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Ability of four potential topoisomerase II inhibitors to enhance the cytotoxicity of *cis*-diamminedichloroplatinum (II) in Chinese hamster ovary cells and in an epipodophyllotoxin-resistant subline*

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Summary. Four drugs known to interact with topoisomerase II were assessed for their ability to enhance the cytotoxicity of *cis*-diamminedichloroplatinum(II) (CDDP) in Chinese hamster ovary (CHO) cell lines sensitive and resistant to VM-26. The combination treatments were analyzed by isobologram methodology. On 24 h exposure, there was no significant difference in the cytotoxicity of novobiocin or ciprofloxacin toward either cell line. The resistant cells were approximately 9-fold more resistant to 4'-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) and approximately 170-fold more resistant to etoposide after a 24-h exposure. The combination of novobiocin and cisplatin produced greater than additive cell kill over the entire dose range of cisplatin tested in both cell lines. *m*-AMSA and CDDP produced cell kill that fell within the envelope of additivity. Etoposide and CDDP resulted in cytotoxicity that was slightly greater than additive at low CDDP concentrations and additive at the highest concentration of CDDP tested in the parental cell line and was slightly greater than additive in the resistant cell line. Ciprofloxacin and CDDP, like novobiocin, resulted in greater than additive cell kill in both cell lines. The enhancement of CDDP cytotoxicity by novobiocin that was seen in exponentially growing cells was lost in stationary-phase cultures. In these studies, novobiocin and, to a lesser degree, ciprofloxacin produced greater than additive cell kill in combination with CDDP in parental and epipodophyllotoxin-resistant CHO cells.

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Abbreviations: CDDP, *cis*-diamminedichloroplatinum(II); *m*-AMSA, 4'-(9-acridinylamino)methanesulfon-*m*-anisidide; IC_{90}, drug concentration require for the inhibition of colony formation by 90%

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Introduction

DNA topoisomerase type II enzymes are proteins found in both prokaryotic and eukaryotic cells that control and modify the topological states of DNA. By transiently breaking a pair of complementary DNA strands and passing another double-stranded segment, topoisomerase type II can catalyze many types of interconversions between DNA topological isomers [27]. DNA topoisomerases have been found to affect a number of vital biological functions, including the replication and repair of DNA [13, 17, 20, 27]. Although the bacterial type II DNA topoisomerase, termed DNA gyrase, and the eukaryotic topoisomerase II show a number of characteristic differences, areas of homology in amino acid sequences have been found in the bacterial and yeast enzymes [27]. Molecules from several families have been shown to interact with DNA topoisomerase II; these include intercalating agents such as Adriamycin and 4'-(9-acridinylamino) methane-sulfon-*m*-anisidide (*m*-AMSA), antitumor epipodophyllotoxins such as etoposide and teniposide, and bacterial DNA gyrase inhibitors such as novobiocin and ciprofloxacin. The actions of these drugs as well as others on mammalian cells may at least in part occur through this mechanism [9, 10, 15, 19].

Previous work from our laboratory has focused on the combination of novobiocin with CDDP because novobiocin can be given at relatively high doses with little toxicity [6, 7]. Both in vitro in Chinese hamster ovary (CHO) cells [6] and in vivo in the FSaIIC fibrosarcoma [7], the administration of novobiocin prior to, during, and for several hours after treatment with CDDP has resulted in greater than additive cell kill by the combination as determined by isobologram analysis. In vitro, this enhanced cytotoxicity correlated with an increased level of DNA-DNA cross-link formation by CDDP [6]. A similar effect was obtained by Tan et al. [25] with novobiocin and nitrogen mustard in a human Raji Burkitt's lymphoma cell line.

An epipodophyllotoxin-resistant subline of a CHO parental cell line was recently produced and characterized [11, 12]; this subline has an altered topoisomerase II [11].

