the resistance, NA given at a nontoxic dose (5 mmol/kg $\times 3$) significantly increased the mean survival time (MST) of the tumor-bearing NMRI nude mice from 20.7 days in the DDP-treated group to 29.0 days in the combination group. Mechanism studies showed that endogenous PARP activity (incorporation of tritiated nicotinamide adenine dinucleotide, [^{3}H]-NAD) was 2.6 times higher in O-342/DDP than in O-342 cells and that the presence of 2.5 m*M* NA during the incubation with the isotope resulted in 73.3% inhibition of the enzyme activity in O-342/DDP cells but in only about 30% inhibition in the sensitive line. However, treatment with NA during and after DDP exposure failed to produce any significant effect on the formation of DNA single-strand breaks (SSB) but decreased the induction of DNA interstrand cross-links (ISCL) by DDP in the sensitive and resistant cell lines. These results suggest that NA might have some clinical potential in reversing DDP resistance, and further studies are therefore warranted to confirm the resistance-reversing effect of NA in other DDP-resistant cell lines.

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Abbreviations: DDP, cisplatin, *cis*-diamminedichloroplatinum(II); ID_{50}, 50% inhibitory dose; ISCL, DNA interstrand cross-links; MST, mean survival time; NAD, nicotinamide adenine dinucleotide; PARP, poly(ADP-ribose) polymerase (formerly ADPRT; ADP-ribosyl transferase); SSB, DNA single-strand breaks; TCA, trichloroacetic acid

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Introduction

Cisplatin (DDP) is a clinically important antineoplastic agent with a wide range of activity against solid tumors, particularly for treatment of patients with testicular and ovarian cancer [32]. In patients with advanced epithelial ovarian cancer, for example, DDP has been demonstrated to be one of the most active drugs when used either as a single agent or in combination with other substances [29, 35]. However, the majority of patients cannot be cured by DDP due to the resistance of tumor cells to this drug [28]. Mechanisms responsible for DDP resistance are multifactorial, including reduced cellular DDP accumulation, increased levels of cellular glutathione (GSH), and increased DNA repair, among others [15]. Although in some cell lines, DDP resistance can in part be reversed by dipyridamole, a membrane-active compound, by buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, or by aphidicolin, an inhibitor of DNA polymerase alpha, by targeting these specific resistance determinants, these observations have not been confirmed in many other cell lines [15, 36], indicating that additional underlying mechanisms are operative in DDP resistance and that these may vary from cell line to cell line. Therefore, the identification of new determinants and codeterminants of DDP resistance for the development of target-oriented strategies to overcome DDP resistance is urgently needed.

Poly(ADP-ribose) polymerase (PARP), a nuclear enzyme (EC 2.4.2.30), synthesizes poly(ADP-ribose) by using the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD) and thereby modifies a number of chromatin proteins, including the enzyme itself [19, 31]. PARP activity is stimulated when DNA breaks are induced after treatment with DNA-damaging agents [1, 23, 39], and the addition of PARP inhibitors such as 3-aminobenzamide (3AB) to this treatment results in increased levels of DNA strand breaks and potentiation of the cytotoxicity of DNA-damaging agents [16, 25]. Therefore, PARP has been postulated to play a role in DNA excision repair, possibly in the ligation step [14, 33], but the exact mechanisms underlying this process remain unclear [3, 13]. Our previous

work showed that 3AB and nicotinamide (NA), another inhibitor of PARP, synergistically enhanced the therapeutic effect of cisplatin (DDP) on sarcoma 180 and Ehrlich ascites carcinoma [8]. Potentiation of DDP's antitumor activity by 3AB has also occurred in a DDP-resistant rat ovarian tumor cell line (O-342/DDP) [10, 11], indicating that PARP inhibitors may have the potential to modulate tumor sensitivity to DDP. To extend further these observations and explore the possible utility of PARP inhibitors against tumor-cell resistance to this important antitumor agent, we studied the effects of combinations of DDP with NA on the O-342/DDP tumor in vitro and in vivo as well as the possible mechanisms of resistance.

