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Sequential Administration of Camptothecin and Etoposide Circumvents the Antagonistic Cytotoxicity of Simultaneous Drug Administration in Slowly Growing Human Colon Carcinoma HT-29 Cells

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We compared the cytotoxicity of simultaneous and sequential combination chemotherapy with camptothecin and etoposide, in slowly growing human colon carcinoma, HT-29 cells. Simultaneous treatments of HT-29 cells with etoposide and camptothecin produced no marked enhancement of cytotoxicity over single agent administration. This finding demonstrates antagonism of one drug's cytotoxicity over the other. When these studies were repeated in sequential treatment protocols, we observed that antagonism could be circumvented if the period between individual drug administration was separated by 6–8 h. The cytotoxicity that was observed with this approach was never more than additive and the order of camptothecin or etoposide administration did not significantly affect the extent of combined cytotoxicity observed. The protective effect of simultaneous camptothecin and etoposide exposure was not due to reduced formation or alterations in the rate of cleavable complex reversal, and protection persisted for a considerably longer period of time than DNA strand breaks. Protection correlated with the kinetics of DNA and RNA synthesis inhibition produced by either drug. Remarkably, full cytotoxic protection could be afforded by one drug over the other, in the presence of only partial inhibition of DNA or RNA synthesis (50–60%). Our findings suggest that sequential rather than simultaneous administration of topoisomerase I and II inhibitors in future cancer chemotherapy schedules will enhance cytotoxicity over single-agent administration.
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INTRODUCTION

DNA TOPOISOMERASE I and II are essential for DNA replication and recombination, RNA transcription and probably DNA repair processes [1]. It has been reported that topoisomerase II can compensate for some, if not all of the functions of topoisomerase I, but the converse has not readily been observed [2, 3]. The antitumour actions of topoisomerase I and II inhibitors is believed to result from the stabilisation of a prelethal transient covalent intermediate between DNA and either topoisomerase I or II [4–6]. The interaction of this stabilised complex with other cellular processes such as DNA replication [7, 8] or RNA transcription [9, 10], seems then essential for cell killing. Drug-induced stabilisation of covalent intermediate between DNA and topoisomerases can be detected in mammalian cells as protein-linked DNA double-strand breaks (topoisomerase II) or DNA single-strand breaks (topoisomerases I and II) [4–6].

Previous studies have reported that the development of cellular resistance to topoisomerase II inhibitors conferred an increased susceptibility to topoisomerase I poisons [11, 12]. The reverse situation, in which resistance developed to camptothecin (a specific inhibitor of topoisomerase I), confers enhanced sensitivity to topoisomerase II inhibitors, has also been reported [13]. The mechanism responsible for this enhanced sensitivity may be related to the compensatory role of one topoisomerase enzyme for the other [11–13]. It follows therefore, that during the period of acute inhibition of one class of topoisomerase, the other class may at least partially compensate for some of its actions in DNA metabolism. One might therefore, expect that simultaneous administration of both topoisomerase I and II inhibitors would increase cytotoxicity over that observed for single agent administration. These possibilities are however in contrast to recent reports [10, 14], that suggest that when topoisomerase I and II inhibitors are administered simultaneously to rapidly proliferating Chinese hamster ovary or human promyelocytic cells, antagonism rather than synergism ensued. These recent findings are also in contrast to combination chemotherapy of topoisomerase inhibitors with other classes of DNA damaging agents, for which a more favourable synergistic cytotoxicity has been reported over single-agent administration [15–18].

In the present study we compared the cytotoxicity of simultaneous and sequential combination chemotherapy with the topoisomerase I inhibitor, camptothecin and the topoisomerase II inhibitor, etoposide, to determine whether, the antagonism reported recently between these two agents in rapidly proliferating cell lines, would also apply to more slowly growing human colon carcinoma, HT-29 cells, and if so, whether, antagonism might be circumvented by sequential administration of these individual drugs in a carefully designed administration schedule.