The major change in the resistant line appears to be related to a decreased ability of topoisomerase II plus enzyme inhibitors to cleave DNA, resulting in the cells' being 10–40 times more resistant to the effects of these agents [11]. The altered enzyme found in these cells is more thermally labile and shows decreased sensitivity to epipodophyllotoxin-mediated DNA re-ligation as compared with the enzyme from the parental cell line [24].

In this study we compared the ability of the four potential topoisomerase II inhibitors novobiocin (an inhibitor of the DNA gyrase binding to subunit B), *m*-AMSA (a DNA intercalator), etoposide (an epipodophyllotoxin), and ciprofloxacin (an inhibitor of the DNA gyrase binding to subunit A) to potentiate the cytotoxicity of CDDP in CHO parental cells and in CHO epipodophyllotoxin-resistant cells, with a goal of determining whether any of these four agents would produce greater than additive cytotoxicity in combination with CDDP in both the parental and resistant cell lines and whether alteration in a specific binding site on topoisomerase II may be implicated.

Materials and methods

Drugs. Novobiocin (Sigma Chemical Co., St. Louis, Mo.) and ciprofloxacin (Miles Pharmaceuticals, Elkhart, Ind.) were prepared as stock solutions in sterile water. *m*-AMSA (obtained as a gift from DCT Program, National Cancer Institute, Bethesda, Md.) and etoposide (Bristol-Myers, Syracuse, N. Y.) were prepared as stock solutions in dimethylsulfoxide. CDDP was prepared in sterile water with *N*-methyl-D-glucamine (Aldrich Chemical Co., Milwaukee, Wis.). Solutions were kept at −100° C. Phosphate-buffered 0.9% saline (0.15 *M* NaCl, 0.7 MKH_2PO_4, 4.3 m*M* K_2HPO_4) was prepared as a stock solution.

Cell lines. CHO parental cells and the resistant subline were generous gifts from Dr. Warren Ross. The resistant subline was developed by the exposure of parental CHO cells to ethylmethane sulfonate followed by teniposide as a selection agent [12]. Both cell lines were grown at 37° C in an atmosphere comprising 95% air/5% CO_2 in alpha minimal essential medium (Grand Island Biologic, Grand Island, N. Y.) supplemented with 5% fetal bovine serum (Hyclone, Logan, Utah), penicillin (250 IU/ml), and streptomycin (250 μg/ml) (Sigma Chemical Co., St. Louis, Mo.).

Survival experiments. For studies in exponentially growing cultures, $10^3-5\times10^4$ cells were plated on 60-mm tissue-culture dishes (Falcon Labware, Oxnard, Calif.) 24 h before drug exposure. For studies in stationary-phase cultures, cells were grown to confluence and then maintained for 3 days prior to their use.

In all experiments, cells were exposed to CDDP for 1 h and to novobiocin, *m*-AMSA, etoposide, or ciprofloxacin for a total of 24 h. In drug-combination studies, cells were exposed to novobiocin, *m*-AMSA, etoposide, or ciprofloxacin for 1 h prior to and during CDDP treatment, then were maintained in serum containing media with novobiocin, *m*-AMSA, etoposide, or ciprofloxacin for an additional 22 h. (Stationary-phase cultures were maintained in depleted media with serum containing novobiocin, *m*-AMSA, etoposide, or ciprofloxacin during the 22-h period following CDDP treatment.) The cells were then washed twice with PBS and suspended by trypsinization, and known numbers of cells were plated for colony formation. Colonies grow to ≥50 cells in 7 days, at which time the cultures were fixed with acetic acid-methanol and stained with 2% methylene blue, and colonies were counted.

Data analysis. Isobologram methodology has previously been used by our laboratory [6, 26]. Briefly, using the method of Deen and Williams [3], 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; it is conceptually identical to the generation of a series of isobolograms and replotting of the results at a constant dose of one agent on a log effect by dose of the second-agent coordinate system. Dose-response curves for each agent alone were first generated. The envelopes of additivity were generated from a series of isoeffect curves derived from the complete dose-response curves for each agent alone. Overall, combinations producing the desired effect that are within the envelope boundaries of modes I and II are considered to be additive; those displaced to the left are supraadditive, whereas those displaced to the right are subadditive [1, 22]. This general approach can be extrapolated to the special case in which the level of an agent is held constant. Under these conditions, an isobologram can be derived that plots the expected effect (modes I and II) for any level of the variable agent plus the agent combinations [6, 26]. Experimentally, this approach is most simple and readily facilitates the determination of additive and nonadditive combinations.

