# Novobiocin Enhances Alkylating Agent Cytotoxicity and DNA Interstrand Crosslinks in a Murine Model

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## Abstract

**DNA–DNA crosslinks are the lethal cellular mechanism of bifunctional alkylating agent cytotoxicity. Novobiocin, an inhibitor of DNA topoisomerase II, impairs eukaryotic DNA repair of alkylating agent adducts and may increase the number of adducts and their resultant cytotoxicity in malignant cells. The effect of novobiocin on clonogenic survival and DNA crosslinking due to cisplatin (*c*DDP) and carmustine (BCNU) was studied. Novobiocin caused synergistic cytotoxicity in Chinese hamster ovary cells exposed to *c*DDP or BCNU. Novobiocin and *c*DDP increased the formation of DNA–DNA interstrand crosslinks sixfold greater than *c*DDP alone. The effect was schedule dependent. Novobiocin and *c*DDP or BCNU markedly reduced in vivo growth of a murine fibrosarcoma without increased host toxicity. As a modulating agent of cytotoxicity due to DNA–DNA crosslinking, novobiocin may enhance the clinical effectiveness of the alkylating agents in human cancer and offer insight into new therapeutic strategies.**

## Introduction

The alkylating agents (AA)[1] are an important class of anti-tumor agents (1) and play a major role in the successful treatment of selected human cancers (2). The introduction of *cis*-diamminedichloroplatinum(II) (*c*DDP) a nonclassical alkylating agent has further extended the efficacy of AA in the clinic (3).

The clinically effective AA are bifunctional; that is, they have the capacity to form two covalent linkages. Although they may combine with many nucleophilic sites within the cell, there is compelling evidence that their cytotoxicity is the result of DNA monoadduct formation followed by inter- or intrastrand crosslinks in DNA (4–7). The kinetics of monoadduct and crosslink formation, the nature and sites of binding to nucleic acid bases, and the efficacy and kinetics of DNA repair vary among the AA (4–7). The net quantity of crosslinks formed is a function of the extent of initial monoadduct formation and their subsequent progression to DNA–DNA crosslinks. An $^{6}$O-alkylguanine-DNA alkyl transferase has been demonstrated to remove nitrosourea-induced monoadducts and thereby impede crosslink formation (8). DNA repair mechanisms are believed to be responsible for DNA–DNA crosslink removal (9). After damage by alkylating agents, enhanced sensitivity of cells from individuals with selective deficiencies of DNA repair activity is observed (10). Furthermore, the disappearance kinetics of DNA–DNA crosslinks after exposure of neoplastic cells to bifunctional alkylating agents are consistent with their removal by a repair process (9). The relationship of the crosslink index to cytotoxicity raises the possibility that the effectiveness of alkylating agents may be enhanced by either increasing the number of alkylation adducts in cellular DNA or decreasing their repair (11).

To investigate this question, we have studied the effects of novobiocin (N), an inhibitor of eukaryotic DNA replication and repair (12), on the formation of DNA–DNA crosslinks and cytotoxicity of the bifunctional AA *c*DDP and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). N inhibits DNA repair synthesis after damage by ultraviolet light irradiation or *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) (12). The DNA repair sensitive target of N is not a DNA polymerase but DNA topoisomerase II (Top II), an enzyme important in DNA supercoiling and catenation/decatenation, which has an essential role in eukaryotic DNA replication, RNA transcription, and mitosis (13–15).

In the present study, the addition of N to either *c*DDP or BCNU produced synergistic cytotoxicity in cultured Chinese hamster ovary (CHO) cells with a marked increase in DNA–DNA crosslinks. Similar cytotoxicity was observed in two independent in vivo animal tumor model systems, the FSaII murine fibrosarcoma and the murine L1210 leukemia.

