REPORTS

Cytotoxic Effects of Topotecan Combined With Various Anticancer Agents in Human Cancer Cell Lines

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Background: Topotecan (TPT) is a topoisomerase I poison that exhibits antineoplastic activity. Analysis of the cytotoxic effects of combinations of TPT and other anticancer agents has been limited. Purpose: We assessed the cytotoxic effects produced by combinations of TPT and other antineoplastic agents in experiments involving multiple human cancer cell lines of diverse histologic origins. Methods: The cytotoxic effects of various antineoplastics (fluorouracil, methotrexate, or cytarabine), anticancer agents (vincristine or paclitaxel [Taxol]), DNA alkylating agents (melphalan, BCNU, and 4HC) also showed drug effects that were less than additive; in most cases, however, nearly additive or even synergistic effects were observed with these same drug combinations at high levels of cytotoxicity (i.e., at >90% inhibition of colony formation). Results obtained with combinations of TPT and cisplatin varied according to the cell line examined. With A549 cells, less than additive effects were seen at low-to-intermediate levels of cytotoxicity, and more than additive effects were found at high levels of cytotoxicity. With NCI-H82rasH cells, synergy was observed over most of the cytotoxicity range. Conclusions and Implications: TPT cytotoxicity appears to be enhanced more by combination with certain DNA-damaging agents than by combination with antineoplastics or antimicrotubule agents. Interactions between TPT and other drugs can vary depending on the cell type examined. Further investigation is required to determine the basis of the observed effects and to determine whether these in vitro findings are predictive of results obtained in vivo. [J Natl Cancer Inst 1996;88:734-41]

Camptothecin and its analogues are being evaluated as potential antineoplastic agents (1-6). A variety of studies [reviewed in (1,3,4,6)] indicate that these agents reversibly inhibit the DNA religation step of the reaction catalyzed by the nuclear enzyme topoisomerase I (topo I). This inhibition increases the number of covalent topo I-DNA complexes within cells, and interaction of these complexes with replication forks results in the formation of small numbers of irreversible double-strand DNA breaks (7,8). These DNA breaks are thought to be the lesions responsible for camptothecin-induced cell death [reviewed in (1,4,9)].

Camptothecin derivatives have shown promising therapeutic activity when used as single agents against carcinomas of the colon, lung, ovary, and cervix, as well as against neuroblastomas and relapsed acute leukemias [reviewed in (1-4)]. Because treatment with these drugs alone is unlikely to be curative, there is considerable clinical interest in combining topo I poisons with other antineoplastic agents. Despite this interest, relatively few preclinical studies evaluating such drug combinations have been performed.

Most combination studies in vitro have focused on the parent compound, camptothecin. In two of the studies (7,10), it was determined that the cytotoxicity of camptothecin was diminished by concurrent treatment with the DNA synthesis inhibitors aphidicolin and hydroxyurea. In contrast, camptothecin and bis[chloroethyl]nitrosourea (BCNU) were reported...
to have additive cytotoxic effects (11). Camptothecin and cisplatin were observed to have synergistic (i.e., more than additive) toxic effects in lymphocytic leukemia cells in vitro (12); however, additive effects were seen with this combination in ovarian cancer cells in vitro (13).

The effects of combining water-soluble camptothecin analogues with other agents have also received limited attention. Kano et al. (14) incubated Mol3 acute lymphocytic leukemia cells with irinotecan (CPT-11) or its active metabolite SN-38 and various compounds for 3 days, after which cell survival was measured using a tetrazolium dye reduction assay; drug interactions were then analyzed using an isobologram method. The results of this study indicated that the effects were additive or synergistic when CPT-11 or SN-38 was combined with a variety of drugs, including fluorouracil (5-FU) and cytarabine, agents that would be expected to inhibit the DNA synthesis required for conversion of topo I-DNA complexes into cytotoxic double-strand DNA breaks.