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MATERIALS AND METHODS

Chemicals

Etoposide (Bristol-Myers) and 20-S-camptothecin (Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute,Bethesda) were dissolved in dimethylsulphoxide (at 10 mmol/l) prior to each experiment. [2-^{14}C]thymidine (1.98 GBq/mmol), [methyl-^{3}H]thymidine (2.99 TBq/mmol) and [5-^{3}H]uridine (1.05 TBq/mmol) were purchased from New England Nuclear. All other chemicals were of reagent grade and purchased either from Sigma or from other local sources.

Cell culture and drug treatment

Human colon carcinoma HT-29 cells were obtained from American Type Cell Culture (Rockville) and grown at 37°C in the presence of 5% CO_2 in Eagle's minimum essential medium (MEM) (ABI, Columbia) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 2 mmol/l glutamine, 1 mmol/l sodium pyruvate, 0.1 mmol/l MEM non-essential aminoacids, 100 U penicillin/ml and 100 ug streptomycin/ml (ABI). The doubling-time of HT-29 cells was 40–44 h.

Prior to all experiments (24 h before), exponentially growing cells were plated at 4×10^5 cells/25 cm^2 flasks. All drug treatment were for 30 min. Sequential treatments were performed at time intervals between the two drug treatments ranging from 0 to 18 h.

Colony formation assay

Following drug-treatment, HT-29 cells were washed twice with 10 ml of complete medium (37°C), trypsinised and 10^2, 10^3 and 10^4 cells were plated in triplicate in 25 cm^2 flasks with 5 ml of fresh medium. Colonies were grown for 14 days. Culture flasks were then washed with ice-cold phosphate-buffered saline (PBS), fixed with methanol (95%) and stained with methylene blue (0.04%) [19]. Results were expressed as log of survival fraction which were calculated by dividing the number of colonies in the treated flasks by the number of colonies in control flasks. Plating efficiencies for control cells ranged from 40 to 60%.

Measurement of DNA single-strand breaks by alkaline elution

HT-29 cells (1×10^5 cells/25 cm^2 flask) were labeled with [^{14}C]thymidine (1.11 kBq/ml) for 48 h and then chased for an additional 24 h in isotope-free medium prior to all drug treatments. At specified times following drug treatment or drug removal, cells were scraped into their culture medium, mixed with internal standard (an aliquot of [^{3}H]-labelled L1210 cells subjected to a fixed dose of gamma radiation [2000 rads]) and assayed by alkaline elution for DNA single-strand breaks under deproteinising conditions [19–21]. Briefly, cells were loaded onto polycarbonate filters (2 μmol/l pore size, 25 mm diameter, Nucleopore), lysed with 5 ml of 2% (w/v) sodium dodecyl sulphate (SDS) containing 0.5 mg/ml proteinase K at pH 10.0. Filters were then washed with 10 ml of 0.02 mol/l EDTA (pH 10.0) and the DNA eluted with a tetrapropylammoniumhydroxide/EDTA solution pH 12.1) containing 0.1% (w/v) SDS at a flow rate of 0.08–0.12 ml/min (5 min fractions). At the end of the elution, filters were processed as described previously [21]. DNA single-strand break frequencies were expressed in rad-equivalents using, as calibrators, irradiated and control HT-29 cells assayed under the same conditions [21]. 1000 rad-equivalents correspond to approximately one break per 10^6 nucleotides.

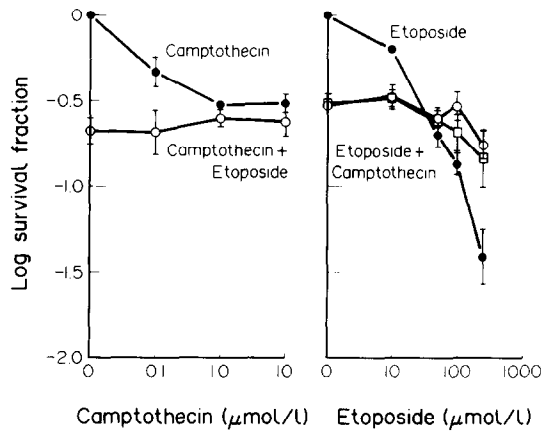

Fig. 1. Cytotoxicity of simultaneous treatments with camptothecin and etoposide in HT-29 cells. HT-29 cells were treated for 30 min with various camptothecin concentrations (left panel) either alone (●) or in the presence of 50 μmol/l etoposide (○).Similarly, HT-29 cells were treated for 30 min with various etoposide concentrations (right panel) either alone (●) or in the presence of 1 μmol/l (○) and 10 μmol/l (□) camptothecin. Cytotoxicity was measured by colony formation assays. Points and vertical bars represent the means (S.E.) of three independent experiments performed in triplicate.