Materials and methods

Materials. RPMI 1640 medium was supplied by Gibco Company (Scotland). DDP solution (0.5 mg/ml) was obtained from Behring Werke AG (Marburg, Germany) and NA powder, from Sigma Chemical Company (USA). Prior to use, they were diluted or dissolved in medium for in vitro study and in 0.9% NaCl for in vivo investigation, respectively. [^{3}H]-NAD (sp. act., 4.0 Ci/mmol; concentration, 0.1 mCi/ml) was purchased from DuPont de Nemours (NEN, Dreiech, Germany). Female BD IX rats (6–8 week old) and female NMRI nude mice (weighing 20–25 g) were supplied by the Zentralinstitut für Versuchstierzucht (Hannover, Germany) and maintained under standard conditions as described elsewhere [10].

Tumor cell lines in vivo and in vitro. An experimental rat ovarian tumor (O-342) was induced by injection of ethylnitrosourea in a female BD IX rat; development of DDP resistance in vivo was achieved by treatment of tumor-bearing rats with DDP in successive passages. DDP-sensitive and -resistant tumors were maintained in vivo by i.p. inoculation at 10- to 12-day intervals, and details about these tumors have previously been reported [10, 43]. In vitro DDP resistance (O-342/DDP) was induced in O-342 cells by stepwise increases of the DDP concentration in medium. The in vitro characteristics and cytogenetic patterns of O-342 and O-342/DDP cells have been described elsewhere [6, 7, 11]. Cells grew in a humidified atmosphere of 95% air/5% CO_2 at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum (Sigma Chemical Co., St. Louis, Mo., USA), 100 U penicillin/ml, 100 µg streptomycin/ml, 1% glutamine, and 0.8% HEPES in the absence (O-342) or presence (O-342/DDP) of 8 µ*M* DDP.

Cytotoxicity test in vitro. The cytotoxicity of DDP and NA in O-342 and O-342/DDP cells was evaluated using the MTT test [26] with minor modifications [9]. Cells were seeded into wells of a 24-well plate (Falcon) at a density of 1×10^5 cells/ml per well. After attachment (about 24 h), the medium was removed and replaced with DDP- and/or NA-containing medium, and the cells were incubated for 48 h before the MTT assay. Then, 0.1 ml MTT reagent [MTT: 3-(4,5-dimethylthiazo-1-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma-Chemie, Deisenhofen, Germany) at 5 mg/ml, dissolved in Dulbecco's phosphate-buffered saline (PBS; Gibco, Scotland)] was added to each well and the plate was incubated for a further 4 h at 37°C. Following this incubation period the bulk of the medium was removed, with care being taken to leave the formazan crystals behind. Then, 0.5 ml acid-isopropanol (10 ml isopropanol plus 0.2 ml 0.04 *N* HCl) was added to each well and mixed thoroughly to dissolve the dark blue crystals. After this mixture had been allowed to stand for a few minutes at room temperature to ensure that all crystals had dissolved, the color suspension was transferred to a 96-well plate (0.1 ml/well), which was read in quadruplicate on a Titertek Multiskan MCC plate reader at 540 nm.

Combination chemotherapy in vivo. Female NMRI nude mice (weight, 20–25 g) were used for in vivo studies. O-342/DDP tumor was removed from the donor mouse and minced mechanically with the aid of scissors and a syringe. About 10^6 O-342/DDP cells (corresponding to about 4 mg minced tumor tissue) were inoculated i.p. per mouse. The mice were then randomly divided into different treatment groups containing ten mice each. On days 1, 4, and 7 after inoculation, mice received either 0.5 ml vehicle (0.9% NaCl) or the same volume of DDP and/or NA solution i.p. For evaluation of the therapeutic effect, the mean survival time (MST) of each group was recorded and analyzed with Student's *t*-test.

