Modulation of the Cell Cycle-dependent Cytotoxicity of Adriamycin and 4-Hydroperoxycyclophosphamide by Novobiocin, an Inhibitor of Mammalian Topoisomerase II

Francis Y. F. Lee, Deborah J. Flannery, and Dietmar W. Siemann
Tumor Biology Division, University of Rochester Cancer Center, Rochester, New York 14642

ABSTRACT

Centrifugal elutriation was used to obtain synchronized cell populations in various cell cycle phases without prior growth-perturbing manipulation. Treatment of these subpopulations with novobiocin (NOVO), a putative inhibitor of the mammalian topoisomerase II enzyme, revealed a unique cell cycle phase-dependent cytotoxicity for this agent. At a concentration of 0.3 mM, NOVO was cytotoxic only to a specific cell subpopulation in the G1-S phase boundary. Cells in other cell cycle phases were completely unaffected. Additionally, S and G2/M phases progressed through the cell cycle relatively unaffected by NOVO but were blocked at the G1/S boundary.

NOVO treatment protected tumor cells from Adriamycin (ADR)-induced lethality but sensitized them to the toxic action of 4-hydroperoxycyclophosphamide, an alkylating agent. These opposing effects of NOVO were demonstrated in all of the four tumor cell lines investigated: A431 and HEp3 (derived from human squamous cell carcinomas); MDA-MB-468; and a Chinese hamster ovary cell line. The degree of protection against ADR was the greatest for S-phase cells, a human ovarian cancer cell line; and a Chinese hamster ovary cell line. In contrast, even though the cytotoxic activity of 4-hydroperoxycyclophosphamide exhibited significant cell cycle dependency, NOVO enhanced 4-hydroperoxycyclophosphamide lethality equally for all cell cycle phases.

INTRODUCTION

There is considerable current interest in the role of Topo II in determining the sensitivity of tumor cells to chemotherapeutic agents, notably the epipodophyllotoxins (1, 2), anthracyclines (3), and alkylating agents (4–6). It has been demonstrated in several studies that tumor cell lines that were resistant to DNA intercalators, whether acquired or innate, had decreased Topo II activity (7–9), while those resistant to alkylating agents had increased Topo II activity (1, 6). Clearly, these directly opposing relationships between Topo II activity and the anti-tumor efficacy of two of the most frequently used classes of anticancer agents have important implications for the optimal use of these agents in polychemotherapy. However, the precise role of Topo II-mediated processes in determining antitumor efficacy is still not well understood. Here we describe experiments, using the putative Topo II inhibitor novobiocin, aiming at defining the possible role of Topo II in the cell cycle-dependent cytotoxicity of ADR and 4-OH-CP.

MATERIALS AND METHODS

Cell Culturing. All cell lines were grown as monolayer cell cultures. Chinese hamster ovary cells were maintained in Ham’s F-10 medium, HEP3 in Ham’s F-12 medium, A543 and MDA-MB-468 in α-minimum essential medium. All media were obtained from Gibco and were supplemented with 10% fetal calf serum, 5 mM glutamine, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Three-day-old exponentially growing cells were used in all experiments.

Drugs and Drug Treatment. ADR was obtained from Adria Laboratory (Columbus, OH); 4-OH-CP was kindly provided by Dr. R. Borch (University of Rochester, Rochester, NY) and Dr. P. Hilgard (Asta-Werke AG, Bielefeld, Germany). NOVO was purchased from Sigma Chemical Co. Cells were generally treated with cytotoxic agents while still attached to Petri dishes. In some experiments, cells were treated in suspensions, at a concentration of 2–3 × 10^5 cells/mL, following standard trypsinization procedures to obtain single cells. Cells were suspended in complete α-minimum essential medium in a type 7 vial at 37°C as described by Whillans and Rauth (10) and were continuously gassed with 95% air-5% CO2. For both treatment procedures, a 10-μL aliquot of a stock solution of the appropriate drug was given per mL of medium.

Clonogenic Cell Survival Assay. At the end of drug exposure, cells were washed and single cell suspensions were obtained. The cell number was counted and a variable number of cells were plated to determine survival by clonogenic assay. At an appropriate interval following plating, the exact length of time being cell line dependent, dishes were stained with crystal violet. Colonies containing more than 50 cells were counted to determine cell survival.