To facilitate these analyses, a flexible, interactive computer program in BASIC was written for the Apple II+ microcomputer. The program first derives the best-fitting dose-response curves using dose or log dose and effect, log effect, probit-percentage effect, or logit-percentage effect relations. For cell-survival dose-response curves, correlations of ≥0.96 have been obtained. The program then calculates an isobologram at a constant level of the selected agent and plots the data.

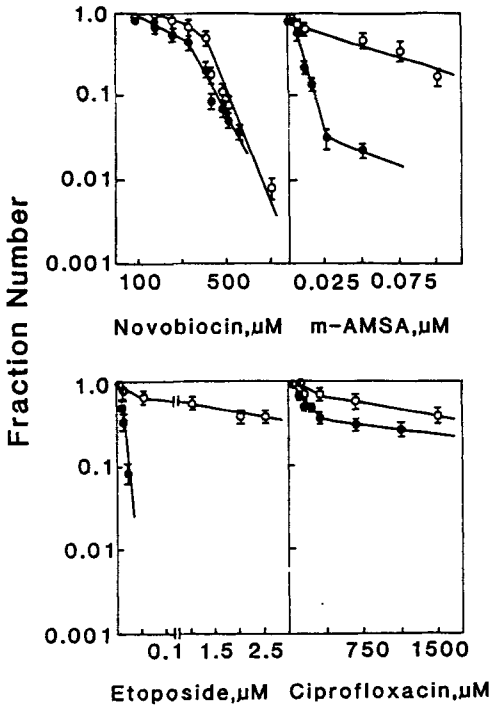

Fig. 1. Survival of parental (●) and resistant (○) cells exposed to various concentrations of novobiocin, *m*-AMSA, etoposide, or ciprofloxacin for 24 h. Points represent the means of at least three independent experiments carried out in triplicate; bars represent the SEM

Results

Survival curves for the parental and resistant cells exposed for 24 h to each of the four potential topoisomerase II interactive agents are shown in Fig. 1. The four drugs displayed cytotoxicity toward parental and resistant cells with a wide range of potencies. Etoposide and *m*-AMSA were much more cytotoxic on a molar basis than were novobiocin and ciprofloxacin. There was no significant difference in the cytotoxicity of novobiocin toward parental and resistant cells over a dose range that killed up to

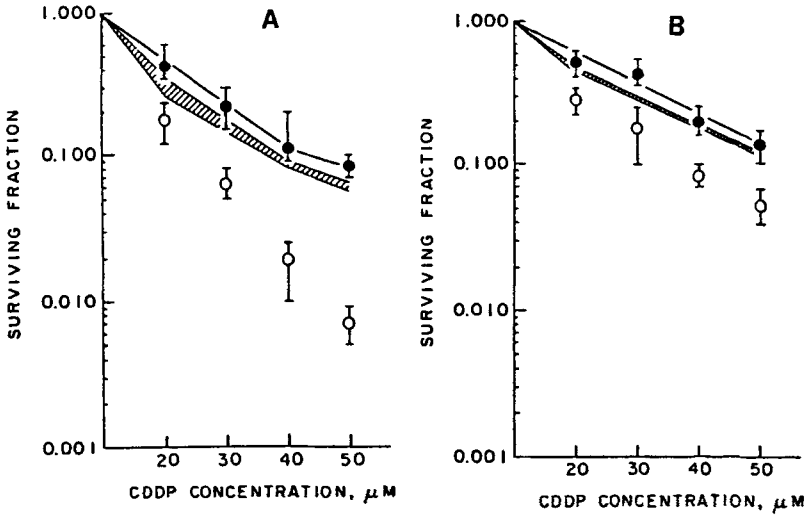

Fig. 2 A, B. A Effect of a minimally inhibitory concentration of novobiocin (80 μ*M*) on CDDP cytotoxicity in parental cells. (●), CDDP toxicity (CDDP concentration in μ*M*); (○), CDDP and 80 μ*M* novobiocin; *shaded area*, envelope of additivity. B Same experiment using resistant cells; symbols are identical