PARP determination. PARP activity was determined essentially as previously described [2]. Only cells in exponential growth (2–3 days after seeding) were used for the PARP-activity assay to avoid any influence of the cell cycle [5]. Cells with or without a previous 2-h exposure to 20 µ*M* DDP were removed from flasks with 0.25% trypsin/0.1% ethylenediaminetetraacetic acid (EDTA) and collected by centrifugation at 240 *g* for 5 min. The pellets were washed twice with cold Dulbecco's PBS. After centrifugation, pellets in duplicate were suspended in 0.5 ml permeabilizing buffer (10 m*M* TRIS-HCl, 1 m*M* EDTA, 4 m*M* $MgCl_2$, 30 m*M* mercaptoethanol, and 0.05% Triton X-100; pH 7.8) at a concentration of 2×10^6 cells/ml at 0°C for 30 min under occasional gentle agitation. Following centrifugation (800 *g*, 4°C, 10 min), cells were resuspended in 0.5 ml reaction buffer (30 m*M* TRIS-HCl, 1 m*M* EDTA, 40 m*M* $MgCl_2$, 20 m*M* mercaptoethanol, 0.25 m*M* NAD, and 0.05% Triton X-100; pH 7.8), to which 0.5 µCi [^{3}H]-NAD was added. The mixture was incubated at 37°C for 1 h in the absence or presence of 2.5 m*M* NA. The reaction was terminated by the addition of 2 ml ice-cold 20% (w/v) trichloroacetic acid (TCA) to the mixture. Acid-insoluble materials were collected on glass-fiber filters (GF/C, Whatman, England), which were washed twice with 10 ml 10% (w/v) TCA containing 2% (w/v) tetrasodium pyrophosphate ($Na_4P_2O_7$) and once with 5 ml 95% ethanol. Following a drying step, the radioactivity on the filter was counted in 5 ml scintillation cocktail (Canberra-Packard GmbH, Germany) by a liquid scintillation analyzer (Packard) with a tritium-counting efficiency of 40%–50%.

DNA-damage assay. Exponentially growing cells were incubated with 20 µ*M* DDP in the absence or presence of 5 m*M* NA at 37°C for 2 h. Thereafter, the drug-containing medium was removed and replaced with fresh medium or medium containing 5 m*M* NA, which remained in cultures throughout the experiment. Alkaline elution was carried out 24 h later in duplicate using Kohn's method [24] as modified in our laboratory [42]. For the introduction of a controlled level of DNA single-strand breaks, a specimen was subjected to gamma irradiation (500 rad) in a dark environment at 0°C (^{137}Cs source, Gammacell 100; Atomic Energy of Canada, Ltd.; dose rate, 16.7 Gy/min). DNA contents of all fractions as well as of filter suspensions were determined fluorimetrically using Hoechst 33258 (Serva, Heidelberg, Germany). DNA single-strand breaks (SSB) and interstrand cross-links (ISCL) were quantified in terms of rad equivalents (rad eq.). This experiment was repeated at least four times in each cell line (one representative result is shown in Figs. 2 and 3 for the resistant and sensitive cells, respectively), and the difference observed in ISCL formation following DDP versus DDP + NA treatment was analyzed by Student's *t*-test.

Results

Overcoming resistance to DDP by NA in vitro and in vivo

The effect of NA on DDP cytotoxicity in O-342 and O-342/DDP cells in vitro is shown in Fig. 1. Continuous exposure to 2.5 m*M* NA alone for 48 h resulted in 17.1% ± 4.5% and 22% ± 8.9% inhibition of cell growth in O-342 and O-342/DDP cells, respectively, as compared with untreated controls. The respective reference groups for calculation of the surviving fractions were therefore untreated and NA-treated cells (both standardized as 100%) for DDP and DDP + NA treatment, respectively. In

