Effect of Novobiocin on the Antitumor Activity and Tumor Cell and Bone Marrow Survivals of Three Alkylating Agents

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ABSTRACT

Our previous in vitro studies demonstrated marked synergy with alkylating agents when novobiocin was present during and after alkylating agent exposure. To determine whether this effect is observed in vivo, novobiocin was administered daily for 3 days prior to alkylating agent treatment, during alkylating agent treatment, and for 2 days after completion of alkylating agent treatment. When combined with cis-diaminedichloroplatinum(II), 1,3-bis(2-chloroethyl)-1-nitrosourea, or cyclophosphamide, there was significant enhancement of the growth delay of the FSaII fibrosarcoma implanted s.c. in C3H mice when compared with alkylating agents alone. In a second assay using ex vivo studies of tumor cells exposed in vivo, single doses of 100 mg/kg of novobiocin followed by cis-diaminedichloroplatinum(II) resulted in a 3- to 4-fold increase in tumor cell killing by cis-diaminedichloroplatinum(II). At a dose of 100 mg/kg of 1,3-bis(2-chloroethyl)-1-nitrosourea there was about a 7-fold increase in tumor cell kill upon addition of novobiocin. Cyclophosphamide showed a dose response effect with novobiocin, reaching 13-fold at a dose of 300 mg/kg of cyclophosphamide. In all cases bone marrow elements were affected less than were neoplastic cells, suggesting that the combination of novobiocin and alkylating agents may be a clinically useful strategy.

INTRODUCTION

The emergence of drug-resistant tumor clones in clinical cancer therapy is a major cause of treatment failure. Strategies to overcome this problem include the use of drug combinations, escalation to maximally tolerated drug doses, and short treatment intervals (1). Despite these strategies, most tumors ultimately exhibit clinical drug resistance which results in progressive disease and death from cancer.

The alkylating agents are among the most useful clinical antineoplastic agents, with cytotoxic activity in many human tumors. These compounds differ in their mechanisms of uptake, intracellular drug binding and metabolism, and site and timing of DNA adduct formation (2). Furthermore, the alkylating agents demonstrate a lack of cross-resistance (3, 4). Novobiocin, a coumermycin antibiotic which inhibits the DNA gyrase (topoisomerase II) of eubacteria (5), potentiated the cytotoxicity of a number of alkylating agents in vitro (6, 7) and was synergistically cytotoxic in combination with CDDP3 and BCNU in Chinese hamster ovary cells. This increased cytotoxicity accrued in association with increased number of DNA interstrand cross-links being formed from monoadducts. Continuous novobiocin exposure was necessary to maximize the cross-link formation. Novobiocin has also been shown to increase the number of DNA interstrand cross-links formed in a nitrogen mustard-resistant human Raji lymphoblastoid cell line which has elevated topoisomerase II activity (7).

Novobiocin has myriad effects which occur at pharmacologically relevant concentrations and likely involve multiple mechanisms. Novobiocin inhibits the bacterial topoisomerase II by interfering with its ATPase and energy transduction processes (5). Novobiocin also inhibits replicative and repair DNA synthesis, but perhaps by different mechanisms (8, 9). The DNA repair target of novobiocin is not the α-polymerase and may be topoisomerase (9). Novobiocin also affects mitochondrial structure and ATP metabolism (10), directly precipitates chromosomes, and impairs RNA polymerase (11, 12). Thus, an unambiguous explanation of novobiocin activity is difficult.

In this study, we have attempted to extend these promising in vitro observations to an in vivo setting. The effect of novobiocin in combination with any of three bifunctional alkylating agents, BCNU, CDDP, and cyclophosphamide, on the TGD and ex vivo tumor cell survival of the FSaII fibrosarcoma and on the toxicity of the drugs to the bone marrow has been investigated. The TGD assay was performed at optimal schedule with novobiocin administered before, during, and for several days after chemotherapy administration. The doses of novobiocin were tolerable to the animals. The tumor excision assay was performed after only a single dose of novobiocin and chemotherapeutic drug, but allowed chemotherapy dose escalations to 3- to 5-fold and concomitant measurement of vital organ function-committed bone marrow progenitors.

MATERIALS AND METHODS

Drugs. Novobiocin was purchased from Sigma Chemical Co. (St. Louis, MO). BCNU (Carmustine) and cyclophosphamide (Cytoxan) were obtained from the Dana-Farber Cancer Institute pharmacy. CDDP was obtained as a gift from Dr. Donald Picker and Dr. Michael Abrams, Johnson Matthey, Inc. (West Chester, PA).

Tumor. The FSaII fibrosarcoma (13) adapted for growth in culture (FSaII(C)) (14) was carried in male C3H/HeJ mice (Jackson Laboratory, Bar Harbor, ME). For the experiments, 2 x 106 tumor cells prepared from a brei of several stock tumors were implanted i.m. into the legs of male C3H/HeJ mice 8 to 10 wk of age.