Measurements of thymidine and uridine incorporation

HT-29 cells were prelabelled with [^{14}C]-thymidine (0.185 kBq/ml) for 24 h and then chased for an additional 16 h in isotope-free medium. Cells were then incubated in drug containing medium for 30 min (either 1 μmol/l camptothecin or 50 μmol/l etoposide). After drug treatment, cells were washed twice with complete medium and further incubated for the specified times after drug removal. Rates of nucleotide incorporation were measured by 10 min pulse experiments with [^{3}H]thymidine (1.37 kBq/ml) or [^{3}H]uridine (370 kBq/ml) [7]. Nucleotide incorporation was stopped by removing the isotope-containing medium and by adding 10 ml of ice-cold PBS. Cell cultures were then quickly scraped on ice, pelleted by centrifugation (500 *g*, 5 min, 4°C) and washed twice with ice-cold PBS. Acid-insoluble nucleotides were precipitated on ice with 10% trichloroacetic acid.The precipitates were dissolved in 0.4 N NaOH and radioactivity was monitored by scintillation spectrometry. Results were expressed as the ratio of [^{3}H]/[^{14}C] for treated cells over the ratio of [^{3}H]/[^{14}C] of untreated cells [7].

RESULTS

The present study compared the cytotoxicity of simultaneous and sequential combination chemotherapy with the topoisomerase I inhibitor, camptothecin, and the topoisomerase II inhibitor, etoposide, in slowing growing human colon carcinoma, HT-29 cells, in order to determine whether the recently reported antagonism between these two agents might be circumvented by sequential administration of these drugs in a carefully designed administration schedule.

Single-agent induced cytotoxicity in HT-29 cells

Brief exposure (30 min) of slowly growing human colon carcinoma, HT-29 cells to camptothecin, produced a dose-dependent increase in cell killing up to doses of 1 μmol (Fig. 1). Maximum cytotoxicity was achieved with camptothecin concentrations of 1 μmol/l and cytotoxicity was not increased further by exposing HT-29 cells to higher concentrations of camptothecin

(10 μmol/l). This cytotoxic plateau for brief camptothecin-exposure (30 min) is consistent with previous studies, and probably reflects saturable DNA lesion production [22]. In contrast to brief camptothecin exposures, but consistent with results obtained with other chemotherapeutic agents [19, 23], 30 min treatment of HT-29 cells with etoposide killed cells proportionally to the logarithm of etoposide concentration employed (Fig. 1). The profound difference in the relationship between drug-exposure and cell killing by either topoisomerase I or II poisons, indicated that the mechanism of cell killing by these two classes of drugs is quite different [7]. Since independent mechanism of action is one of the criteria used in the selection of chemotherapeutic agents for combination chemotherapy protocols, one might expect that combined exposure of HT-29 cells to camptothecin and etoposide would enhance chemotherapeutic response. To explore this possibility in slowly growing human colon carcinoma, HT-29 cells, we compared combination chemotherapy administered both in simultaneous and sequential drug-exposure protocols.