Centrifugal Elutriation. Cells in various cell cycle phases were isolated by centrifugal elutriation, which separates cells according to size and density. Briefly, single cell suspensions of tumor cells were elutriated in ice-cold complete media containing 10% fetal calf serum. During the elutriation, the reservoir, rotor, and collection flasks were kept at 4°C. After 10^6 cells were loaded into a Beckman JE-6 elutriator rotor, subfractions were collected at decreasing rotor speeds. Cell volume and number were determined with a Coulter Channelizer. Since most tumor cells are aneuploid, flow cytometry was used as a second measure of tumor cell purity. Cells were fixed with 70% methanol and stained with mithramycin or propidium iodide, and the fluorescence intensity was quantitated by a Coulter EPICS V flow cytometer.

Glutathione Assay by High Performance Liquid Chromatography. Cellular GSH content was analyzed by the high performance liquid chromatography technique described previously (11). Briefly, tumor cells (2–5 × 10^6) were washed with phosphate-buffered saline, and centrifuged at 400 × g for 10 min at 4°C, and the cell pellet was stored at −70°C before analysis. Thawed cell pellets and frozen-solid tumor cubes (1 mm³) were homogenized with 200 μL or 20 volumes (w/v), respectively, of a 20 mM S-sulfosalicylic acid solution and centrifuged in an Eppendorf microcentrifuge for 40 s. The supernatant was derivatized using the fluorescent reagent monobromobimane. The fluorescent GSH conjugate was eluted in a Waters Radial-PACK reversed-phase bonded octadecylsilane (C18) cartridge column (8 mm inside diameter) using isocratic conditions consisting of a mobile phase of 23% acetonitrile in 40 mM ammonium phosphate buffer, pH 7.2, containing 5 mM tetrabutylammonium hydroxide, and a flow rate of 3 mL/min. Fluorescence of the effluent was detected at 410 nm with excitation at 340 nm. GSH concentration was determined from peak height with reference to a linear calibration curve constructed using synthetic GSH standards.

Received 1/2/91; accepted 4/21/92.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work is supported by NIH Grant CA-36858 and BRSG Sub S4RR05403-27.

2 To whom requests for reprints should be addressed, at Experimental Therapeutics Department, Pharmaceutical Research Institute, Bristol-Myers Squibb Co., 5 Research Parkway, P. O. Box 5100, Wallingford, CT 06492-7660.

3 The abbreviations used are: Topo II, topoisomerase II; NOVO, novobiocin; ADR, Adriamycin; GSH, glutathione; 4-OH-CP, 4-hydroperoxycyclophosphamide.
Typical performance characteristics of this assay are: lower limit of detections, 8.0 pmol on column; coefficient of variation, 4-8%; accuracy, 92-109%.

RESULTS

Cell Cycle Selective Cytotoxicity of NOVO

NOVO exhibits pronounced cell cycle phase selectivity in its toxic action. Following a 4-h treatment with 0.3 mM NOVO, lethality was observed only for a small population of cells in the G1-S boundary of the cell cycle (Fig. 1). For this particular subpopulation, clonogenic cell survival was reduced to ~30% of control. Cells in other parts of the cell cycle were completely unaffected.

![Cell cycle phase distribution](image)

Fig. 1. Cell cycle-specific cytotoxicity of NOVO. Monolayer cultures of MLS cells in exponential growth were treated with 0.3 mM NOVO or phosphate-buffered saline for 4 h. Single cell suspensions were then prepared for centrifugal elutriation. Cell cycle phase distribution was determined by flow cytometry following DNA labeling with propidium iodide.