Tumor Growth Delay Experiments. Treatment was initiated when the tumors were approximately 50 mm3 in volume. Novobiocin (50 mg/kg or 100 mg/kg) was administered i.p. once daily on Days 6 through 15 post tumor implantation. CDDP (4.5 mg/kg) was administered i.p. once daily on Days 9 through 13. BCNU (8 mg/kg) was also administered i.p. once daily on Days 9 through 13. Cyclophosphamide (100 mg/kg) was administered i.p. once daily on Days 9, 11, and 13. The progress of each tumor was measured with calipers 3 times weekly until it reached a volume of 500 mm3. Tumor growth delay was calculated as the days taken by each individual tumor to reach 500 mm3 compared to the untreated controls. Each treatment group had 7 animals, and the experiment was repeated 3 times. Days of tumor growth delay were the mean ± SE for the treatment group compared to the control.

Tumor Excision Assay. When the tumors were approximately 50 mm3 in volume (about 1 wk after tumor cell implantation), single doses of novobiocin (100, 200, or 300 mg/kg), CDDP (15, 20, or 25 mg/kg), BCNU (50, 100, or 200 mg/kg), or cyclophosphamide (100, 200, or 300 mg/kg) were administered i.p., or novobiocin (100 mg/kg) was given 1 h prior to each of the alkylating agents to ensure the presence of novobiocin in the circulation at the time of alkylating agent treatment. Mice were sacrificed and soaked briefly in 95% ethanol 24 h...
after treatment to allow for full expression of drug cytotoxicity and repair of potentially lethal damage. The tumors were excised under sterile conditions in a laminar flow hood and minced to a fine brei with 2 scalpels. Four tumors were pooled to make each treatment group. Approximately 1 g of tumor brei was used to make each single-cell suspension. All reagents were sterilized with 0.22-μm Millipore membranes and were added aseptically to the tumor cells. Each sample was washed in 20 ml of α-MEM (Grand Island Biological Co., Grand Island, NY) in a 50-ml centrifuge tube, after which the liquid was gently decanted and discarded. The samples were resuspended in 450 units/ml of collagenase (Sigma) and 0.1 mg/ml of DNase (Sigma) and incubated for 10 min at 37°C in a shaking water bath. The samples were centrifuged at 200 x g, and the supernatant was discarded. The samples were resuspended as above and incubated for another 15 min at 37°C. One ml of 1 mg/ml of DNase was added, and incubation was continued for 5 min at 37°C. The samples were then filtered through 2 layers of sterile gauze. The samples were washed twice and then resuspended in α-MEM supplemented with 10% FBS (Sterile Systems, Logan, UT). These single-cell suspensions were counted and plated at three different cell concentrations in duplicate for the colony-forming assay. One wk later the plates were stained with crystal violet, and colonies of more than 50 cells were counted. The untreated tumor cell suspensions had a plating efficiency of 10 to 16%. The results are expressed as the surviving fraction ± SE of cells from treated groups compared to untreated controls.

Bone Marrow Toxicity. Bone marrow was taken from the same animals used for the tumor excision assay. A pool of marrow from the femurs of 2 animals was obtained by gently flushing the marrow through a 23-gauge needle using ice-cold McCoy's Medium 5A (Grand Island Biological Co.), supplemented with 2% FBS and nonessential amino acids (100x; 2 ml/liter) (Grand Island Biological Co.), sodium pyruvate (50 μg/ml), L-glutamine (146 μg/ml), L-asparagine (8 μg/ml), L-serine (4.2 μg/ml), vitamins (100x; 2 ml/liter), penicillin (50 units/ml), and streptomycin (50 μg/ml) (Grand Island Biological Co.) (15). The cells were washed and resuspended in supplemented medium. Granulocyte-macrophage colony-forming units were measured as follows. Bone marrow cells were suspended in supplemented McCoy's Medium 5A containing 15% FBS, 0.3% agar (Difco, Detroit, MI), and 10% L-cell-conditioned medium as a source of colony-stimulating activity. The colony-stimulating activity supplement was prepared by incubating L-929 mouse fibroblasts (2500 cells/ml; Microbiological Associates, Bethesda, MD) with 30% FBS in McCoy's Medium 5A for 7 days in a humidified 5% CO₂ atmosphere at 37°C. The colony-stimulating activity-containing supernatant was obtained by centrifugation of the medium at 10,000 x g for 10 min at 4°C and then filtration under sterile conditions (16). The bone marrow cell cultures were incubated for 7 days in a humidified 5% CO₂ atmosphere at 37°C and then fixed with 10% glutaraldehyde. Colonies of at least 50 cells were scored on an Acculile Colony counter (Fisher, Springfield, NJ). The results from 3 experiments, in which each group was measured in triplicate, were averaged. The results are expressed as the surviving fraction of treated groups compared to untreated controls.