Cytotoxicity of simultaneous camptothecin and etoposide exposure in HT-29 cells

The cytotoxicity of simultaneous treatment of HT-29 cells with camptothecin and etoposide was investigated by two different approaches. In the first, HT-29 cells were treated for 30 min with a fixed concentration of etoposide (50 μmol/l), while increasing camptothecin concentration from 0 to 10 μmol/l (Fig. 1, left panel). In this case, etoposide treatment alone killed approximately 80% of exponentially growing cells. When simultaneous combined treatments of etoposide and camptothecin were employed however, we observed no marked enhancement of etoposide-induced cytotoxicity in HT-29 cells. In the second series of experiments, HT-29 cells were first treated with a fixed concentration of camptothecin for 30 min followed by increasing etoposide concentrations ranging from 0 to 250 μmol/l (Fig. 1, right panel). At concentrations below 50 μmol/l etoposide, the cytotoxicity of combined treatments was that of camptothecin alone (non additive). However, at 100 and 200 μmol/l etoposide, the cytotoxicity of combined treatments was markedly less than that of etoposide alone. Similar results were obtained when HT-29 cells were pre-treated with a much higher dose of camptothecin (10 μmol/l) (Fig. 1, right panel). This however, possibly reflects the cytotoxic-plateau effect observed for brief camptothecin-exposure in HT-29 cells, that commences at doses in excess of 1 μmol/l camptothecin. Nonetheless, these results demonstrate that simultaneous exposure of slowly growing human colon carcinoma, HT-29 cells to topoisomerase I and II poisons is antagonistic *in vitro*, and our findings predict that similar clinical dose-schedules would be unfavourable for future investigation.

Cytotoxicity of sequential camptothecin and etoposide exposure in HT-29 cells

To determine whether combination chemotherapy with topoisomerase I and II inhibitors might be improved by sequential drug administration, we investigated the time-dependence schedule for the antagonism observed between camptothecin and etoposide. For this purpose, we treated HT-29 cells initially with camptothecin (1 μmol/l) for 30 min and then with etoposide (50 μmol/l, 30 min) administered at various times after camptothecin removal. Figure 2 shows that camptothecin pretreatment of HT-29 cells completely protected them against etoposide-induced cytotoxicity for up to 2 h following camptothecin

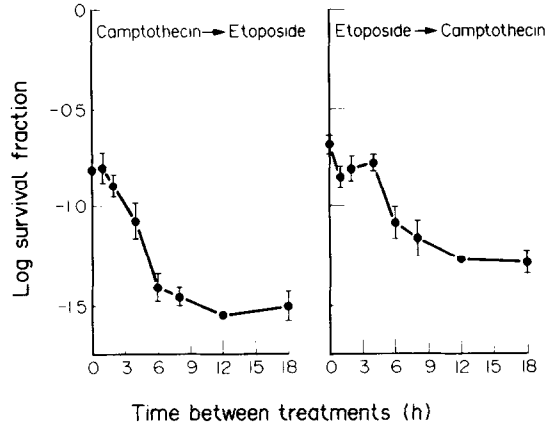

Fig. 2. Cytotoxicity of sequential treatments with camptothecin and etoposide on HT-29 cells survival. HT-29 cells were first treated for 30 min with camptothecin (1 μmol/l) (left panel) followed by 50 μmol/l etoposide for 30 min at the identicated times after camptothecin removal. Similarly, cells were treated for 30 min with etoposide (50 μmol/l) (right panel) followed by 1 μmol/l camptothecin for 30 min at the indicated times after etoposide removal. At the end of the second treatment, drug was washed away and cytotoxicity was measured by colony formation assays. Points and vertical bars represent the means (S.E.) of two (left panel) and three (right panel) independent experiments performed in triplicate (left panel).

removal. Thereafter, however, the cytotoxicity of etoposide increased progressively with time, such that etoposide and camptothecin induced cytotoxicity became approximately additive by 6–8 hours after camptothecin removal. Additivity persisted for 18 h after camptothecin withdrawal without any apparent synergism. Figure 2 also shows that etoposide (50 μmol/l) pre-treatment of HT-29 cells also protected against camptothecin-induced cytotoxicity for up to 4 h after removal of etoposide. Thereafter, camptothecin-induced cytotoxicity became apparent, also reaching additive cytotoxicity by 8 h after etoposide removal. These results indicate, that in slowly growing human colon carcinoma HT-29 cells, the antagonism of simultaneous treatment with topoisomerase I and II inhibitors can be circumvented by sequential drug treatment. This can be achieved *in vitro* by spacing drug treatments by at least 6 h apart from one-another, and presumably these results can be repeated *in vivo* by separating drug-administration based on pharmacokinetics considerations for the clearance of each drug. Interestingly, no apparent synergy was detectable for this drug combination in HT-29 cells, within the first 18 h of administration. Furthermore, the order of topoisomerase I or II administration did not seem to significantly affect the additive effect of combined cytotoxicity observed for these agents.