Effects of NOVO on Cell Cycle Progression

NOVO at a concentration of 0.3 mM inhibited the cell cycle progression from G1 to S phase (Fig. 2). Fig. 2, A and C, shows that under normal conditions the number of G1 cells in a G1 phase-enriched cell population declined progressively with time as individual cells transited the cell cycle. At 16 h, the number of G1 cells reached a minimum (31%), while 44 and 25% of the cells were in S and G2M phase, respectively. With NOVO, the G1 to S transition was blocked (Fig. 2, A and D). At 16 h, 73% of the cells remained in the late G1 phase of the cell cycle while 17 and 10% were in S and G2M phases, respectively. The effects of NOVO on cell cycle progression are confirmed by cell growth measurement (Fig. 2B). Under control conditions the number of cells roughly doubled 30 h following the plating of G1 cells. With NOVO in the growth medium, however, no increase in cell number was apparent.

The effects of NOVO (0.3 mM) on cell cycle progression appeared to be specific for cells in the G1-S transition phase. Cells in S and G2M phases progressed normally through the cell cycle at similar rates as cells under control conditions (Fig. 3). However, at very high NOVO concentration (1.8 mM), cell cycle progression was blocked at the G2M phase (Fig. 3).

Cell Cycle-dependent Cytotoxicity of ADR and 4-OOH-CP

ADR. The four different cell lines investigated in this study all exhibited similar patterns of cell cycle phase-dependent responses to ADR (Figs. 4A and 5B). Cells in the G1 phase of the cell cycle were always the most resistant, whereas S and
NOVOBIOCIN MODULATION OF ANTICANCER DRUG ACTION

**Effects of NOVO on the Cell Cycle-dependent Cytotoxicities of ADR and 4-OHH-CP**

ADR. NOVO protected exponentially growing cells (both Chinese hamster ovary and A431) against the cytotoxicity of ADR (Fig. 5A). The protective efficacy of NOVO was concentration dependent; significant protection was observed at a concentration of 0.01 mM. In addition, the protective efficacy of NOVO was cell cycle dependent; cells in S phase were protected to a greater degree than cells in G1 phase (Fig. 5B).

4-OHH-CP. NOVO enhanced the cytotoxicity of 4-OHH-CP (Fig. 6A). The sensitizing activity of NOVO was dose dependent; its effectiveness increased with increasing NOVO concentration, from 0.01 to 0.3 mM (Fig. 6B). However, in contrast to its effects on ADR, the enhancement by NOVO of cellular 4-OHH-CP chemosensitivity was not cell cycle dependent (Fig. 7). The timing of NOVO treatment also appeared to be critical for its activity. Simultaneous presence of NOVO and 4-OHH-CP was essential for interaction; treatment of cells with NOVO after 4-OHH-CP was removed produced no sensitization effect (Fig. 6B).

**Effects of NOVO on Glutathione Metabolism**

Because GSH is known to be a critical determinant of the cytotoxicity of 4-OHH-CP (12-14), we have carried out studies on the effects of NOVO on the status of GSH metabolism using the MLS tumor cell line. As depicted in Fig. 8A, NOVO treatment alone did not affect the GSH level of cells in all phases of the cell cycle. These results show that the enhancement of 4-OHH-CP cytotoxicity by NOVO was not simply due to a direct effect on the level of GSH per se. However, the sensitizing action of NOVO may nevertheless involve GSH. This possibility was suggested by the observation that NOVO enhanced the GSH depletion induced by 4-OHH-CP. Previous dose-response studies where tumor cells were incubated with various concentrations of 4-OHH-CP have shown that depletion of GSH occurred only with cytotoxic concentration of 4-OHH-CP. Significant depletion invariably indicates a breakdown of the cellular defense mechanism which protects cells from damage by the toxic metabolites of 4-OHH-CP (14). Additional data suggested that in treated cells GSH was lost through conjugation reaction with the GSH-reactive toxic metabolites of 4-OHH-CP, acrolein and phosphoramid mustard. The present finding, shown in Fig. 8B, that cells treated with

---

**Fig. 4.** A, cell cycle-dependent cytotoxicity of ADR on the survival of clonogenic cells of three cell lines. O, CHO; △, A431; •, HEp3. Cells were treated with 0.5 μg/ml ADR for 3 h. B, cell cycle-dependent cytotoxicity of 4-OHH-CP on the survival of clonogenic cells of three cell lines. O, CHO; △, A431; △, MLS. Cells were treated with 10, 50, and 25 μM 4-OHH-CP for 3 h, respectively. G2M phase cells were invariably more sensitive.