## Methods

*Clonogenic survival studies.* CHO cells were grown in Ham's F12 media with 10% fetal bovine serum (FBS), penicillin (250 U/ml), and streptomycin (250 μg/ml) at 37°C and 5% $CO_2$. $10^2$ or $10^3$ cells from subconfluent cultures were plated on 60-mm tissue culture dishes (Falcon Labware, Oxnard, CA) and exposed to the appropriate drugs 12–18 h after plating (cell cycle time 11–14 h). N (Sigma Chemical Co., St. Louis, MO) was prepared as an aqueous stock solution of 10 mg/ml ($1.5 \times 10^{-2}$ M), *c*DDP (Sigma Chemical Co.) was prepared as a solution of $10^{-3}$ M in saline, BCNU (Bristol Pharmaceuticals, Syracuse, NY) was prepared as a $1.5 \times 10^{-2}$ M stock solution in 0.009% alcohol as a stabilizer. Stock solutions were stored at −70°C. After drug exposure the cells were washed three times with phosphate-buffered saline (PBS, 0.015 M NaCl, 0.7 M $KH_2PO_4$, 4.3 mM $K_2PO_4$) and incubated in standard culture media for 10–14 d. At this time the colonies were fixed with methanol and formalin,

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1. *Abbreviations used in this paper:* AA, alkylating agents; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CHO, Chinese hamster ovary; CLF, crosslinking factor; *c*DDP, *cis*-diamminedichloroplatinum(II); $IC_{50}$, inhibitory concentration 0.50; $IC_{90}$, inhibitory concentration 0.90; mAMSA, (4′-[9-acridinyl aminomethanesulfon-m-anisidide); MNNG, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine; Top II, DNA topoisomerase II.

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stained with 2% methylene blue, and counted. The data reported represent the results of experiments performed in triplicate, at a minimum.

*Data analysis.* Using the method of Deen and Williams and Dewey et al. (16, 17), isobolograms were generated for the special case in which the dose of one agent is held constant. This method produces envelopes of additive effect for different levels of the variable agent. This is conceptually identical to generating a series of isobolograms and replotting the results at a constant dose of one agent on a log effect by dose of the second agent coordinate system to give a complete dose-response curve. The envelopes of additivity shown in the figures were generated from a series of iso-effect curves derived from the complete dose-response curves for each agent alone. Overall, combinations that produce an effect that is within the boundaries of the shaded envelope are considered additive. Those displaced to the left are supra-additive while those displaced to the right are sub-additive (18, 19).

To facilitate these analyses a flexible interactive computer program in BASIC was written for the Apple II+ microcomputer (Cupertino, CA). The program first derives the best fitting dose-response curves using dose or log dose, and effect, log effect, probit percent effect, of logit percent effect relations. For cell survival dose-response curve correlations of 0.96 or greater have been obtained. The program then calculates isobologram at a constant level of the selected agent, and plots the data. The figures show log survival versus dose on a linear scale.

*Alkaline elution assays.* The alkaline elution assay was performed in triplicate after the method of Kohn et al. (20). Approximately $5 \times 10^5$ to $1 \times 10^6$ [$^{14}$C]thymidine-labeled CHO cells plus a similar number of $^3$H-labeled reference cells were used. After the appropriate incubation period (1, 12, or 24 h), the cells were scraped from plates in unlabeled media, placed on ice, and diluted in 20 ml of cold PBS and collected on a polyvinyl chloride filter (pore size 2 μm, diam 24 mm) (Millipore Corp., Bedford, MA). The cells were washed with cold PBS and lysed on the filter with 6 ml of 0.2% Sarkosyl (Ciba Geigy Co., Summit, NJ), 2 M sodium chloride, and 0.04 M EDTA (pH 10) at room temperature (22–24°C). The lysis solution was allowed to flow through by gravity and the filter was washed with 3 M of 0.02 M EDTA (pH 10). Elution was carried out in the dark at a flow rate of 0.035–0.045 ml/min. The elution solution consists of 0.02 M EDTA (acid form) and 10% tetrapropyl ammonium hydroxide (Eastman Kodak Co., Rochester, NY) 10% solution in water, pH 12.1 to 12.2. Diluted fractions were collected at 90-min intervals mixed with 3.3 vol of Aquasol (New England Nuclear, Boston, MA) containing 0.075% acetic acid for scintillation counting. Radioactivity remaining unfiltered was determined by treating filters with 0.4 ml of 1 N HCl at 65°C for 1 h followed by 2.5 ml of 0.4 N NaOH at room temperature for 1 h, and then adding 12 ml of Aquasol.