Kinetics of camptothecin- and etoposide-induced DNA single-strand breaks in HT-29 cells

To determine whether the antagonistic activity of simultaneous treatment with camptothecin and etoposide in HT-29 cells was due to an alteration in DNA lesion production by either drug, we measured the kinetics of DNA single-strand break production in cells treated with camptothecin and etoposide by alkaline elution methodology. Figure 3 shows that independent of pre-treatment with 50 μmol/l etoposide for 30 min, camptothecin induced approximately 1600 rad-equivalents of DNA single-strand breaks (approximately one DNA break per 7×10^5 nucleotides) in HT-29 cells. Furthermore,

camptothecin-induced DNA single-strand breaks completely resealed within approximately 1 h after removal of camptothecin from the culture medium, regardless of etoposide pre-treatment. These findings indicate that the protective effect of etoposide pre-treatment against camptothecin-induced cytotoxicity is not explainable on the basis of quantitative alterations in the kinetics of camptothecin-induced DNA single-strand breaks.

When these studies were repeated in camptothecin (1 μmol/l) pretreated HT-29 cells, we found a small but non-significant decrease in the mean level of etoposide induced DNA breaks compared with that observed with etoposide administered alone (Fig. 3). Furthermore, the protective effect of camptothecin pretreatment against etoposide-induced cytotoxicity was not due to modulation in the kinetics of etoposide-induced DNA single-strand break reversal since the rate of etoposide induced DNA single-strand breaks was similar or even slower to that observed in cells treated with etoposide alone.

Consistent with our previous studies in camptothecin-treated HT-29 cells [22], camptothecin- and etoposide-induced DNA single-strand breaks completely reversed within 1 h of drug removal. Since the protective-effect of one topoisomerase inhibitor over the cytotoxicity of the other, persisted for several hours after drug removal, it is highly unlikely that the protective-mechanism is due directly to topoisomerase I or II inhibition. Furthermore, our results provide a clear demonstration that protein-linked DNA single-strand breaks are in themselves only potentially lethal lesions, and that other cellular processes are essential for translating DNA breaks into cell killing by topoisomerase inhibitors [6].

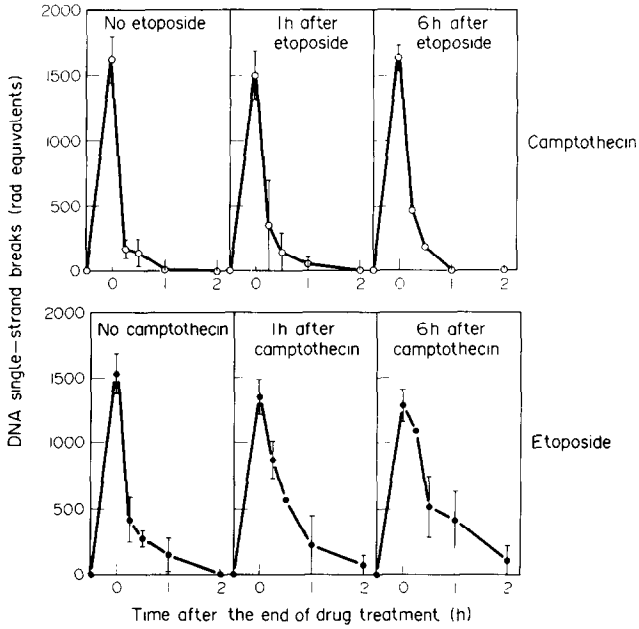

Fig. 3. Kinetics of camptothecin- and etoposide-induced DNA single-strand breaks at various time intervals between drug treatments in HT-29 cells. In the upper panel, HT-29 cells were treated with 1 μmol/l camptothecin (30 min) and single-strand breaks were assayed by alkaline elution at the indicated times after the end of drug treatment. Secondly, camptothecin-induced DNA single-strand breaks were measured in HT-29 cells that have been pretreated with etoposide (50 μmol/l) either 1 or 6 h before camptothecin exposure. In the lower panel, HT-29 cells were treated with 50 μmol/l etoposide (30 min) and single-strand breaks assayed by alkaline elution at the indicated times. Secondly, etoposide-induced DNA single-strand breaks were then measured in cells that have been pretreated with camptothecin (1 μmol/l) either 1 or 6 h before etoposide exposure. Points and vertical bars represent the means (S.E.) of three independent experiments.