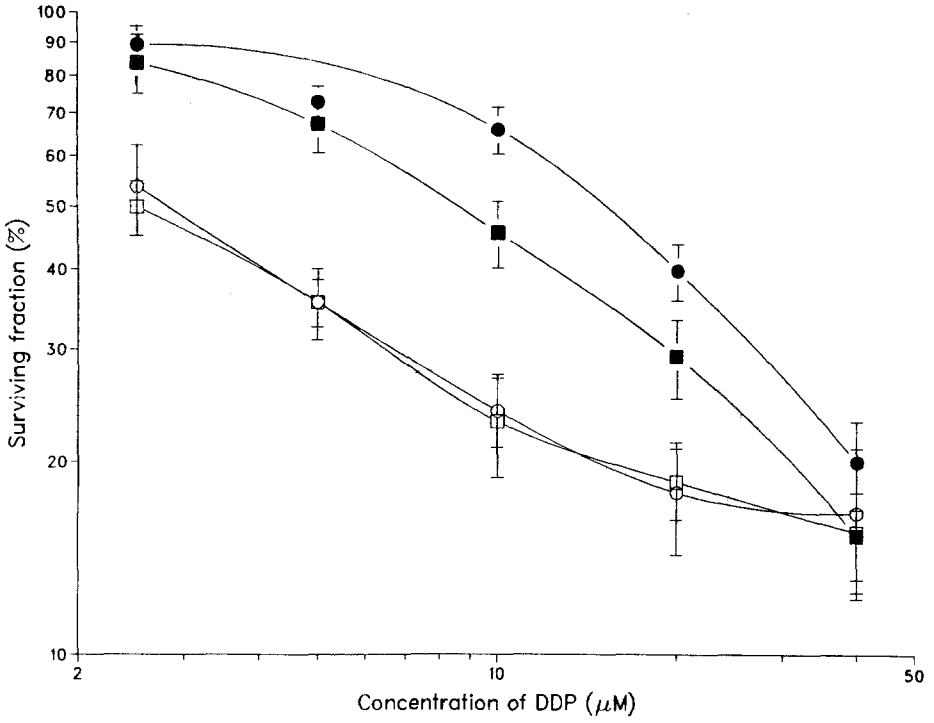

Fig. 1. Survival of O-342 (○, □) and O-342/DDP (●, ■) cells treated with either DDP alone (○, ●) or DDP + 2.5 m*M* NA (□, ■) as determined by MTT assay. Points represent mean values ± SE of 5 independent experiments

Table 1. Combination effect of DDP and NA on the survival of NMRI nude mice bearing O-342/DDP tumor

Treatment	Schedule[a]	Survival in days ($\bar{X}$ ± SD)[b]	ILS (%)	*P*[c]
0.9% NaCl	On days 1, 4, 7	21.5±2.0	–	–
NA (5 mmol/kg)	On days 1, 4, 7	22.0±2.6	2.3	>0.05*
DDP (1 mg/kg)	On days 1, 4, 7	20.7±3.2	–3.7	>0.05*
NA (5 mmol/kg)	On days 1, 4, 7			
+		29.0±2.6	34.9	<0.001*
DDP (1 mg/kg)	On days 1, 4, 7			<0.001**

[a] 1×10^6 cells were inoculated i. p. on day 0; drugs were simultaneously injected i. p.
[b] 10 mice/group
[c] *Versus control, **vs DDP treatment alone; evaluated using Student's *t*-test

Table 2. PARP activity in untreated and DDP-treated O-342 and O-342/DDP cells as well as its inhibition by NA

Treatment[a]		[^{3}H]-NAD incorporation (dpm/10^6 cells; $\bar{X}$ ± SD)[b]		
DDP	NA	O-342	O-342/DDP	*P*[c]
–	–	339± 65 (100%)	878±34 (100%)	<0.001
–	+	236± 63 (69.6%)	234±71 (26.7%)	>0.05
+	–	342± 68 (101%)	609±96 (69.4%)	<0.01
+	+	285±145 (84.1%)	181±21 (20.6%)	>0.05

Numbers in parentheses indicate percentages of untreated control values
[a] Cells in exponential growth (untreated or following 2 h exposure to 20 μ*M* DDP) were permeabilized as described in Materials and methods and incubated with 0.5 μCi [^{3}H]-NAD/10^6 cells in the reaction buffer at 37° C for 1 h in the absence or presence of 2.5 m*M* NA
[b] Results of 3 and 4 independent determinations (each carried out in duplicate) in O-342 and O-342/DDP cells, respectively
[c] O-342 vs O-342/DDP, evaluated using Student's *t*-test

the sensitive cells, no substantial difference was observed in the cytotoxicity produced by DDP treatment and that caused by the combination, whereas in the resistant cells, DDP cytotoxicity was significantly enhanced by NA by a dose-modification factor of 1.81 [50% inhibitory dose (ID_{50}) in the absence of NA (15.6 μ*M*)/ID_{50} in the presence of NA (8.6 μ*M*)] (Fig. 1).