As an internal standard, $^3$H-labeled CHO cells irradiated with 150 rad at 0°C were used. To assay for either DNA strand breaks or crosslinks, the cells were eluted both directly and after exposure to 600 rad to 0°C. After x-ray exposure the cells were kept at 0°C to avoid repair of DNA breaks. X-irradiation introduces a controlled number of strand breaks in the DNA, and crosslinking is manifested by an inhibition of the effect of the x-ray dose on the elution of kinetics. The experiments distinguished DNA protein links from DNA interstrand crosslinks by using proteinase K (0.5 mg/ml) in the lysis solution. The crosslinking factor (CLF) was calculated as CLF = [log (irradiated control/control)]/[log (irradiated drug/control)].

*Antitumor activity in vivo.* Antitumor activity of N in combination with *c*DDP or BCNU was assessed in two transplantable mouse tumors: (*a*) L1210 leukemia growing in DBA mice and (*b*) FSaII fibrosarcoma growing in C3HFe/J mice. Tumor cells were obtained from stock tumors growing in DBA or C3HFe/J hosts, respectively (all animals were purchased from Jackson Laboratories, Bar Harbor, ME). For intraperitoneal tumors, $10^6$ L1210 cells were implanted on day 0. Treatment with N (100 μg/kg, i.p.) was begun late on day 0 and continued daily for 8 d. Treatment with *c*DDP (4.5 mg/kg, i.p.) or BCNU (8 mg/kg, i.p.) was begun on day 1 and continued daily for 5 d. For subcutaneous tumors, $2 \times 10^6$ FSaII cells were implanted on day 0. Treatment with N (100 μg/kg, i.p.) was begun on day 6 and continued daily for 8 d. Treatment with *c*DDP (4.5 mg/kg, i.p.) or BCNU (8 mg/kg, i.p.) was begun on day 7 and continued for 5 d. These treatments did not produce weight loss in the animals over the treatment period. For the L1210 experiments, outcome was assessed as increase in life span of treated compared to untreated control animals (T/C). For the FSaII experiments, outcome was assessed by comparison of the number of days until treated tumors reached 500 mm$^3$ in volume compared to the number of days for untreated control tumors to reach the same volume (tumor growth delay). The size of each tumor was measured thrice weekly. Untreated FSaII tumors reach 500 mm$^3$ in ~ 12 d. Each experiment was repeated three times with seven animals per group ($n$ = 21).

## Results

*Clonogenic cell survival studies.* Dose-response curves for BCNU, *c*DDP, and N were obtained in CHO cells following a 1-h exposure in the case of the AA, and up to 24 h for N. The inhibitory concentration $(IC)_{50}$ and the $IC_{90}$ for BCNU was 50 μM and 90 μM, respectively, and for *c*DDP, 60 and 90 μM. N was nontoxic up to concentrations of 150 μM for a 24 h exposure. Above this level reduced clonogenic survival was observed with N. To evaluate the effects of N plus AA exposure, a variety of schedules were evaluated empirically, as follows. Concurrent N and AA (*c*DDP or BCNU at varying concentrations) exposure for 1 h, pretreatment for 1 h with N followed by concurrent exposure to AA, N for 1 h prior to AA exposure, and then for 22 h after AA removal, N exposure at 2, 4, or 8 h for 24 h after AA treatment.