Examination of topotecan (TPT) in combination with other treatments in vitro revealed that antagonism (i.e., less than additive effects) resulted from simultaneous exposure to TPT and etoposide (15), whereas synergy was found when TPT was combined with γ irradiation (16) or 2′-deoxy-5-azacytidine (17). In more recent studies, TPT was also examined in combination with paclitaxel (Taxol) (18), cisplatin (18,19), and other DNA-damaging agents (19). In the former study (18), which was limited to continuous-exposure experiments using teratocarcinoma cell lines, the combinations of paclitaxel and TPT and cisplatin and TPT exhibited synergistic effects. In the latter study (19), more Chinese hamster lung fibroblasts were killed when TPT was combined with an arbitrary, fixed dose of alkylating agent than were killed with TPT alone; however, a formal mathematical analysis of this interaction was not performed.

In our study, the interaction between TPT and various antineoplastic agents was re-examined. The experiments described herein differ in two respects from those presented in previous reports. First, the median effect method of Chou and Talalay (20) was used to analyze the potential interactions between cytotoxic treatments. Second, multiple human cancer cell lines of diverse histologic origins were examined to rule out the possibility that unexpected interactions were unique to one cell line.

Materials and Methods

Materials

TPT and 4-hydroperoxycyclophosphamide (4HC) were provided by R. K. Johnson (SmithKline Beecham Pharmaceuticals, Philadelphia, PA) and O. Michael Colvin (Duke University Cancer Center, Durham, NC), respectively. Cisplatin, methotrexate, paclitaxel, 5-FU, cytarabine, BCNU, and melphalan were purchased from Sigma Chemical Co. (St. Louis, MO).

The following stock solutions were prepared and stored at ~20 °C: TPT (20 mM), methotrexate (10 mM), and cytarabine (10 mM) in dimethyl sulfoxide (DMSO); melphalan (10 mM) in 25 mM HCl; and BCNU (20 mM) in 95% ethanolic. Cisplatin, 5-FU, and 4HC were prepared immediately before use as 1000-fold concentrated solutions in DMSO (cisplatin) or ice-cold water (5-FU and 4HC).

Cell lines were cultured at 37 °C in an atmosphere of 95% air and 5% CO2 in the following media, which all contained 50 U/mL penicillin G, 50 μg/mL streptomycin, and 2 mM glutamine: A549 non-small-cell lung cancer cells (provided by R. Casero, Johns Hopkins Oncology Center, Baltimore, MD) and HCT8 ileocecal adenocarcinoma cells (American Type Culture Collection, Rockville, MD) in RPMI-1640 with 5% (vol/vol) fetal bovine serum (FBS) (medium A); NCI-H82rasH1 lung cancer cells (provided by M. Mabry, Johns Hopkins Oncology Center) in RPMI-1640 with 10% FBS; T98G glioblastoma cells (American Type Culture Collection) in minimal essential medium containing Earle’s balanced salt solution plus 10% (vol/vol) FBS, nonessential amino acids, and 1 mM sodium pyruvate (medium B); and MCF-7 breast cancer cells in medium B containing 10 μg/mL insulin.

Clonogenic Assay

Aliquots containing 300-500 A549 or HCT8 cells were plated in 35-mm tissue culture plates containing 2 mL medium A and incubated for 12-16 hours to allow the cells to attach to the plastic. Drugs or diluent were added to triplicate plates at each indicated concentration. After 7-8 days of incubation, the plates were washed with calcium/magnesium-free phosphate-buffered saline and stained with Coomassie brilliant blue so that visible colonies could be counted. This procedure yielded 100-200 colonies on diluent-treated plates. The following modifications were made in assays using T98G and MCF-7 cells: 500 (T98G) or 750 (MCF-7) cells were plated to yield 100-200 colonies on control plates; the media described above were substituted for medium A; and the cells were incubated with drugs from 8 (T98G) to 14 (MCF-7) days to allow colony formation.