To extend these observations further, we studied the effects of the combination of DDP and NA on the O-342/DDP tumor in NMRI nude mice. The results (Table 1) showed that treatment of this highly resistant tumor with DDP alone (1 mg/kg ×3) produced no antitumor activity, resulting in a mean survival time (MST) of 20.7 days in the treated group vs 21.5 days in the control group. Likewise, NA (5 mmol/kg ×3) alone was not active. Simultaneous administration of DDP and NA, however, increased the MST from 20.7 days in the DDP group to 29.0 days in the DDP + NA group (P <0.001). This result was confirmed by a further independent experiment in vivo, in which the MST increased from 21.1 days in the DDP group to 27.1 days in the DDP + NA (10 mmol/kg) group. Therefore, we concluded that NA can overcome the resistance of the O-342/DDP tumor to DDP both in vitro and in vivo.

PARP activity in O-342 and O-342/DDP cells

To clarify whether the chemosensitization of NA might be mediated by PARP, we determined the enzyme activity in the sensitive and resistant cells. By measuring the incorporation of [^{3}H]-NAD, a substrate for PARP, in permeabilized cells, a 2.6-fold increase in endogenous PARP activi-

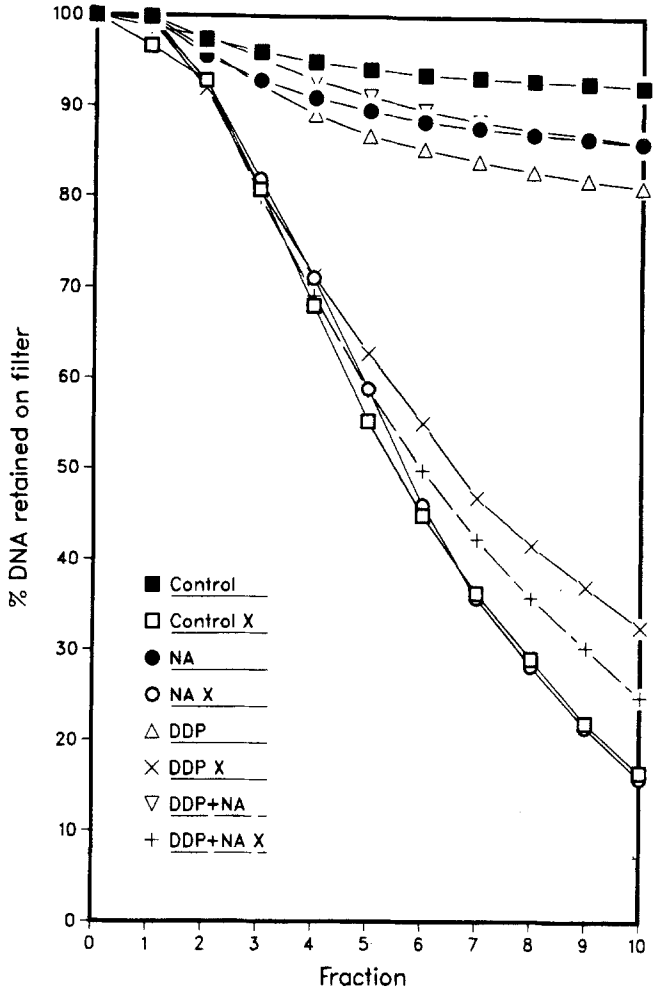

Fig. 2. Reduction of DNA-ISCL in O-342/DDP cells treated with DDP plus NA over DDP alone. X, Cells irradiated with 500 rad