Subinhibitory concentrations of N (150 μM) and increasing doses of either BCNU or *c*DDP resulted in a marked reduction in clonogenic survival of CHO cells when compared to each AA alone (Figs. 1, 2). The degree of synergy increased as the concentration of BCNU or *c*DDP was increased and exceeded the expected envelope of additivity as determined by construction of an isobologram. This marked synergistic effect was schedule

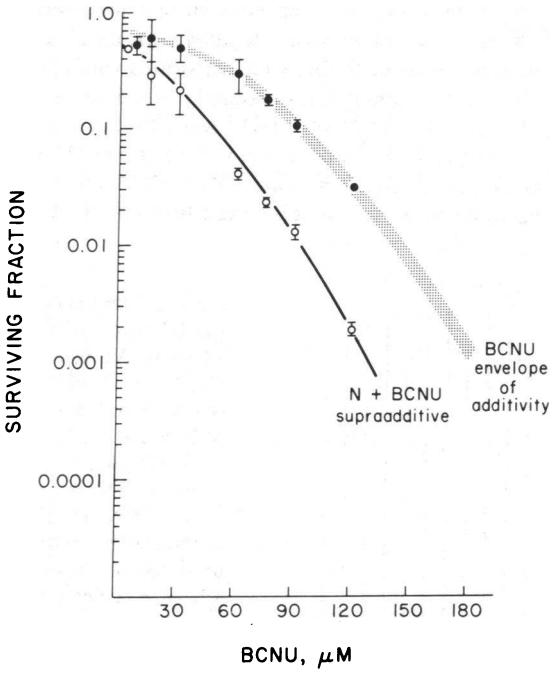


*Figure 1.* Isobologram of the synergy produced by N at 150 μM with increasing doses of BCNU. Leftward displacement from envelope of additivity denotes synergy. Schedule of N and BCNU was pretreatment, concurrent, and 24 h posttreatment with N.

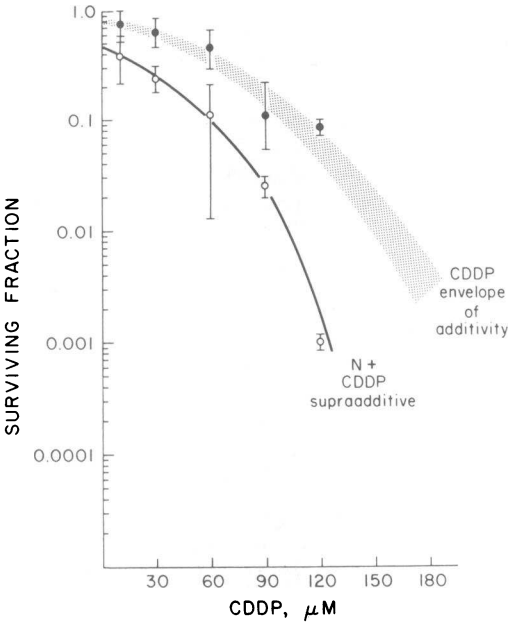


*Figure 2.* Isobologram of the synergy produced by N at 150 μM with increasing doses of *c*DDP. Leftward displacement from envelope of additivity denotes synergy. Schedule of N and *c*DDP was pretreatment, concurrent, and 24 h posttreatment with N.

dependent. Maximal cytotoxicity was observed when cells were treated with N from 1 h prior to the addition of either alkylating agent through 24 h following exposure (Fig. 3). If N was removed prior to the addition of the alkylating agent, the reduction in clonogenic survival was diminished and synergistic cytotoxicity was not observed if the cells were exposed to N after treatment with either of the alkylating agents.