2 logs of cells. At a level of 90% cell kill (IC_{90}), the resistance to *m*-AMSA by the resistant cells was approximately 9-fold that of the parental cells. The resistant cell line was approximately 170 times more resistant to etoposide than was the parental cell line at a level of 1 log of cell kill by this schedule of drug exposure. Ciprofloxacin was very nontoxic in this assay system, with drug levels of ≥ 1 m*M* (the limit of solubility) for 24 h resulting in slightly less than 1 log of cell kill. At a level of 50% cell kill, the resistant cell line was approximately 5 times more resistant to ciprofloxacin than was the parental cell line.

Previous experiments in CHO K_1 cells demonstrated that minimally toxic concentrations of novobiocin could potentiate the cytotoxic effects of CDDP [6]. To determine whether topoisomerase II might mediate the effect of novobiocin on CDDP cytotoxicity, we compared the ability of three different topoisomerase II inhibitors to produce a similar effect, using novobiocin in the parental CHO cell line and in a CHO subline with an altered topoisomerase II. Survival curves for CDDP exposure showed a 2.5-fold degree of resistance in the resistant cells as compared with the parental line (Fig. 2). Glutathione and metallothionein levels in the resistant line were 2- to 3-fold those found in the parental line, which could account for this mild degree of resistance (B. Teicher, personal communication). At a minimally cytotoxic concentration of novobiocin (80 μ*M*) (surviving fraction, >0.95) in combination with increasing concentrations of CDDP, there was greater than additive cytotoxicity in both cell lines, although the slopes of the lines were different (Fig. 2), with a 10-fold difference being observed at the IC_{90} of CDDP. The resistant subline was slightly less sensitive to the potentiation of CDDP cytotoxicity by novobiocin when low concentrations of novobiocin were used.

Experiments were then undertaken to examine the effect of each of the four agents added at equicytotoxic concentrations on the cytotoxicity of CDDP in each of the cell lines. The treatment combinations were assessed for additivity using isobologram methodology. On exposure of parental cells for 24 h to novobiocin, *m*-AMSA, etoposide, and ciprofloxacin, approximately 10% cell survival was observed at concentrations of 350, 0.015, 0.02, and 1,120 μ*M*, respectively. These topoisomerase II-inhibitor concentrations were selected for study in combination with a range of CDDP doses (Fig. 3). All combinations of *m*-AMSA and CDDP produced cell kills that fell within the envelope of additivity for this combination. The combination of etoposide and CDDP was slightly greater than additive at low CDDP concentrations and was additive at the highest concentration of CDDP tested. The combination of novobiocin and CDDP produced greater than additive cell kill over the entire dose range of CDDP tested, with cell kill that was 5–10 times greater than additivity being observed. In combination with CDDP, ciprofloxacin, like novobiocin, resulted in greater than additive cell kill