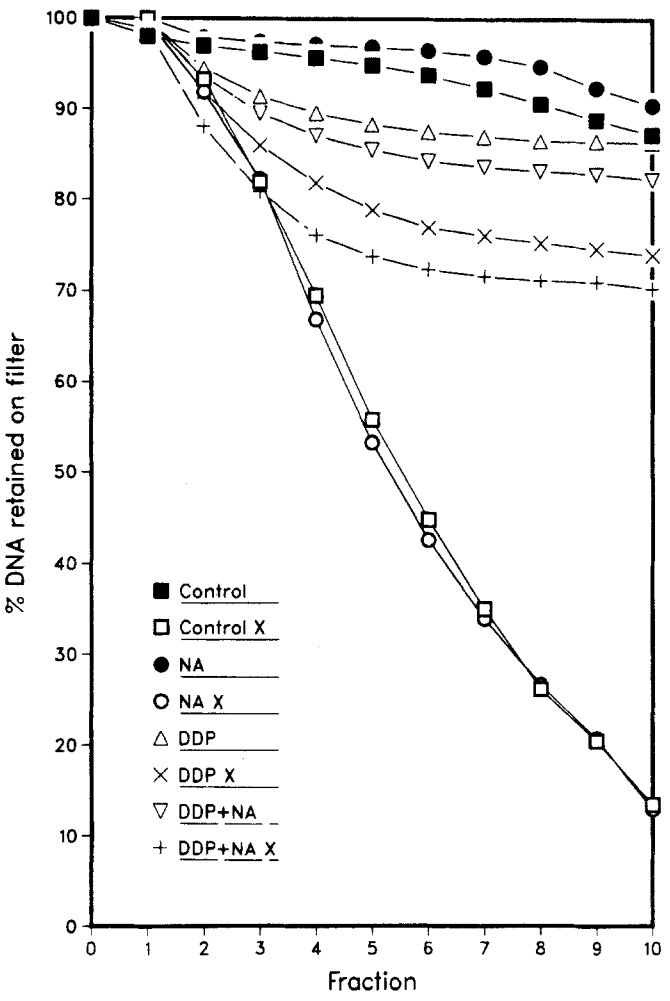

Fig. 3. Effect of NA on DNA-ISCL formation by DDP in O-342 cells. X, Cells irradiated with 500 rad

ty was observed in the DDP-resistant line as compared with the sensitive line (878 dpm/10^6 cells in O-342/DDP cells vs 339 dpm/10^6 cells in O-342 cells, $P<0.001$; Table 2). The addition of 2.5 m*M* NA to the incubation mixture of cells with the isotope caused a 73% inhibition of the incorporation in the resistant line ($P<0.001$) but only a 30% inhibition in the sensitive counterpart ($P>0.05$). Exposure of O-342/DDP cells to 20 μ*M* DDP for 2 h resulted in a significant decrease in PARP activity (from 878 to 609 dpm/10^6 cells, $P<0.01$), whereas in O-342 cells the same treatment had no effect on the enzyme activity. When NA was added following DDP exposure, the activity further decreased significantly only in O-342/DDP cells (Table 2).

Effect of NA on DNA damage induced by DDP

Since PARP inhibitors increase DNA strand breaks in alkylating agent-treated cells [25] and DDP induces DNA ISCL to produce lethal damage to the cells [30, 44], alkaline elution was carried out to explore the possible effects of NA on DDP-induced DNA damage in these two lines. As depicted in Figs. 2 and 3, in neither O-342/DDP nor O-342 cells a significant effect of NA on the frequency of SSB induction by DDP was observed, whereas NA produced a decrease in the numbers of DNA-ISCL induced by DDP in both lines [from 50 ± 14 to 28 ± 12 in O-342/DDP cells (mean $\pm$ SD) and from 315 ± 60 to 247 ± 57 in O-342 cells; $P<0.05$ in both cases], although at this time point following DDP treatment there was a significant difference of the amounts of ISCL between these two cell lines.

Discussion

In the present study we demonstrated a partial reversal of the resistance of O-342/DDP cells to DDP by NA in vitro. More importantly, this resistance-reversing effect of NA was also present in the intact mouse model, which should be far more representative of human tumors in situ. These results are potentially relevant to clinical practice, since NA is an old drug (vitamin PP) that is relatively nontoxic in both animals [20] and humans [41] and has been used in

the clinical setting for treatment of psoriasis [40], pellagra [17], and schizophrenia [18]. The dose of NA used in the present study is achievable in humans [18]. Another main concern for the use of DDP and NA in combination is the possible toxicological interactions of these two compounds in vivo. Our earlier investigation showed that the simultaneous administration of DDP (1 mg/kg) and NA (5 m*M*/kg) every other day for five cycles in mice significantly increased the antitumor activity of DDP against sarcoma 180 without obviously influencing its toxicity in terms of body weight [8]. Furthermore, 5 m*M*/kg NA reduced DDP single-dose lethality and nephrotoxicity [8]. Moreover, NA has been demonstrated to increase radiation sensitivity in several tumor models [20, 21]. Therefore, it is possible that as a well-established drug, NA might find a new application as a chemo- and radiosensitizer in the clinical setting. Certainly, these results need to be further confirmed in vitro and in vivo in other DDP-resistant lines, especially human tumor cell lines.