*Alkaline elution studies.* Since DNA crosslinks correlate with cytotoxicity of BCNU and *c*DDP, the biochemical consequences of the interaction of N and AA was investigated using the alkaline elution technique. Detailed studies were performed with *c*DDP because of the ease with which it is measured in alkaline elution studies. At 1 h the CLF for N and *c*DDP was 30% higher than for *c*DDP alone (Fig. 4). N did not result in formation of DNA–DNA crosslinks alone. At 12 h, when DNA–DNA crosslinks are expected to be maximal, the difference between N treated

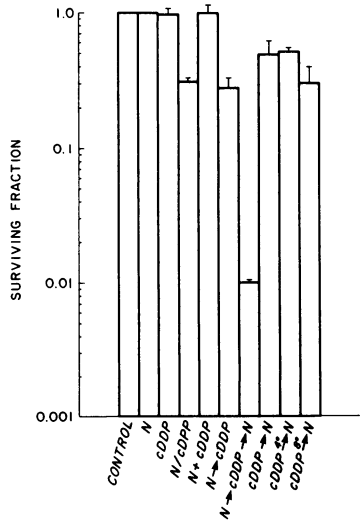


*Figure 3.* Effect of N scheduling on *c*DDP cytotoxicity. An identical effect was seen with BCNU. N = N alone for 24 h. *c*DDP = 1 h *c*DDP (25 μM) only. N/*c*DDP = N for 1 h, removed before *c*DDP. N and *c*DDP = both drugs concurrently for 1 h. N → *c*DDP = N preceding and concurrent with *c*DDP. N → *c*DDP → N = pre-, concurrent, and posttreatment with N. *c*DDP → N = N added 0, 2, 4, or 8 h after *c*DDP for 24 h.

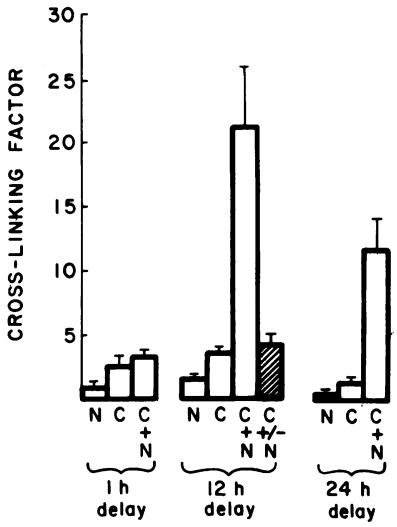


*Figure 4.* The effect of novobiocin (N) treatment on DNA–DNA interstrand crosslinks produced by cisplatin (*c*DDP) is illustrated. N = 150 μM N until cell harvest. C = 100 μM *c*DDP for 1 h. C + N = 150 μM N for 1 h preceding, during *c*DDP and N in postexposure media until cell harvest. C+/−N = 150 μM N 1 h before and with 100 μM *c*DDP but no N in postexposure media. The CLF measures the inhibition of DNA elution due to interstrand crosslinks relative to the untreated control. CLF = [log (irradiated control/control)]/[log (irradiated drug/control)].

and untreated cells was sixfold (21.2 vs. 3.6 for N/*c*DDP vs. *c*DDP alone). The CLF for *c*DDP increased 35% over 12 h, whereas the combination was associated with a rise of 533% during the same time. At 24 h the CLF was 37% of maximal for *c*DDP alone, and 56% for N/*c*DDP, indicating that the most striking differences were observed during the period of crosslink formation, rather than their disappearance. Studies that employed N before and during *c*DDP exposure, but in which N was removed during the postincubation period failed to show an increase in DNA crosslink formation beyond that which was observed at 1 h.

*In vivo studies.* The effect of the addition of N to treatment with *c*DDP or BCNU was assessed in two murine tumor systems. The L1210 leukemia is a rapidly growing ascites tumor. N increased the life span of mice bearing the L1210 leukemia when added to treatment with either *c*DDP or BCNU (Table I). There was an additional 1.3-fold increase in life span in mice treated with N and *c*DDP and additional 1.2-fold increase in life span in animals treated with N and BCNU ($P$ value not significant). N alone had a small effect on this tumor; therefore, it is possible that the increases in life span seen with the drug combinations may represent an additive response to each agent. The FSaII fibrosarcoma is a solid tumor growing subcutaneously in the flank. N at the same total dose used in the leukemia experiment had very little effect on the growth of this tumor; however, the combination of N with *c*DDP produced a 2.8-fold increase in tumor growth delay compared to *c*DDP alone ($P < 0.0005$) and the combination of N with BCNU produced a 9.0-fold increase in tumor growth delay compared with BCNU alone ($P < 0.001$).