RESULTS

Our previous studies with Chinese hamster ovary cells in tissue culture indicated that the presence of novobiocin was required during and after alkylating agent exposure to obtain enhancement in the cell killing by the alkylating agent (6). To mimic this effect in vivo, novobiocin was administered daily for 3 days prior to alkylating agent treatment, during alkylating agent treatment, and for 2 days after completion of alkylating agent treatment. In humans, steady-state levels are achieved in 48 h with twice daily p.o. preparations (17). Novobiocin at doses of 50 mg/kg or 100 mg/kg had very little effect on tumor growth (Table 1). The dose of 100 mg/kg of novobiocin daily for Days 6 to 15 was the maximum tolerated dosage of this drug. However, when combined with CDDP, BCNU, or cyclophosphamide at optimal dosage and schedules for these drugs in the FSAIC fibrosarcoma, there was significant enhancement of the tumor growth delay produced by the alkylating agent. With each of the alkylating drugs, it appeared that the higher dose of novobiocin was much more effective in increasing the growth delay of the tumor. There was about a 3-fold increase in the tumor growth delay produced by the alkylating agent. Table 1 shows the results from experiments, in which each group was treated with various doses of novobiocin. Points, mean of three independent determinations; bar, SE.

Table 1. Tumor growth delay of the FSAIC fibrosarcoma treated with novobiocin alone or in combination with CDDP, BCNU, or cyclophosphamide

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Tumor growth delay (days)</th>
</tr>
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<tbody>
<tr>
<td>Novobiocin (50 mg/kg)</td>
<td>1.0 ± 0.5*</td>
</tr>
<tr>
<td>Novobiocin (100 mg/kg)</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>CDDP*</td>
<td>10.6 ± 0.7</td>
</tr>
<tr>
<td>Novobiocin (50 mg/kg) + CDDP</td>
<td>14.0 ± 1.2</td>
</tr>
<tr>
<td>Novobiocin (100 mg/kg) + CDDP</td>
<td>29.6 ± 2.5</td>
</tr>
<tr>
<td>BCNU*</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>Novobiocin (50 mg/kg) + BCNU</td>
<td>4.1 ± 0.6</td>
</tr>
<tr>
<td>Novobiocin (100 mg/kg) + BCNU</td>
<td>26.2 ± 2.0</td>
</tr>
<tr>
<td>Cyclophosphamide*</td>
<td>6.8 ± 1.0</td>
</tr>
<tr>
<td>Novobiocin (50 mg/kg) + cyclophosphamide</td>
<td>9.0 ± 1.5</td>
</tr>
<tr>
<td>Novobiocin (100 mg/kg) + cyclophosphamide</td>
<td>14.4 ± 1.7</td>
</tr>
</tbody>
</table>

* Novobiocin was given once daily on Days 6 to 15 post tumor cell implantation.
* Mean ± SE of the days required for treated tumor to reach 500 mm² compared to untreated controls. Controls reached 500 mm² in 15.0 ± 0.2 days.
* CDDP (4.5 mg/kg) was given once daily on Days 9 to 13 post tumor cell implantation.
* BCNU (8 mg/kg) was given once daily on Days 9 to 13 post tumor cell implantation.
* Cyclophosphamide (100 mg/kg) was given once daily on Days 9, 11, and 13 post tumor cell implantation.

When single doses of novobiocin were administered to tumor-bearing animals and tumor cell survival was measured in vitro, some toxicity of novobiocin toward tumor cells was seen (Fig. 1). There was only a slight level of toxicity of this drug seen from animals treated with various doses of novobiocin. Points, mean of three independent determinations; bar, SE.

in the FSAIC fibrosarcoma, there was significant enhancement of the tumor growth delay produced by the alkylating agent. With each of the alkylating drugs, it appeared that the higher dose of novobiocin was much more effective in increasing the growth delay of the tumor. There was about a 3-fold increase in the tumor growth delay produced by CDDP, about a 9-fold increase in tumor growth delay produced by BCNU, and about a 2-fold increase in the tumor growth delay produced by cyclophosphamide with the addition of novobiocin (100 mg/kg) to the drug treatment.
NOVOBIOCIN AND ALKYLATED AGENTS

1.000
0.100
0.010
0.001
0.000
15 20 25 15 20 25
CDDP, mg/kg

Fig. 2. Survival of FSAIC cells from FSAIC tumors (left) and bone marrow (right) from animals treated with various doses of CDDP (•) or with various doses of CDDP preceded 1 h earlier by a single dose of novobiocin (100 mg/kg) (○). Points, mean of three independent determinations; bars, SE.