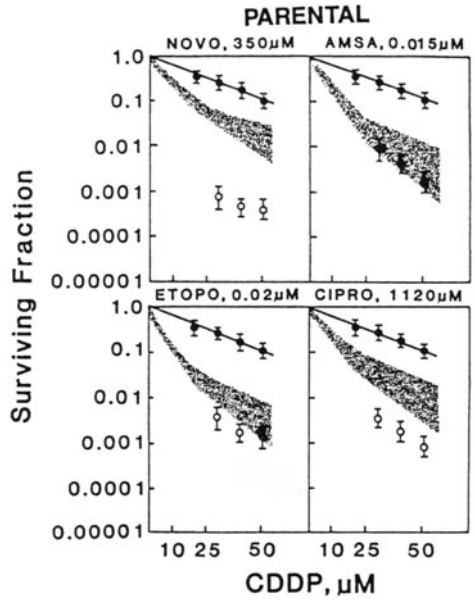

Fig. 3. Survival of parental cells exposed to various concentrations of CDDP (●) for 1 h or to an IC_{90} concentration of novobiocin (*NOVO*, 350 μ*M*), *m*-AMSA (*AMSA*, 0.015 μ*M*), etoposide (*ETOPO*, 0.02 μ*M*), or ciprofloxacin (*CIPRO*, 1,120 μ*M*) for 1 h prior to and during exposure to various concentrations of CDDP for 1 h and then for 22 h following CDDP treatment (○). *Shaded areas* indicate the envelope of additivity for each treatment combination. Points represent the means of three independent experiments; bars represent the SEM

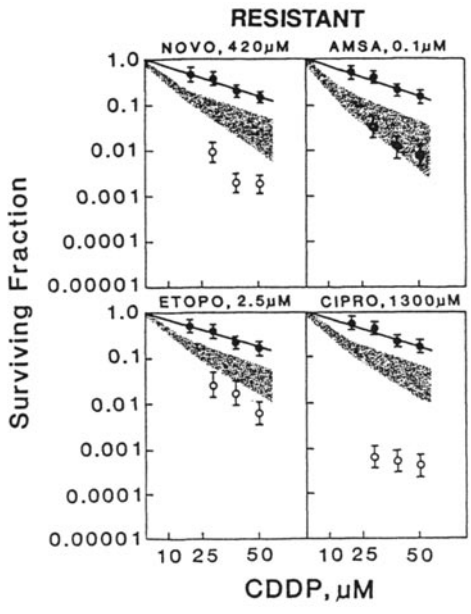

Fig. 4. Survival of resistant cells exposed to various concentrations of CDDP (●) for 1 h or to an IC_{90} concentration of novobiocin (*NOVO*, 420 μ*M*), *m*-AMSA (*AMSA*, 0.1 μ*M*), etoposide (*ETOPO*, 2.5 μ*M*), or ciprofloxacin (*CIPRO*, 1,300 μ*M*) for 1 h prior to and during exposure to various concentrations of CDDP for 1 h and then for 22 h following CDDP treatment (○). *Shaded areas* indicate the envelope of additivity for each treatment combination. Points represent the means of three independent experiments; bars represent the SEM

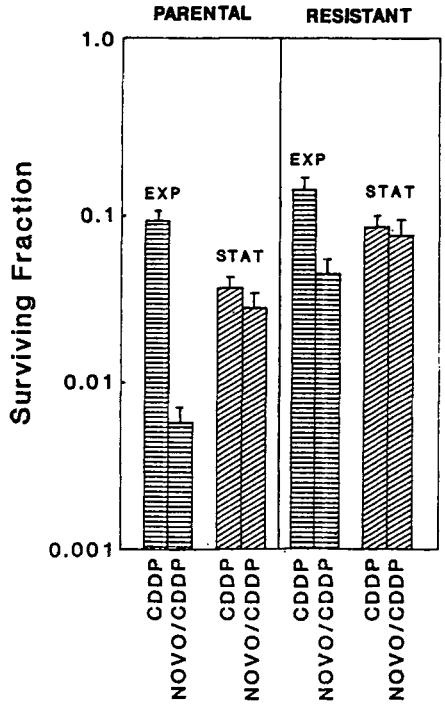

Fig. 5. Survival of exponentially growing (*EXP*) or stationary-phase (*STAT*) parental and resistant cells exposed for 1 h to 50 μ*M* CDDP or to 80 μ*M* novobiocin (*NOVO*) for 1 h prior to and during exposure to 50 μ*M* CDDP and then for 22 h following CDDP treatment. Data represent the means of three independent experiments; bars represent the SEM

over the entire range of CDDP concentrations examined, but the level of synergy was lower than that seen with novobiocin, being about 2–3 times greater than additivity.