4-OHH-CP. A radically different pattern of cell cycle-dependent responses was observed for 4-OHH-CP. For this cytotoxic agent, cells in the early G1 phase of the cell cycle were always the most sensitive to the cytotoxicity of 4-OHH-CP. As cells traversed the cell cycle from G1 through S to G2M their sensitivities to 4-OHH-CP became progressively reduced (Fig. 4B).

---

**Effects of NOVO on the Cell Cycle-dependent Cytotoxicities of ADR and 4-OHH-CP**

ADR. NOVO protected exponentially growing cells (both Chinese hamster ovary and A431) against the cytotoxicity of ADR (Fig. 5A). The protective efficacy of NOVO was concentration dependent; significant protection was observed at a concentration of 0.01 mM. In addition, the protective efficacy of NOVO was cell cycle dependent; cells in S phase were protected to a greater degree than cells in G1 phase (Fig. 5B).

4-OHH-CP. NOVO enhanced the cytotoxicity of 4-OHH-CP (Fig. 6A). The sensitizing activity of NOVO was dose dependent; its effectiveness increased with increasing NOVO concentration, from 0.01 to 0.3 mM (Fig. 6B). However, in contrast to its effects on ADR, the enhancement by NOVO of cellular 4-OHH-CP chemosensitivity was not cell cycle dependent (Fig. 7). The timing of NOVO treatment also appeared to be critical for its activity. Simultaneous presence of NOVO and 4-OHH-CP was essential for interaction; treatment of cells with NOVO after 4-OHH-CP was removed produced no sensitization effect (Fig. 6B).

**Effects of NOVO on Glutathione Metabolism**

Because GSH is known to be a critical determinant of the cytotoxicity of 4-OHH-CP (12-14), we have carried out studies on the effects of NOVO on the status of GSH metabolism using the MLS tumor cell line. As depicted in Fig. 8A, NOVO treatment alone did not affect the GSH level of cells in all phases of the cell cycle. These results show that the enhancement of 4-OHH-CP cytotoxicity by NOVO was not simply due to a direct effect on the level of GSH per se. However, the sensitizing action of NOVO may nevertheless involve GSH. This possibility was suggested by the observation that NOVO enhanced the GSH depletion induced by 4-OHH-CP. Previous dose-response studies where tumor cells were incubated with various concentrations of 4-OHH-CP have shown that depletion of GSH occurred only with cytotoxic concentration of 4-OHH-CP. Significant depletion invariably indicates a breakdown of the cellular defense mechanism which protects cells from damage by the toxic metabolites of 4-OHH-CP (14). Additional data suggested that in treated cells GSH was lost through conjugation reaction with the GSH-reactive toxic metabolites of 4-OHH-CP, acrolein and phosphoramid mustard. The present finding, shown in Fig. 8B, that cells treated with
both NOVO and 4-OOH-CP exhibited greater degrees of GSH depletion than cells treated with 4-OOH-CP alone suggests that NOVO may act through a mechanism which resulted in an increase in the level of toxic 4-OOH-CP metabolites. The precise mechanism by which this may occur is not yet known.

**DISCUSSION**

The problem of acquired drug resistance to antineoplastic agents by cancer cells is currently attracting a good deal of research interest. For the DNA intercalator class of antineoplastic agents, a number of biochemical mechanisms of acquired resistance have been identified (for a review see Ref. 13). However, the existence of innately resistant cell subpopulations within the solid tumor mass may also be an important cause of treatment failure in patients receiving first-line chemotherapy. A good case in point is the innate resistance of nonproliferating cells to Topo II-interactive antineoplastic agents (e.g., Adria-

---

**Fig. 6.** A, effects of NOVO on the cytotoxicity of 4-OOH-CP in the MLS ovarian cancer cell line. Cells were incubated with NOVO (0.3 mM) for 1 h followed by coincubation with various concentrations of 4-OOH-CP for an additional 3 h. NOVO enhanced the cytotoxicity of 4-OOH-CP by a factor of 2.1 at the 10% survival level. Bars, SD. B, effects of incubation with NOVO (0.3 mM), simultaneously with or following 4-OOH-CP exposure, on the cytotoxicity of the alkylating agent. The MLS human ovarian cancer cells were treated either simultaneously with 4-OOH-CP and NOVO for 4 h (○), or in two stages: first with 4-OOH-CP for 4 h, washed and then incubated with NOVO for an additional 4 h (△). Bars, SD.

