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The significance of the sequence of administration of topotecan and etoposide

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Abstract *Purpose*: Often the best method of integrating chemotherapeutic agents is unknown. Recently there has been interest in the use of combinations of the topoisomerase II inhibitors and the topoisomerase I inhibitors as these agents have shown individual activity in malignancies such as non-small-cell lung cancer. This study examined the interaction of the topoisomerase II inhibitor etoposide with the topoisomerase I inhibitor topotecan (Tpt) in V79 cells (hamster lung fibroblast cells) to determine the optimal method of delivering these agents. *Methods and results*: Cell survival was assessed by colony formation. Synergistic interactions were assessed by the median effect principle in which a combination index (CI) of less than one suggests a synergistic interaction. The V79 cells were exposed to sequential 24-h incubations with the two chemotherapeutic agents. Initially, equitoxic doses of the two agents were delivered (i.e. 0.0275 μg/ml of topotecan alone or 0.089 μg/ml of etoposide alone resulting in a surviving fraction of 70%; Tpt : etoposide ratio 1 : 3.2). It was determined that a sequence-dependent synergistic interaction (CI < 1) resulted at a lower level of cytotoxicity if the etoposide exposure followed the Tpt exposure compared to the opposite sequence. This same effect was seen after treatment of cells with various concentration (μg/ml) ratios of Tpt : etoposide (1 : 4.0, 1 : 1, 2.5 : 1). *Conclusions*: These results suggest that maximum synergy occurs for the delivery of etoposide following Tpt exposure (compared to the opposite sequence) and these findings may have important clinical implications.

Key words Topotecan · Etoposide · Synergy

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Introduction

The topoisomerase II-reactive agents are commonly used for the treatment of a variety of malignancies, and include the epipodophyllotoxins (etoposide, VM-26) and the intercalators (m-AMSA, doxorubicin). These agents stabilize the formation of a complex between the enzyme topoisomerase II and DNA (cleavable complexes) and this stabilized complex is believed to be a critical event in the pathway leading to cytotoxicity [5, 7, 17]. Recently, several topoisomerase I-reactive agents (camptothecin, topotecan, CPT-11) have been shown to have significant cytotoxic activity in various malignancies and are currently being investigated in multiple clinical trials [1, 9, 13]. The topoisomerase I-reactive agents stabilize a complex between the enzyme topoisomerase I and DNA (cleavable complex) and, like the stabilized DNA topoisomerase II complex, this event is believed to be critical for cytotoxicity [15, 16].

Since topoisomerase I and topoisomerase II are involved in different catalytic processes (relaxation of torsionally strained DNA by allowing single-strand DNA passage or double-strand DNA passage, respectively), investigations were initiated to determine whether simultaneous administration of these agents could result in additive or synergistic cytotoxicity. In fact, early investigations suggested that the simultaneous administration of these agents may result in a less than additive effect and it was hypothesized that the inhibition of DNA or RNA synthesis by one of the agents may inhibit the transformation of the stabilized cleavable complex induced by the other agent into a lethal event [4, 11, 15]. However, it has been demonstrated that the sequential administration of topoisomerase I- and topoisomerase II-reactive agents can result in additive or synergistic interactions [4, 12].

Currently, the optimal method of sequencing these agents is unknown and may have important clinical

ramifications as studies exploring combinations of these agents are ongoing [8, 10]. This study was performed to explore this question utilizing V79 hamster lung fibroblast cells exposed to different sequences of the topoisomerase I inhibitor, topotecan, and the topoisomerase II inhibitor, etoposide. The potential synergistic nature of the drug interactions were assessed by the method of Chow and Talalay [6]. Briefly, this technique allows for the linearization of cell survival data. Following the linearization of the data, calculations can be made to determine the expected contribution of an agent to the overall cytotoxicity of a combination of various agents. A combination index (CI) can be calculated at various levels of cytotoxicity and a value less than one suggests a synergistic interaction. The potential benefits and shortcomings of the application of this model to the interaction of topoisomerase I- and topoisomerase II-reactive agents are discussed.

Materials and methods

Cell culture

Hamster lung fibroblasts (V79) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with nonessential amino acids, penicillin, streptomycin and 10% heat-inactivated (56 °C for 30 min) fetal bovine serum as previously described [5] The protein concentration of this medium was 3.8 g/l. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells grown in this manner had a doubling time of approximately 10 h.

Drug treatment

Etoposide (Bristol Laboratories, Evansville, lowa) for clinical use (100 mg/5 ml) was diluted in phosphate-buffered saline (PBS) to a concentration of 1 mg/ml and stored in polypropylene freezing tubes at − 70 °C. Topotecan was diluted in PBS and stored in polypropylene freezing tubes at a concentration of 100 μg/ml and at − 70 °C.