NCl-H82rasH1 cells were plated in soft agar to assess colony formation. After log-phase cultures were triturated into single-cell suspensions, aliquots containing 103 cells were plated in 1 mL 0.3% (wt/vol) agar in the medium of Pike and Robinson (21), which was supplemented with drug at the indicated final concentration immediately before plating. After the plates were incubated for 7-10 days at 37 °C in an atmosphere containing 7.5% (vol/vol) CO2, colonies containing more than 32 cells were counted with the aid of an inverted phase contrast microscope. Diluent-treated plates generally contained 200-400 NCI-H82rasH1 colonies using this procedure.

Analysis of Combined Drug Effects

In each experiment, cells were treated with serial dilutions of each drug individually and with a fixed ratio of both drugs simultaneously at doses that typically corresponded to 1/2, 5/8, 3/4, 7/8, 1, and 1.5 times the individual IC50 values. Fractional survival (f) was calculated by dividing the number of colonies in drug-treated plates by the number of colonies in control plates. Data were subsequently analyzed by the method of Chou and Talalay (20) using a QuatroPro spreadsheet program (Borland, Scotts Valley, CA). In brief, log([f/f - 1]) was plotted against log(drug dose). From the resulting median effect lines, the x-axis intercept (log IC50) and slope m (a measure of sigmoidicity) were calculated for each drug and for the combination by the least-squares method. These parameters were used as described (20) to calculate the doses of the individual drugs and the combination required to produce varying levels of cytotoxicity (f = 0.95, 0.90, 0.85, ... 0.05) according to equation 1:

\[ \text{Dose}_{\text{IC50}} = \text{Dose}_{\text{IC50}} \left( 1 - \frac{1}{f} \right)^{1/m} \]

When the two drugs were administered at a fixed ratio, the dose of the combination required to produce fractional survival f could be separated into the component doses (D1) and (D2) of drugs 1 and 2, respectively. For each level of cytotoxicity (f = 0.95, 0.90, 0.85, ..., 0.05), a parameter called the combination index (CI) (20) was then calculated according to equation 2:

\[ \text{CI} = \left( D_1/D_{1f} \right)^{\alpha} + \left( D_2/D_{2f} \right)^{\alpha} + \alpha \left( D_1/D_{1f} \right)^{\alpha} \left( D_2/D_{2f} \right)^{\alpha} \]

where (D1) and (D2) are the concentrations of the combination required to produce survival f, (D1f) and (D2f) are the concentrations of the individual drugs required to produce f, and α = 1 or 0 depending on whether the drugs are assumed to be mutually nonexclusive or mutually exclusive, respectively, in

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their action (20). According to this method, synergy is indicated by a CI less than 1, additivity by a CI equal to 1, and antagonism by a CI greater than 1 (20).

Unless otherwise indicated, the experiments were repeated until three replicates yielded correlation coefficients (R) greater than 0.9 for all three median effect lines (20). Results of multiple CI plots were summarized by indicating the mean ± standard deviation of the CI at the indicated level of colony inhibition.

Results

Interaction Between TPT and Antimetabolites

The cytotoxic effects of TPT and other antineoplastic agents were examined by exposing cells to TPT and a second agent continuously for 7-14 days and assessing colony formation. This approach was used because of its ease of implementation and because it approximated the prolonged TPT exposure expected with several treatment schedules that have been investigated clinically, e.g., daily infusions for 5 consecutive days and continuous infusions for greater than or equal to 5 days (reviewed in (/2,22)).

Fig. 1 shows results obtained when HCT8 cells were exposed to TPT and 5-FU. Each agent by itself inhibited the formation of colonies (Fig. 1, A and B). When cells were exposed to a fixed 1:150 ratio (TPT:5-FU) of the two, the inhibition was greater than that observed with either drug alone (Fig. 1, A). Transformation of the data as described by Chou and Talalay (20) resulted in linear median effect curves (Fig. 1, C). For each line in Fig. 1, C, the x-axis intercept indicates the log(IC50) for the treatment and the slope provides a measure of the sigmoidicity of the dose–response curve. Chou and Talalay (20) have suggested that it is possible to calculate from these parameters the effect that would be expected if the individual effects of the two agents were additive. The same slopes and intercepts can also be used to calculate the CI, a parameter that indicates whether the doses of the drugs required to produce a particular effect in combination are larger than (CI>1), equal to (CI=1), or smaller than (CI<1) the doses required to produce that same degree of toxicity if the effects of the two drugs were additive (20).