The patterns of cytotoxicity demonstrated by the drug combinations in the resistant cell line were similar to those seen in the parental cell line (Fig. 4). Equicytotoxic concentrations of the four topoisomerase inhibitors were selected that produced a surviving fraction of approximately 0.10 in the resistant cell line. The drug concentrations used were 420, 0.1, 2.5, and 1,300 μ*M* for novobiocin, *m*-AMSA, etoposide, and ciprofloxacin, respectively. *m*-AMSA and CDDP in combination produced additive cell kill of the resistant cells. The cytotoxicity of the combination of CDDP and etoposide was only slightly greater than additive over the concentration range of CDDP tested, with the synergy achieved being 2–3 times greater than additivity. Novobiocin in combination with CDDP produced greater than additive cell kill in the resistant cells over the entire concentration range of CDDP tested; the cell kill observed with this drug combination was 3–7 times greater than additivity. A larger degree of supraadditivity was seen with the combination of ciprofloxacin and CDDP; this effect was approximately 2 logs in magnitude at the lower doses of CDDP and approximately 1 log in magnitude at the highest dose of CDDP.

For further definition of the effect of novobiocin in this system, the effect of the combination of CDDP (50 μ*M*) and novobiocin (80 μ*M*) was compared in exponentially growing and stationary-phase parental and resistant cells (Fig. 5). The greater than additive effect that was observed in exponentially growing parental cells at a higher concentration of novobiocin (350 μ*M*) was also seen at the essentially nontoxic concentration of 80 μ*M* novobiocin. CDDP was somewhat more toxic to stationary-phase than to exponentially growing parental cells, as has previously been reported for this drug [8, 18]. The effect of novobiocin on CDDP cytotoxicity that was seen in exponentially growing parental cells was lost in stationary-phase cultures. The combination of novobiocin (80 μ*M*) and CDDP was also greater than additive in exponentially growing resistant cells, although the level of synergy was less than that observed in parental cells. As in stationary-phase parental cells, the effect of novobiocin on CDDP cytotoxicity was lost in stationary-phase resistant cells.

Discussion

The clinically effective alkylating agents are bifunctional alkylators that form two covalent linkages to DNA. Although CDDP is an inorganic molecule that forms two coordinate bonds with DNA, it is most often grouped with the antitumor alkylating agents. The resulting cytotoxicity is attributable to DNA monoadduct formation followed by inter- or intrastrand DNA cross-link formation [28]. The quantity of cross-links formed is a function of initial nucleotide monoadduct formation and subsequent progression to DNA cross-links. Enzyme-mediated removal of nitrosourea monoadducts and the disappearance of DNA cross-links formed by several alkylating agents are consistent with repair processes [16, 18, 21]. Novobiocin, an inhibitor of topoisomerase II, increases CDDP interstrand cross-links, perhaps by inhibition of monoadduct repair(s). To determine whether CHO epipodophyllotoxin-resistant

cells would show different patterns of response to combinations of potential topoisomerase II inhibitors and CDDP, potential topoisomerase II inhibitors of different pharmacologic types were studied. To equalize the effect of the resistance, equicytotoxic concentrations of the drugs were used.

The four topoisomerase II inhibitors used in this study vary widely in their cytotoxic potency. Consequently, a fixed biologic reference point, the drug concentration that inhibits colony formation by approximately 90% was used as a standard. The resistant cell line exhibited marked resistance to etoposide that was approximately 170-fold that shown by the parental cell line. This degree of resistance was considerably greater after a 24-h continuous exposure than after short (1-h) exposures and may reflect a schedule -dependent effect. Moderate cross-resistance to m-AMSA was seen in the resistant line as compared with the partial line. By contrast, novobiocin showed no significant cross-resistance between the two cell lines. Ciprofloxacin was remarkably nontoxic under these assay conditions.