Cell survival

Log phase V79 cells were exposed to etoposide or topotecan for 24 h either alone or in sequential fashion. The cells were washed twice with PBS between drug exposures for the sequential treatments. After treatment, the medium was aspirated, monolayers were washed with PBS, and cells were detached with PBS containing 0.03% trypsin and 0.27 m*M* EDTA. Appropriate controls of drug alone were performed in all cases. Cells were plated in triplicate in 60 mm × 10 mm tissue culture dishes in numbers sufficient to yield 20–200 colonies. Dilutions were performed in triplicate. Colony formation was then assessed after incubation for 6–7 days by previously described methods [5]. Briefly, the cells were fixed with methanol-acetic acid and stained with crystal violet. Colonies containing more than 50 cells were counted. Individual experiments were performed in triplicate and standard errors were typically less than 15% of the mean and are contained within the size of the symbols unless otherwise shown. All experiments were repeated at least three times, with the results shown representing single experiments.

Median effect principle analysis to assess synergy

Experiments were performed at a fixed ratio of topotecan to etoposide, and the data were analyzed according to the median effect principle as described by Chou and Talalay [6]. Median effect plots were created by using the equation $\log[(1 - SF)/SF] = m[\log(D/D_m)]$, in which SF is the surviving fraction, D is the concentration of drug, D_m is the concentration of drug required to produce the median effect (50% SF), and m is a Hill-type coefficient that describes the sigmoidicity of the survival curve. A plot of $y = \log[(1/SF) - 1]$ versus $x = \log(D)$ was constructed to linearize the dose-response data with a slope m and y intercept of $-\log(D_m)$. In all cases the data resulted in a linear plot with regression coefficients ≥ 0.9. This level of correlation is required to appropriately use this model to assess synergy. The interaction of the two chemotherapeutic agents was then quantitated by the calculation of the CI according to the equation:

$$CI = D_1/D_{x1} + D_2/D_{x2} + n(D_1)(D_2)/(D_{x1})(D_{x2})$$

$$D_{x1} = D_{m1}(1/SF - 1)^{1/m_1}$$

$$D_{x2} = D_{m2}(1/SF - 1)^{1/m_2}$$

$$D_{x1,2} = D_{m1,2}(1/SF - 1)^{1/m_{1,2}}$$

$D_1 = D_{x1,2}$ (fraction of mixture that is agent 1)

$D_2 = D_{x1,2}$ (fraction of mixture that is agent 2)

n = 0 for mutually exclusive interactions and 1 for mutually nonexclusive interactions

The CI was calculated for surviving fractions of 0.75, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001, 0.0003, and 0.0001. A CI versus surviving fraction plot was then constructed. The model suggests an antagonistic relationship between the agents when the CI value was greater than 1 and an additive relation with a value of 1. A synergism interaction was suggested for CI values less than 1. The above form of the equation is for mutually nonexclusive agents and tends to overestimate antagonism and underestimate synergism as compared to the formula for mutually exclusive agents in which the final term of the equation is dropped [6]. Since this issue regarding the mechanism of the topoisomerase I-reactive agents and topoisomerase II-reactive agents is not resolved, the calculation was performed with and without the last term.

Results

V79 hamster lung fibroblast cells were used to test the appropriate sequencing of topotecan and etoposide. It is known that brief exposures (< 4 h) to the topoisomerase I-reactive agents result in dose-dependent cytotoxicity that often plateaus at low levels of cytotoxicity. This effect has been attributed to the S phase specificity of these agents as well as the saturability of the stabilized complexes [4, 5]. Therefore, 24-h exposure times were used for the experiments of topotecan alone or etoposide alone. The drug concentrations that were utilized are comparable to the plasma concentrations that may be obtained at 12–24 h after clinical treatments with commonly used bolus regimens [8–10, 13]. Subsequently, combination experiments were performed with etoposide (24 h) followed by topotecan (24 h), or topotecan (24 h) followed by etoposide (24 h). As stipulated by the mathematical

modeling technique of Chou and Talalay, the sequential experiments were performed using fixed ratios of etoposide to topotecan. Additionally, appropriate controls of early drug exposure (the first 24 h of 48-h experiments) alone and late drug exposure (the second 24 h of 48-h experiments) alone were performed for the combination drug experiments. The dose-response curves for the individual agents (topotecan or etoposide) given alone for 24 h are shown in Fig. 1.

Subsequently, the combination experiments (etoposide followed by topotecan or topotecan followed by etoposide) were performed in the following fixed ratios of topotecan to etoposide: 1:4, 1:2.5, 1:1 and 2.5:1. Experiments were performed for all drug ratios at least three times. The data from these cell survival experiments were fitted by the linearizing median effect equation and median effect plots were constructed for each drug alone and the various combination treatments as described in Materials and methods. CI values were then calculated for the above conditions. Figure 2 illustrates the CI values for the combination of topotecan followed by etoposide or etoposide followed by topotecan delivered at a fixed ratio of topotecan to etoposide of 1:4. The combination of topotecan followed by etoposide resulted in lower CI values at all levels of cytotoxicity compared to the opposite sequence, and this result and the shape of the CI versus surviving fraction graphs were consistent for all the ratios (mentioned above) of topotecan to etoposide explored (data not shown).