In applying this method to the data in Fig. 1, C, we assumed that TPT and 5-FU were mutually nonexclusive, i.e., that the action of TPT on topo I did not affect the action of 5-fluorodeoxyuridine monophosphate on thymidylate synthase and vice versa. This assumption was based on current understanding of the initial steps in the mechanisms of action of these agents and on the observation that the single-agent median effect lines (Fig. 1, C) had different slopes (20). However,
for completeness, the data were also analyzed assuming that the mechanisms of action were mutually exclusive. The CI calculated on the assumption of mutually nonexclusive action is drawn as a solid line in Fig. 1, D. In three separate experiments, the CI for TPT and 5-FU was 1.9 ± 0.2 (n = 3) at the IC50 of the combination and greater than 1 at all levels of toxicity, suggesting that the effects of these agents were less than additive. Treatment of A549 lung cancer cells with the same combination likewise revealed a CI of 1.5 ± 0.2 at the IC50, although the CI was 1.0 ± 0.2 at the IC90 of the combination in this cell line (Fig. 1, E).

To determine whether these results were unique to 5-FU, combinations of TPT and methotrexate and TPT and cytarabine were also tested. When A549 cells were treated with TPT and methotrexate, the CI was 2.4 ± 0.5 at the IC50 and greater than 1 at all levels of toxicity (Fig. 1, F). Likewise, when TPT and cytarabine were combined, the CI was 2.2 ± 0.6 at the IC50 and greater than 1 at all levels of toxicity (data not shown).

Interaction Between TPT and Antimicrotubule Agents

To assess the effects of other cell cycle phase-specific agents on TPT toxicity, A549 cells were treated with combinations of TPT and drugs that target microtubules. Results obtained with the combination of TPT and vincristine are shown in Fig. 2, A-D. More toxicity was observed in the presence of both drugs than with either drug alone (Fig. 2, A and B). Nonetheless, calculations performed assuming that the mechanisms of action of TPT and vincristine are mutually nonexclusive revealed that the CI was 1.4 ± 0.2 at the IC50 and greater than 1 at all levels of cytotoxicity (Fig. 2, D, solid line). Antagonism was similarly observed when A549 cells were treated with TPT and paclitaxel (Fig. 2, E; CI = 1.7 ± 0.2 at the IC50).

Interaction Between TPT and DNA Alkylating Agents

The effect of combining TPT with DNA alkylating agents, which are used extensively in combination chemotherapy, was examined next. Fig. 3, A-D, shows the effects of TPT and melphalan on A549 cells. Once again, the addition of a second agent enhanced the cytotoxicity of TPT (Fig. 3, A). Analysis by the median effect method revealed that the CI was 1.3 ± 0.2 (n = 5) at the IC50 of the combination but dropped to less than 1 at the IC90 in four of five similar experiments (Fig. 3, D), suggesting that the interaction was at least additive at high levels of toxicity. These results were not unique to the A549 cell line or to this pair of drugs. When MCF-7 cells were exposed to TPT and melphalan, the CI was
likewise 1.7 ± 0.5 at the IC₅₀ and 1.0 ± 0.2 at the IC₉₀ (data not shown). Similarly, the CI approached or dropped below unity at the IC₅₀ in MCF-7 cells treated with TPT and 4HC (Fig. 3, E) or T98G cells treated with TPT and BCNU (Fig. 3, F). This trend was not, however, observed in all cell lines treated with all combinations. When A549 cells were treated with TPT and 4HC, the CI at the IC₉₀ of the combination was 1.2 ± 0.2. Likewise, treatment of K562 human leukemia cells with TPT and 4HC resulted in a CI of 1.5 ± 0.02 (n = 2) at the IC₉₀, suggesting that the interaction between TPT and particular alkylating agents might vary in different cell lines.