Novobiocin and ciprofloxacin are clinically useful antibacterials with a substantial therapeutic ratio of bacteriocidal: host eukaryotic cell cytotoxicity. Although novobiocin and ciprofloxacin have similar clinical uses as antibacterials and both act on bacterial DNA gyrase (topoisomerase II), their mechanisms as well as sites of action are different [4, 27]. In eubacteria in which DNA gyrase is a tetramer, ciprofloxacin and novobiocin act on different protein subunits that are the products of different genes [4,27].

Novobiocin acts on topoisomerase II by competitive inhibition of an ATPase site that is essential for enzyme-DNA dissociation [4, 9]. This drug does not cause formation of a "cleavable complex" as do the antitumor agents etoposide and *m*-AMSA (Byron Long, personal communication). In HL-60 leukemia cells, novobiocin has also been shown to induce differentiation that contributes to the growth inhibition of these cells by the drug [2].

Ciprofloxacin causes a DNA-protein precipitable complex in eubacteria, as do etoposide and *m*-AMSA [4, 27]. It is not clear that the inhibition of topoisomerase function is, in fact, the lethal event in cells associated with any of these agents: however, the formation of a stable intermediate ternary complex (topoisomerase II/DNA/drug) may elicit a second cytotoxic process that acts as a cellular poison [4]. The inhibition of topoisomerase function accounts for the toxicity of novobiocin in bacteria [9].

In parental cells, the approximate IC_{90} concentrations of novobiocin and ciprofloxacin produced marked synergy in combination with CDDP over the concentration range examined. Etoposide was slightly synergistic at lower CDDP concentrations, but at the highest concentration the cytotoxicity of the combination was only additive, as it was for m-AMSA over the entire range of CDDP concentrations tested. In the resistant cell line, the use of approximately equicytotoxic concentrations of the inhibitors with the same concentration range of CDDP produced very similar results. Novobiocin and ciprofloxacin in combination with CDDP produced synergistic cytotoxicity over the entire range of CDDP concentrations studied. The combination of etoposide and CDDP was slightly greater than additive in the resistant cell line. Again, *m*-AMSA produced only an additive cytotoxic effect with CDDP in the resistant as well as the parental cell line. At an essentially noncytotoxic concentration, novobiocin still produced greater than additive cytotoxicity in both parental and resistant cells over the entire CDDP concentration range examined, although the level of enhancement was somewhat lower in the resistant cell line.

If topoisomerase II is important for the enhancement of CDDP cytotoxicity by novobiocin, the effect should be at least partly dependent on the proliferative state of the cells. Topoisomerase II content is greatest in exponentially growing cells and is markedly reduced or undetectable in growth-arrested, stationary-phase cells [23]. When stationary-phase parental and resistant CHO cells were treated with CDDP and novobiocin, no synergy was observed; this is in marked contrast with the effect observed in exponentially growing cells. Many other enzymes are also decreased in resting or stationary cells, but DNA polymerase alpha, another potential target of novobiocin action [5], is not decreased significantly in G_1/resting CHO cells [14]. This observation is consistent with but does not prove the hypothesis that the interaction of novobiocin with topoisomerase II mediates the enhancement in CDDP cytotoxicity produced by this combination.

Qualitatively, there appears to be a difference in the abilities of these four potential topoisomerase II inhibitors to enhance the cytotoxicity of CDDP to a level greater than additivity. It may be that etoposide and *m*-AMSA, which are extremely cytotoxic drugs, are more efficient initiators of the cascade of biochemical events that eventually lead to mammalian cell death than are ciprofloxacin or novobiocin. This secondary lethal pathway is most likely not involved after the use of drugs in combination with CDDP. Another cellular process, which novobiocin and ciprofloxacin inhibit more selectively, results in the synergy of these two drugs with CDDP: the strand-passing activity of topoisomerase II.

In conclusion, CDDP cytotoxicity can be markedly enhanced by novobiocin and ciprofloxacin. The use of available techniques to study DNA repair and topoisomerase II function at the DNA level may enable further clarification of the mechanism of this effect. Concurrently, rational combinations of these drugs with bifunctional alkylators such as CDDP may play some role in the clinical treatment of cancer.

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