1.000
0.100
0.010
0.001
0.000
50 100 200 50 100 200
BCNU, mg/kg

Fig. 3. Survival of FSAIC cells from FSAIC tumors (left) and bone marrow (right) from animals treated with various doses of BCNU (•) or with various doses of BCNU preceded 1 h earlier by a single dose of novobiocin (100 mg/kg) (○). Points, mean of three independent determinations; bars, SE.

a 2-fold increase in cell killing with the addition of novobiocin to the drug treatment.

BCNU gave a more complex pattern (Fig. 3). At the lowest dose of BCNU, there was no significant effect of novobiocin. At a dose of 100 mg/kg of BCNU there was about a 7-fold increase in tumor cell kill upon addition of novobiocin to the drug treatment; however, at the highest dose of BCNU (200 mg/kg), this effect fell to about a 2-fold increase in tumor cell kill with the combination treatment. As with CDDP, there was overall less than a 2-fold increase in bone marrow killing with the combination of BCNU and novobiocin compared to BCNU alone.

Cyclophosphamide showed the increasing effect of novobiocin with the increasing dose of alkylating agent (Fig. 4). The presence of novobiocin increased the tumor cell kill of 100 mg/kg of cyclophosphamide by approximately 3-fold. However, at a dose of 300 mg/kg of cyclophosphamide there was a 13-fold increase in tumor cell kill when the alkylating agent was preceded by a dose of novobiocin. Novobiocin increased the toxicity of cyclophosphamide to the bone marrow by 4- to 5-fold over the dosage range of cyclophosphamide examined.

DISCUSSION

Chemotherapeutic drug resistance had emerged as a major unresolved problem in cancer chemotherapy. The determination of specific mechanisms of resistance in a specific tumor cell type or the ability to determine the presence of a specific mechanism of resistance for an individual tumor would allow for specific countermeasures to be taken to increase the therapeutic effectiveness of available cancer chemotherapeutic agents. Known mechanisms of resistance include elevated levels of pleiotropic resistance due to enhanced expression of the mdr gene, increased glutathione levels, amplification of target genes like dihydrofolate reductase, and enhanced DNA repair (18). Recent work indicates that elevated activity or altered enzyme properties of topoisomerase II may mediate some forms of drug resistance (7, 19).

While experimental protocols may produce a substantial amount of resistance by virtue of a single mechanism, other protocols produce multiple mechanisms of resistance. Where a high degree of resistance is due to intrinsic expression of a gene constitutively present in a cell type (such as mdr in the liver, kidney, or intestinal cells), a specific pharmacological modulation may have therapeutic benefit (20). In human cells made resistant to alkylating agents by gradual, incremental exposure, however, multiple mechanisms of resistance are observed and may limit the ability of any single intervention to overcome it (3, 21). Thus, multiple pharmacological interventions may be required.

These recent discoveries regarding the nature and mechanisms of drug resistance offer new treatment strategies directed at specific mechanisms. The use of calcium channel blockers to reverse the rapid efflux associated with the multiple drug resistance phenotype is already in clinical trials (22). Lipid-soluble analogues can overcome resistance due to decreased drug transport (23). Buthionine sulfoxime, which inhibits glutathione synthesis, can restore drug sensitivity in cell lines with enhanced glutathione levels as a mechanism of resistance (24).
Novobiocin inhibits eukaryotic topoisomerase II and specifically overcomes resistance in an alkylating agent-resistant human tumor cell line (7). We were able to demonstrate therapeutic synergism between novobiocin and the alkylating agents CDDP and BCNU (6). We then chose to see if any therapeutic benefit could be found in vivo in experimental tumors. The TGD and tumor excision assays were used so that studies could be performed in solid tumors. The TGD assay was done at therapeutically active doses which allow normal animal survival and allowed an optimal schedule of novobiocin. The doses of novobiocin used (50 to 100 mg/kg) can be safely given to humans. The therapeutic effect seen was significant and suggested a dose response effect for the alkylating agent. Moreover, since topoisomerase II is a proliferation-dependent enzyme which could be affected by an antiproliferative but not cytotoxic effect of the drug combination, the tumor excision assay directly measures cell kill.

By treating animals with novobiocin prior to, during, and after treatment with each of three alkylating agents, we observed significant increases in tumor growth delay. In a single dose protocol measuring tumor cell survival, again a significant effect of the addition of novobiocin was seen. Since there was, in general, less increase in the toxicity to the bone marrow than to the tumor, the addition of novobiocin to treatment with these alkylating drugs represents an increase in the therapeutic effect of the drugs if bone marrow is the dose-limiting tissue. It may be possible that extending the treatment period with novobiocin both prior to and after the alkylating agent administration would further increase the enhancement effect of novobiocin.

The mechanism underlying this effect may be multifactorial, involving topoisomerase II inhibition and/or other enzyme systems. Considering the magnitude of the novobiocin-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.

REFERENCES

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