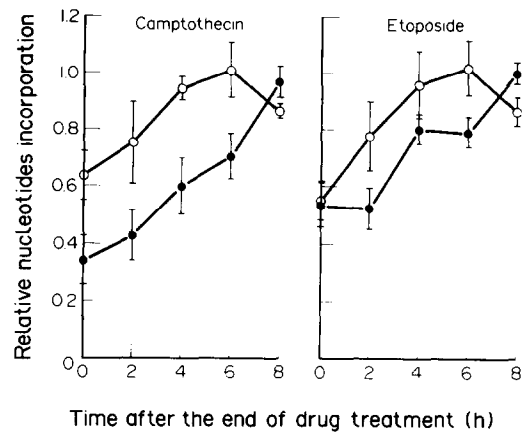

Fig. 4. Kinetics of DNA and RNA synthesis inhibition by camptothecin and etoposide in HT-29 cells. Cells were treated with 1 μmol/l camptothecin (left panel) or 50 μmol/l etoposide(right panel) for 30 min. At indicated times after drugs removal, [^{3}H]thymidine (●) and [^{3}H]uridine (○) incorporation were determined by 10 min pulse experiments. Points and vertical bars represent the means (S.E.) of four independent experiments performed in triplicate.

Kinetics of DNA and RNA synthesis inhibition by camptothecin and etoposide in HT-29 cells

Both DNA replication and RNA transcription have been among the most widely explored processes to be implicated in the cytotoxic mechanism of action of topoisomerase poisons. In order to determine whether, there was a correlation between the effects of camptothecin and etoposide on DNA and RNA synthesis inhibition and the cytotoxic potency of either simultaneous or sequential combination chemotherapy protocol employed, we measured the incorporation of radiolabeled thymidine and uridine into acid-insoluble material in drug-treated HT-29 cells. Figure 4 shows that immediately following camptothecin exposure (1 μmol/l, 30 min), both DNA and RNA synthesis were inhibited to approximately 60% and 40% of the control untreated samples, respectively. Following camptothecin removal, DNA and RNA synthesis returned progressively to control values within an 8 h period of camptothecin removal. Similar inhibitions were also seen in the case of etoposide. For etoposide however, the initial extent of DNA and RNA synthesis inhibition in HT-29 cells was the same. These observations indicate that both camptothecin (1 μmol/l) and etoposide (50 μmol/l) only partially inhibited DNA and RNA synthesis within 30 min of drug treatment of HT-29 cells. Moreover, RNA synthesis recovered to near control levels within 4 h of either camptothecin or etoposide removal, while DNA synthesis inhibition was much more protracted, not returning to control levels until 8 h after drug removal. Furthermore, both RNA and especially DNA synthesis inhibitions lasted much longer than that of drug-induced DNA strand-breaks, which reversed within 1 h of drug washout. The protracted inhibition of replication and transcription processes following camptothecin-induced DNA break reversal, has recently been suggested to ensue from the inability of drug-treated cells to reform competent replication and transcription complexes on damaged DNA strands [24].

DISCUSSION

The present study compared the cytotoxicity of simultaneous and sequential combination chemotherapy with the topoisomerase I inhibitor, camptothecin and the topoisomerase II inhibitor, etoposide to determine whether the antagonism reported recently between these two agents, would also apply to more slowly growing human colon carcinoma, HT-29 cells [10, 14], and if so, whether antagonism might be circumvented by sequential administration of these individual drugs in a carefully designed administration schedule. HT-29 cells are relatively sensitive to camptothecin [22] and resistant to etoposide [19].

The present study clearly demonstrates that simultaneous treatments of slowly growing human colon carcinoma, HT-29 cells with camptothecin and etoposide produced an antagonistic response. These observations are in excellent agreement with recently published studies performed in more rapidly proliferating chinese hamster ovary [10] and human promyelocytic leukaemia [14] cells. These combined observations suggest that antagonism observed with simultaneous topoisomerase I and II poisons is independent of species or tissue origin and growth fraction of drug-treated cells.