Stimulation of PARP activity by DNA breaks following DNA damage as mentioned above is well documented [3]. The increased enzyme activity observed in DDP-resistant O-342/DDP cells (Table 2) may have occurred as a consequence of the chronic treatment of cells with DDP during the induction and maintenance of resistance. The elevation of the activity can be explained in part by higher levels of PARP enzyme protein in this line as detected by Western blotting (Chen and Zeller, manuscript in preparation). Although no acute change in the enzyme activity following 2 h exposure to DDP was observed in the sensitive line (Table 2), the kinetic investigation did show that DDP stimulated PARP activity in O-342 cells at 24 h and thereafter following the exposure, possibly mediated by DNA breaks produced during DNA repair [7]. This result was further confirmed by indirect immunofluorescence of in vivo-stimulated poly(ADP-ribose) polymerase, in which poly(ADP-ribose) staining was increased significantly by DDP treatment as detected by a monoclonal antibody directed specifically against the polymer [4].

Similar to our results, a lack of activation of PARP by peplomycin has been found in a peplomycin-supersensitive lung-cancer cell line [27], and a higher activity of the enzyme has been reported in a bleomycin-resistant HeLa cell line [37]. Wassermann et al. [38] have also reported an increased stimulation of PARP by X-ray irradiation in a radiation-resistant human carcinoma cell line. In the present study, the same level of enzyme activity in the resistant line upon the addition of NA as in the sensitive cells (Table 2) conferred only partial reversal of DDP resistance (Fig. 1), indicating that the increased PARP activity may represent only one of the contributing factors in the development of DDP resistance in this system. However, it cannot be ruled out that NA might potentiate DDP antitumor activity in vivo through mechanisms other than PARP inhibition, since (a) mechanisms operative for alkylating agent resistance differ between in vivo and in vitro systems [34], (b) in vivo PARP inhibitors enhance DDP antitumor activity not only in a DDP-resistant tumor (3AB) [10] but also in DDP-sensitive tumors (3AB, NA) [8, 10], and (c) NA is believed to exert its radiosensitizing effect in animals by increasing the tumor oxygenation status [21]. Determination of the exact mechanisms involved in this process awaits further investigations.

Since most of the DNA repair in O-342/DDP cells occurred within the first 24 h following DDP removal [7], attempts were made to identify whether the inclusion of NA during this period after DDP exposure might have any effect on DNA SSB and ISCL formation induced by DDP. As shown in Figs. 2 and 3, NA had no effect on DNA SSB production but reduced ISCL formation in both O-342/DDP and O-342 cells treated with DDP. This result is somewhat different from that obtained with 3AB in that at this time point following DDP exposure, 5 m*M* 3AB significantly increased the frequency of SSB formation by 1.5 and 2.0 times in the sensitive and resistant cells, respectively [12]. Institoris et al. [22] recently found that benzamide, another PARP inhibitor, enhanced the cytotoxicity and incidence of DNA SSB at 24 h following the exposure of cells to 1,2:5,6-dianhydrogalactitol (DAG) in the resistant cells but not in the sensitive cells. The selective inhibition of PARP activity (Table 2) and the selective enhancement of DDP cytotoxicity (Fig. 1) by NA in O-342/DDP cells but not in O-342 cells suggest that the decrease in the number of DNA ISCL caused by NA in the DDP-treated sensitive and resistant cells might occur through mechanisms other than PARP inhibition and may not be directly related to the chemosensitizing effect of NA in the resistant cells.

The major finding in this study is that NA can reverse DDP resistance both in vitro and in vivo. We can also provide some evidence that this effect might occur through modulation of poly(ADP-ribose) synthesis. Since NA is relatively nontoxic and has long been used in clinical practice, this result would have some clinical potential for the treatment of patients with DDP-resistant malignancies. The exact roles of PARP in DDP resistance as well as ISCL repair, however, need to be explored further.

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