Interaction Between TPT and Cisplatin

The effect of treating A549 cells with TPT and cisplatin is shown in Fig. 4, A-D. The CI was 1.4 ± 0.2 at the IC₅₀ of this combination but fell to 0.7 ± 0.1 at the IC₉₀ (Fig. 4, D, solid line). Results obtained with NCI-H82ras² cells, which display a large-cell lung cancer phenotype (23), were even more striking (Fig. 4, E). Synergy was observed over most or all of the range of toxicity, with a mean CI of 0.7 ± 0.2 (n = 4) at the IC₅₀ and 0.8 ± 0.2 at the IC₉₀ of the combination.

Discussion

In this report, the effects of combining TPT with other antineoplastic agents were examined. The optimum approach in performing this type of analysis has been the subject of considerable discussion (20,24,25). Options for evaluating the combined effects of two drugs include the fractional product method, the isobologram method, the response surface method, and the median effect method. The fractional product method has been criticized for failing to take into account the sigmoidicity of dose–response curves (20,25). The isobologram method has been criticized because of its implicit assumption that drugs are mutually exclusive in their action and its requirement for relatively large datasets (20). The response surface method (24) is appealing, but it requires large datasets and relatively sophisticated computational capabilities to compare the experimental and predicted response surfaces. At the outset of these experiments, we elected to use the median effect method because it requires datasets that are of manageable size (experiments involving 50-100 plates).

Various aspects of the median effect method have been recently debated. Berenbaum (24) questioned the distinction between mutually exclusive and mutually nonexclusive drugs and suggested that the value of α in equation 2 (see “Materials and Methods” section) should be zero for both types of interac-
Fig. 4. Interaction of topotecan (TPT) and cisplatin. A and B) Inhibition of A549 cell colony formation by TPT alone (A, closed symbols), cisplatin alone (B), or a 1:40 ratio of TPT and cisplatin (A, open symbols). C) Median effect plot of the data in A and B. D) Combination index (CI) calculated from the data in C under the assumption that agents are mutually nonexclusive (solid line) or mutually exclusive (dotted line) in their mechanisms of action. E) CI plot for TPT and cisplatin at a 1:100 ratio in NCI-H82rasH cells.

Greco et al. (25) supported the distinction between mutually exclusive and mutually nonexclusive drugs but questioned whether the third term in equation 2 was correctly derived. Because the proper way for calculating additivity (and hence synergy or antagonism) remains somewhat contentious, panels A and B of each figure contain survival data from the respective experiments so that alternative mathematical models can be applied to the experimental results.

Bearing these issues in mind, we employed the method of Chou and Talalay (20) to analyze the cytotoxic effects observed when TPT was combined with various antineoplastic agents. When the data were analyzed under the assumption that TPT and the other agents are mutually nonexclusive, the effects of TPT and either antimetabolites or antimicrotubule agents were determined to be less than additive (Figs. 1 and 2), the effects with DNA alkylating agents were determined to be less than additive at the IC50s of the combinations but more than additive at the IC50s in several cell lines (Fig. 3), and the effects with cisplatin were determined to be more than additive over a broad range of toxicity (Fig. 4).

In view of the observation that camptothecin analogues cause cell cycle perturbations (26,27), the decision to consider antimetabolites and TPT as mutually nonexclusive might be questioned. Likewise, the observation that DNA damage stalls cells in G2 (28), a phase where they are relatively resistant to camptothecin (29), might also call into question the assumption that TPT and DNA damaging agents are mutually nonexclusive. The dotted lines on the CI plots in Figs. 1-4 show CIs calculated when the mechanisms of drug action are assumed to be mutually exclusive. The mean CI values at the IC50s of the combinations shown in Figs. 1 and 2 were 1.4 ± 0.1 and 1.2 ± 0.1 for TPT and 5-FU in HCT8 and A549 cells, respectively; 1.7 ± 0.3 for TPT and methotrexate in A549 cells; 1.1 ± 0.1 for TPT and vincristine in A549 cells; and 1.2 ± 0.1 for TPT and paclitaxel in A549 cells. Because the precision of CI measurements is difficult to quantitate, we cannot rule out the possibility that the effects of TPT and antimicrotubule agents are additive when effects of additive agents are predicted according to this alternative hypothesis. The rank order of the interactions, however, does not change. With this alternative assumption, most TPT and antimetabolite combinations still appear antagonistic, and the
TPT and DNA damaging agent combinations appear even more synergistic.