In order to investigate this antagonistic behaviour further, the effects of sequential drug treatments were examined in HT-29 cells. Sequential treatments with equitoxic doses, showed that either camptothecin or etoposide could antagonise the cytotoxicity of the other drug, when either agent was administered within 6 h of each other. Moreover, when treatments with either agent were separated by more than 6 h, cytotoxicity became additive. The present study provides new data that shows that the cytotoxic antagonism of simultaneous treatments with camptothecin and etoposide can be circumvented by carefully designed administration schedules. Also, the present observations failed to find any detectable synergism between camptothecin and etoposide within 18 h of drug administration. This finding is contrary to combination chemotherapy protocols in which topoisomerase II poisons and DNA crosslinking agents are administered concurrently [15–18]. The observations made in the present study might therefore, have important implications on the choice of chemotherapeutic agents to be used in combination protocols.

The use of sequential treatments with camptothecin and etoposide made it relatively accessible to carefully follow the kinetics of drug-induced DNA breaks for either agent. By doing so, we found that the mutually protective effect of camptothecin and etoposide was not due to reduced formation of drug-induced DNA breaks or alterations in the rate of cleavable complex reversal.

Reduction of topoisomerase inhibitor induced cytotoxicity without alteration in the extent or kinetics of drug-induced DNA lesions has previously been reported in tumour cells pretreated with aphidicolin [7, 8], cordycepin [9, 10], dinitrophenol [8, 25, 26] and novobiocin [26, 27]. More recently, we have also shown that depletion of intracellular calcium pools abrogates camptothecin and etoposide-induced cytotoxicity without affecting drug-induced DNA single-strand breaks [28]. Our present findings and those of other workers [6] provide a clear demonstration that protein-linked DNA single-strand breaks are pre-lethal DNA lesions, and that other cellular processes are essential for translating DNA breaks into cell killing by topoisomerase inhibitors.

The present studies also noted that both RNA and especially DNA synthesis inhibitions lasted much longer than that of either topoisomerase I or II induced DNA strand breaks, which reversed within 1 h of drug wash-out. This protracted inhibition of replication and transcription processes following DNA break reversal, might indicate a marked inability of drug-treated cells to reform competent replication and transcription complexes on damaged DNA strands. Our present results are consistent with the view that active DNA replication processes participate in cell killing by camptothecin, since the reversal of etoposide-induced DNA synthesis inhibition correlated well with the extent of cytotoxic protection afforded by etoposide treatment. Furthermore, the reversal of etoposide induced DNA synthesis inhibition correlated better with the window of protection against camptothecin-induced cytotoxicity than RNA synthesis. Our findings are also consistent with the view that active transcription may be more important in the cytotoxic mechanism of action of topoisomerase II inhibitors than DNA replication processes. These observations are consistent with our own unpublished observations with aphidicolin and cordycepin in treated cells with topoisomerase II inhibitors and with several other recent reports [7–10]. What seems quite striking from our studies however, is that full cytotoxic protection can be afforded by one drug over the other, in the presence of only partial inhibition of DNA or RNA synthesis (50–60%). This important observation suggests that the antagonistic activity of simultaneous administration of topoisomerase I and II inhibitors might be associated with only a subset of the total DNA and RNA synthesis inhibition produced by these agents.

In summary, the present data suggests that the antagonistic activity of simultaneous combination chemotherapy with topoisomerase I and II inhibitors in slowly growing human colon carcinoma, HT-29 cells can be achieved in the presence of only partial inhibition of DNA or RNA synthesis by either camptothecin or etoposide. Furthermore, the antagonistic activity of simultaneous combination chemotherapy can be circumvented, provided camptothecin and etoposide are administered in a sequential manner, 6–8 h apart. A sequential topoisomerase I and II administration protocol provided an additive rather than a synergistic enhancement of cytotoxicity in HT-29 cells. These findings suggest that sequential administration of topoisomerase I and II inhibitors in future cancer chemotherapy schedules might enhance the cytotoxic response of single-agent administration in relatively camptothecin sensitive tumours, provided the drugs are separated according to individual pharmacokinetics considerations.

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