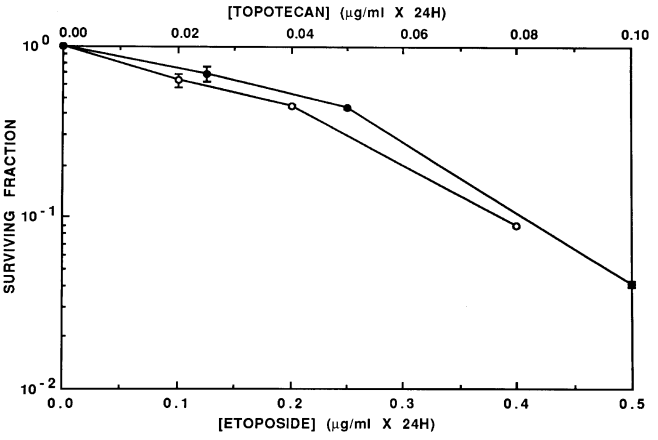

Fig. 1 Topotecan (*closed circles*) and etoposide (*open circles*) resulted in dose-dependent cytotoxicity in V79 hamster lung fibroblast cells (*top abscissa* concentration of topotecan, *bottom abscissa* concentration of etoposide). Cells were exposed to the drug for 24 h, washed twice with phosphate buffered saline and subsequently assessed for colony formation

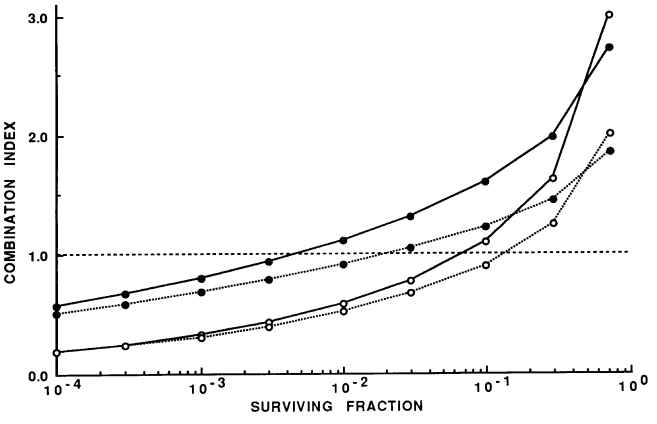

Fig. 2 Synergistic interactions (CI < 1) occurred at a lower level of cytotoxicity when V79 cells were exposed to topotecan followed by etoposide (*open circles*) compared to the opposite sequence (*closed circles*) for a topotecanto etoposide ratio of 1:4. Cells were exposed to the individual drugs alone or in combinations of sequential 24-h exposures, washed twice with phosphate buffered saline (in between drug exposures and at the completion of the exposure) and assessed for cell survival by colony formation. CI values were calculated by the method of Chou and Talalay [6]. *Dotted lines* indicate the mutually exclusive assumption and the solid lines indicate the mutually nonexclusive assumption. An example of the surviving fractions of the cells following exposure to the individual agents alone and in combination from a single experiment (in triplicate) is as follows: topotecan (0.1 μg/ml) 24 h, 0.046 ± 0.001; etoposide (0.4 μg/ml) 0.0885 ± 0.007; topotecan 24 h followed by etoposide 24 h, 0.00049 ± 0.00018; etoposide 24 h followed by topotecan 24 h 0.002 ± 0.001

Discussion

These results suggest that cytotoxicity may be greater using etoposide after topotecan than using the opposite sequence of administration. These results should be confirmed in vivo and may play a role in the design of future clinical trials. Recently, Bertrand et al. explored the interaction of camptothecin (another topoisomerase I-reactive compound) and etoposide in HT-29 human colon cancer cells [4]. Although formal mathematical modeling was not employed and the authors merely concluded that sequential treatment (either sequence) resulted in greater cell kill compared to simultaneous treatment, examination of the cell survival data presented suggests greater cytotoxicity for the sequence of camptothecin followed by etoposide compared to the opposite sequence. The study by Kim et al. [12] may suggest a possible mechanistic link for the results of our study as they found that topoisomerase II mRNA is increased 24 h after CPT-11 (another topoisomerase I-reactive compound) exposure. Further work will be necessary to decipher the mechanism of the interaction of the topoisomerase I- and topoisomerase II-reactive agents as well as the clinical ramifications of these results.