---

**Fig. 7.** Effects of NOVO on the cell cycle-dependent cytotoxicity of 4-OOH-CP in the MLS ovarian cancer cell line. Cells were treated with NOVO (0.3 mM) for 1 h followed by coincubation with 25 μM 4-OOH-CP for another 3 h. Single cell suspensions were then prepared for centrifugal elutriation. Bars, SD.

---

**Fig. 8.** A, effects of NOVO on the GSH content of MLS human ovarian cancer cells in different phases of the cell cycle. Exponentially growing cells were treated with NOVO (0.3 mM) for 4 h prior to separation into the various cell cycle phases by centrifugal elutriation. Bars, SD. B, NOVO enhanced the GSH content-depleting effects of 4-OOH-CP. MLS cells were incubated with NOVO (0.3 mM) for 1 h followed by coincubation with various concentrations of 4-OOH-CP for another 3 h. Bars, SD.
mycin, etoposide). Several recent studies have associated the “resistance” of nonproliferating cells to Topo II-interactive antineoplastic agents with reduced Topo II activity in these cells (7, 9, 15). Similarly, the greater sensitivity of cells in active DNA synthesis (S phase) has been correlated with increased Topo II activity in this cell cycle phase (9, 16). The present results obtained using a variety of different tumor models clearly support this hypothesis.

An important feature of the present investigation is the use of countercurrent centrifugal elutriation to obtain cell populations in different cell cycle phases. This technique has two advantages: (a) cells in different cell cycle phases can be obtained simultaneously from the same exponentially growing cell populations; (b) prior manipulation of growth conditions is not required, thus avoiding complications resulting from growth perturbation (11). The characteristic cell cycle-dependent cytotoxicity of ADR demonstrated for the four different cell lines in this study is in agreement with previously published data (17, 18). Cells in S phase as a rule showed the greatest and G1 cells the least sensitivity to ADR (Figs. 4A and 5B). The findings that NOVO abolished the cell cycle-dependent cytotoxicity of ADR (Fig. 5B) are consistent with the hypothesis that the cytotoxicity of ADR is partly caused by DNA strand breakages resulting from the stabilization of DNA “cleavable complexes” (19, 20). According to this hypothesis the greater sensitivity of proliferating cells to ADR, as compared to stationary phase cells, is a consequence of the higher Topo II activities in actively dividing cells (9, 15). The present findings suggest that NOVO may abrogate the Topo II-mediated cell cycle-dependent cytotoxicity of ADR. This interpretation is in agreement with other studies using DNA intercalators other than ADR (9, 15, 21). It should be noted, however, that the work of Smith and Bell (22) suggested that at least part of the effects of NOVO may be attributed to a reduction in cellular accumulation of ADR.

The effects of NOVO on 4-OOH-CP cytotoxicity have not been previously reported, although a number of studies have investigated its effects on other alkylating agents (4, 5). In this study we showed that NOVO augmented the cytotoxicity of 4-OOH-CP in exponentially growing MLS human ovarian cancer cells by a dose enhancement factor of 2.1 at the isoeffect of 1 log cell kill (Fig. 6A). Unlike its effects on ADR, however, the enhancement of 4-OOH-CP cytotoxicity appeared not to be cell cycle dependent (Fig. 7). In addition, 4-OOH-CP treatment alone did not exhibit the same cell cycle-dependent cytotoxicity as described above for ADR (cf. Figs. 4A and 5B). Furthermore, for the two cell lines investigated thus far, namely A431 and MLS, significant differences in sensitivity to 4-OOH-CP were not observed for exponential versus stationary cells (results not shown). These findings argue against the direct involvement of Topo II per se in the mechanism of alkylating agent toxicity enhancement by NOVO. Other known effects of NOVO may play important roles in this respect. NOVO has been shown to inhibit replicative and repair DNA syntheses (23), affect mitochondrial structure and ATP metabolism (24), and impair DNA and RNA polymerases activity (25). The observation in this study, that NOVO treatment immediately following a 3-h exposure to 4-OOH-CP did not enhance the activity of the cytotoxic agent (Fig. 6B), suggests that NOVO probably effected a process(es) which occurs concurrently with 4-OOH-CP exposure. We have obtained preliminary results showing that combined 4-OOH-CP and NOVO caused a greater degree of glutathione depletion than 4-OOH-CP alone. In previous studies we have established that glutathione depletion is an effective indicator of cytotoxicity because it accurately reflects the amount of alkylating toxic species formed from the parent 4-OOH-CP (14, 26). Therefore, the greater depletion of GSH by combined NOVO and 4-OOH-CP may indicate that the formation of the toxic phosphoramidate mustard and acrolein from the parent 4-OOH-CP was enhanced by NOVO. However, the mechanism by which this occurs is not known at present.