## Discussion

The biochemical basis for N synergistic effects is not understood. The compound is known to inhibit bacterial DNA gyrase, a Top II that relaxes and supercoils double-stranded DNA (21). N has been shown to inhibit eukaryotic Top II in vitro (22), and its effects are ATP dependent (21). An unambiguous interpretation of N effects is confounded by the compound's other known actions. It will bind histones at pharmacologic concentrations and may change chromosomal conformation independent of Top II

*Table I. Effect of N on the Outcome of Treatment with cDDP or BCNU of Animals Bearing L1210 Leukemia or FSaII Fibrosarcoma*

| | Tumor | | | |
|---|---|---|---|---|
| | L1210 | | FSaII | |
| Treatment group* | T/C‡ | D + N/D§ | TGD‖ | D+N/D¶ |
| N | 1.22±0.05 | | 1.00±0.48 | |
| *c*DDP | 2.48±0.20 | | 10.63±1.38 | |
| N + *c*DDP | 3.41±0.35 | 1.3 (NS)* | 29.60±3.19 | 2.8 ($P < 0.0005$) |
| BCNU | 3.09±0.25 | | 2.92±0.37 | |
| N + BCNU | 3.78±0.45 | 1.2 (NS) | 26.24±2.83 | 9.0 ($P < 0.0001$) |

* For the L1210 experiments treatment with N (100 mg/kg) was begun late on day 0, the day of tumor implantation and continued daily until day 8. Treatment with N was begun on day 6 in mice bearing the FSaII fibrosarcoma and continued for 8 d. *c*DDP (4.5 mg/kg) or BCNU (8 mg/kg) was administered on days 1–5 of the N treatment. All drugs were injected intraperitoneally.

‡ T/C is the ratio of survival for treated versus control animals. The control animals survived 8.48±0.22 d ($n$ = 14).

§ D + N/D is the ratio of survival for animals treated with N in addition to *c*DDP or BCNU versus those treated with *c*DDP or BCNU alone ($n$ = 14 for each group).

‖ TGD is the tumor growth delay in days produced by each treatment compared with untreated control tumors. The point of observation is the number of days required for tumors to reach 500 $mm^3$ in volume ($n$ = 14 for each group).

¶ D + N/D is the ratio of the tumor growth delay produced in animals treated with novobiocin in addition to *c*DDP or BCNU compared to drug treatment alone. Statistical comparisons were carried out with the Dunn multiple comparisons test.

inhibition (23). The latter effect is associated with inhibition of transcription in *Xenopus* (24). N will inhibit the eukaryotic DNA polymerase alpha (25), bacterial DNA polymerase and DNA ligase (26). In addition to interfering with semiconservative replication, N has been demonstrated to inhibit DNA repair synthesis following damage with ultraviolet light or MNNG (12).

By reversible breakage and union of two DNA strands and the passage of double-stranded DNA through the enzyme bridged gap, Top II can alter the conformation of constrained nuclear DNA (27). As such, it is important in a number of essential cellular functions such as mitosis, DNA repair, DNA replication, and possibly transcription. Furthermore, Top II has been identified as the target of several antineoplastic agents (28–30). These agents, such as adriamycin, the epipodophyllotoxins and m-AMSA (4′-[9-acridinyl amino]methanesulfon-m-anisidide), stabilize the Top II-DNA cleaved strand complex that results in DNA strand scission (28–30).

N exerts a far more potent enhancement effect on alkylating agents than etoposide (Teicher, B. A., unpublished data) and is minimally toxic by comparison. The mechanism underlying this effect may be multifactorial, involving Top II inhibition and or other enzyme systems. Considering the magnitude of the N associated enhancement observed in vitro and in vivo, a complete understanding of this agent's effects may lead to the advent of novel treatment strategies to be evaluated in the clinic.

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