Further experiments are required to establish the mechanistic basis for the interactions described above in mathematical terms. In general, the observation of synergy or antagonism between two cytotoxic drugs may reflect any of a number of different mechanisms, including an effect of one agent on 1) uptake or metabolism of the second agent, 2) binding of the second agent to its target, 3) ability of the second agent to damage cells, or 4) ability of cells to respond to damage induced by the second agent.

Control experiments have indicated that the drugs used in combination with TPT do not alter the pH of the medium (a determinant of the equilibrium between TPT lactone and hydroxyacid (30)) or cellular TPT uptake (Kaufmann SH, Svingen PA: unpublished results). Therefore, other explanations for the observed interactions must be invoked. On the basis of current knowledge of the action of camptothecin analogues, the following tentative explanations can be proposed. The antagonism between TPT and antimetabolites (Fig. 1) might reflect two phenomena. First, a TPT-induced G2 arrest might diminish the number of cells entering S phase and hence the efficacy of antimetabolites. Second, antimetabolites might slow DNA synthesis in cells that enter S phase, diminishing conversion of TPT-stabilized topo I-DNA complexes into cytotoxic double-strand breaks. Conversely, the synergy between TPT and alkylating agents at high levels of toxicity (Fig. 3) might reflect interaction between topo I-DNA complexes and polymerases repairing alkylator-induced damage. Alternatively, because poly(adenosine diphosphate [ADP]-ribose) polymerase plays a role in resistance to camptothecin analogues (31,32) and depletion of the poly(ADP-ribose) polymerase substrate NAD+ by other agents sensitzes hamster lung cells to camptothecin (33), it is plausible that alkylating agents might activate poly(ADP-ribose) polymerase, deplete NAD+ (34), and thereby sensitize cells to TPT. Further experiments are required to evaluate these explanations.

In considering the potential implications of this study, two additional limitations must be kept in mind. First, when drugs are combined at their IC50 ratios, their combined effects appear to vary between different cell lines (Figs. 1, D, versus 1, E, 3, D, versus 3, E; and 4, D, versus 4, E). These variations, which might reflect differences in the way different cell lines handle drug-inflicted damage, could contribute to differences in the conclusions reached in individual studies. Second, the data for each cell line were generated using fixed ratios of each pair of drugs. If a different ratio had been evaluated, a different CI plot would have resulted. Because the entire response surface (25) was not mapped, the conclusions are limited to the ratios of drugs that were actually studied.

Despite these limitations, it will be interesting to see whether the present results have any predictive value. Trials of several TPT-containing drug combinations are currently nearing completion. It is unclear whether the antagonism observed between TPT and antimetabolites in vitro will also be observed in vivo. In this context, it is important to realize that antagonism in the strict mathematical sense was observed even with combinations that killed a larger number of cells than either drug alone (Fig. 1, A). This antagonism indicates that the effects of the drugs are less than additive but does not necessarily mean that the action of one drug abolishes the action of the other. Depending on the effects of these agents on normal tissues versus tumor tissues, it is possible that some of the "antagonistic" combinations might prove to be useful clinically. Conversely, our data suggest that combining TPT with certain alkylating agents or platinating agents results in synergy in vitro. If, however, the synergistic cytotoxicity of these combinations is observed in normal tissues as well as in tumor cells, this synergy might not be advantageous. The results of clinical trials evaluating the efficacy of some of the combinations described in this study are awaited with interest.

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Notes

1Unless otherwise indicated, all combination index values specified in the text are means and standard deviations resulting from three independent experiments.
2Results obtained using this assumption have been shown to correspond to results obtained using isobologram techniques (20,24,25).

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