There is strong evidence that NOVO impairs Topo II activity by inhibiting the ATPase portion of this enzyme (27, 28). The present study shows that treatment of tumor cells with NOVO at concentrations that can inhibit cell cycle progression (from G1 to S phase), protect against ADR cytotoxicity, and produce synergistic cell killing with 4-OOH-CP did not in fact cause significant cytotoxicity. It appears therefore that NOVO is comparatively nontoxic for mammalian cells. It should be noted that at extremely high concentrations (>500 μM) NOVO has been reported to be toxic to mammalian cells (5, 22) and to cause the accumulation of cells in G2M phase of the cell cycle (22, 29). However, as shown in this study, NOVO was effective in protecting against ADR cytotoxicity at the concentration of 50 μM or less (Fig. 5B); additionally, Utsumi et al. (21) found that 100 μM NOVO can abrogate the cytotoxicity of the DNA intercalator 4′-(9-accidinylamino)methanesulfon-m-anisidide. Thus, assuming that the above protective effects of NOVO were the result of its inhibition of Topo II, it is likely that the toxic action of NOVO at high concentrations is not a direct consequence of Topo II inhibition. The only toxicity that we observed with low NOVO exposure (0.3 mM for 4 h), as revealed in the cell fractionation experiments, was uniquely cell cycle phase specific and therefore would not otherwise be detected in an unfractionated cell population. Only cells in the G1-S boundary (which usually constitute less than 5% of the whole population of cells) appeared to be killed by NOVO (Fig. 1). These results suggest that the NOVO treatment may be deleterious to survival only for cells the DNA of which was in the process of being unwound in preparation for DNA synthesis. In addition, NOVO prevented the cell cycle transition from G1 to S phase (Fig. 2). Presumably, cells were blocked in the G1-S boundary at a point immediately before the unwinding of the supercoiled DNA. Additional studies concerning the exact position in the G1-S boundary at which cells were blocked, relative to those produced by hydroxyurea and nutrient deprivation, are in progress in this laboratory.

In conclusion, we have demonstrated that NOVO can abolish the cell cycle-dependent cytotoxicity of ADR and enhance the toxicity of 4-OOH-CP in a variety of tumor cell lines. Preliminary evidence suggests that these two effects of NOVO may be mediated by different mechanisms. Further investigations into the effects of NOVO on glutathione metabolism, drug uptake and accumulation, and DNA repair may provide important information into the mode of action of this clinically safe agent. This may ultimately aid in the design of combined regimens of NOVO and cytotoxic alkylating agents for the treatment of cancer.

ACKNOWLEDGMENTS

The authors wish to thank the Cell Separation Facility of the University of Rochester Cancer Center for technical support; Dr. R. Borch and Dr. P. Hilgard for the supply of 4-hydroperoxycyclophosphamide; and B. King for excellent technical assistance.
REFERENCES


Modulation of the Cell Cycle-dependent Cytotoxicity of Adriamycin and 4-Hydroperoxycyclophosphamide by Novobiocin, an Inhibitor of Mammalian Topoisomerase II

Francis Y. F. Lee, Deborah J. Flannery and Dietmar W. Siemann