The distribution of the CI values with respect to surviving fraction (Fig. 2) deserves further comment. Although these results suggest greater synergy with increasing levels of cytotoxicity for either sequential combination of the two chemotherapeutic agents, the significance of the increasingly antagonistic

relationship (for both sequences of administration) as the level of cytotoxicity is reduced remains unclear. A similar phenomenon has been demonstrated when the median effect principle has been used to analyze the interactions of numerous other chemotherapeutic agents, and it is possible that these combinations result in antagonism at lower levels of cytotoxicity or, alternatively, this consistent finding may represent an inability of the model to adequately characterize the lower levels of cytotoxicity [2, 3, 14]. It is of note that antagonism was seen at low levels of cytotoxicity even for median effect experiments in which all concentrations of both agents given alone resulted in less than 50% cytotoxicity. However, the magnitude of the antagonism was reduced when just this portion of the curve was examined (data not shown). The complete interpretation of this type of mathematical modeling with respect to the level of cytotoxicity will require a better understanding of the mechanism of interaction of the specific agents under investigation. However, this study does show that the CI values for the sequence of topotecan followed by etoposide are reduced compared to the opposite sequence of administration at all levels of cytotoxicity.

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References

1. Abigerges D, Armand J-P, Chabot GG, et al (1994) Irinotecan (CPT-11) high-dose escalation using intensive high-dose loperamide to control diarrhea. J Natl Cancer Inst 86(6):446–449
2. Ackland SP, Schilsky RL, Beckett MA, Weichselbaum RR (1988) Synergistic cytotoxicity and DNA strand break formation by bromodeoxyuridine and bleomycin in human tumor cells. Cancer Res 48:4244–4249
3. Anzai H, Frost P, Abbruzzese JL (1992) Synergistic cytotoxicity with 2'-deoxy-5-azacytidine and topotecan in vitro and in vivo. Cancer Res 52:2180–2185
4. Bertrand R, O'Conner PM, Kerrigan D, Pommier Y (1992) Sequential administration of camptothecin and etoposide circumvents the antagonistic cytotoxicity of simultaneous drug administration in slowly growing human colon carcinoma HT-29 cells. Eur J Cancer 28A(4/5):743–748
5. Bonner JA, Liengswangwong V (1994) Topoisomerase II-independent doxorubicin-induced cytotoxicity in an extremely doxorubicin-resistant cell line. Biochem Biophys Res Commun 198(2):582–589
6. Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27–55
7. D'Arpa P, Liu LF (1989) Topoisomerase-targeting antitumor drugs. Biochim Biophys Acta 989:163–177
8. Eckardt JR, Burris HA, Von Hoff DD, et al (1994) Measurement of tumor topoisomerase I and II levels during the sequential administration of topotecan and etoposide. J Clin Oncol 13:141
9. Hochster H, Liebes L, Speyer J, Sorich J, Taubes B, Oratz R, Wernz J, Chachoua A, Raphael B, Vinci RZ, et al (1994) Phase I trial of low-dose continuous topotecan infusion in patients with cancer: an active and well-tolerated regimen. J Clin Oncol 12(3):553–559
10. Karato A, Sasaki Y, Shinkai T, Eguchi K, Tamura T, Ohe Y, Oshita F, Nishio M, Kunikane H, Arioka H, et al (1993) Phase I study of CPT-11 and etoposide in patients with refractory solid tumors. J Clin Oncol 11:2030–2035
11. Kaufmann SH (1991) Antagonism between camptothecin and topoisomerase II-directed chemotherapeutic ages in a human leukemia cell line. Cancer Res 51:1129–1136
12. Kim R, Hirabayashi N, Nishiyama M, Jinushi K, Toge T, Okada K (1992) Experimental studies on biochemical modulation targeting topoisomerase I and II in human tumor xenografts in nude mice. Int J Cancer 50:760–766
13. Pratt CB, Stewart C, Santana VM, Bowman L, Furman W, Ochs J, Marina N, Kuttesch JF, Heideman R, Sandlund JT, et al (1994) Phase I study of topotecan for pediatric patients with malignant solid tumors. J Clin Oncol 12(3):539–543
14. Ratain MJ, Schilsky RL, Wojack BR, Simon T, Senekjian EK, Vogelzang NJ (1988) Hydroxyurea and etoposide: in vitro synergy and phase I clinical trials. J Natl Cancer Inst 80:1412–1416
15. Slichenmyer WJ, Rowinsky EK, Donehower RC, Kaufmann SH (1993) The current status of camptothecin analogues as antitumor agents. J Natl Cancer Inst 85(4):271–291
16. Tanizawa A, Fujimorei A, Fujimorei Y (1994) Comparison of topoisomerase I inhibition, DNA damage, and cytotoxicity of camptothecin derivatives presently in clinical trials. J Natl Cancer Inst 86(11):836–842
17. Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF (1984) Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 226:466–468