Pergamon

European Journal of Cancer, Vol. 35, No. 6, pp. 984–993, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(99)00018-0

Original Paper

Characterisation of a Synergistic Interaction Between a Thymidylate Synthase Inhibitor, ZD1694, and a Novel Lipophilic Topoisomerase I Inhibitor Karenitecin, BNP1100: Mechanisms and Clinical Implications*

S.-i. Matsui,[1] W. Endo,[1] C. Wrzosek,[1] K. Haridas,[2] P. Seetharamulu,[2] F.H. Hausheer[2] and Y.M. Rustum[1]

[1]Department of Pharmacology and Therapeutics, Grace Cancer Drug Center, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, New York 14263; and [2]BioNumerik Pharmaceuticals, Inc., San Antonio, Texas, U.S.A.

We developed a combination protocol for inhibitors of thymidylate synthase (TS) and DNA topoisomerase I (Topo I) that can exert highly lethal effects *in vitro* against HCT-8 human colorectal cancer cells. The specific schedule was constructed so that a TS inhibitor could induce not only primary DNA damage but also cellular conditions optimal for the efficient action of a Topo I inhibitor. The initial drug treatment consisted of a brief exposure to a quinazoline-based antifolate, ZD1694. After an interval of approximately one cell-doubling time, cells were exposed for 8–24 h to BNP1100, a Karenitecin-class 7-thiomethyl-camptothecin, in the presence of 1–10 μM thymidine; the latter acted as a crucial factor to promote the collision of moving replication forks with the drug-stabilised DNA–Topo I cleavable complexes even under continuous TS inhibition. Clonogenic analyses confirmed that these mechanistically distinct drugs at clinically achievable concentrations worked in a highly synergistic manner, with a maximum effect abolishing the viability of virtually all cancer cells (> 99.9%). The pretreatment with ZD1694 increased the amount of DNA-bound Topo I by up to 4-fold and the DNA-damaging capability of BNP1100 by up to 15-fold. The possibility of at least four DNA-damaging pathways is proposed which might have resulted from the individual actions of TS and Topo I inhibitors as well as their concerted actions. Taken together, the present findings provided a logically permissible explanation as to why TS and Topo I inhibitors in concerted interactions induced a highly lethal effect which was more than a simple additive effect. Since these drugs are effective specifically on actively proliferating cancer cells, but not on non-cycling G_0/G_1 cells, this mechanism-based protocol may warrant consideration for clinical verification. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: ZD1694, thymidylate synthase inhibitor, BNP1100, topoisomerase I inhibitor, drug synergism, clonogenicity, DNA damage, colorectal cancer
Eur J Cancer, Vol. 35, No. 6, pp. 984–993, 1999

INTRODUCTION

To date, 5-fluorouracil/leucovorin (5-FU/LV)-based regimens have been the mainstay of first-line chemotherapy in the treatment of advanced colorectal cancers [1–3]. Several research groups previously attempted to enhance the efficacy of 5-FU by combining it with cisplatin (CDDP), *N*-(phosphonoacetyl)-*L*-aspartic acid (PALA), methotrexate, interferon, etc. [4–7], but follow-up studies seem to have failed to demonstrate any compelling advantages of these strategies over 5-FU/LV [1]. Although a quinazoline-based antifolate, ZD1694 (*N*-[5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl]-L-glutamic acid), has also emerged as one of the most promising stoichiometric

*Presented in parts at the Annual Meetings of the American Association for Cancer Research, New Orleans, Louisiana, 28 March–1 April 1998 and the American Society of Clinical Oncology, Los Angeles, California, U.S.A. 16–19 May 1998.

Correspondence to S.-i. Matsui.

Received 4 Sep. 1998; revised 16 Dec. 1998; accepted 18 Dec. 1998.

thymidylate synthase (TS) inhibitors with less adverse side-effects on patients [8–14], its response rate in clinical phase II–III trials, as with the 5-FU/LV regimen, is under 26% [11–14]. Because of this, the development of a novel scheme for combining TS inhibitors and other drugs has been urged [13].

DNA topoisomerase I (Topo I) inhibitors have recently entered clinical trials as novel agents to induce lethal DNA damage in cancer cells using a mechanism distinct from that of TS inhibitors [15, 16]. Among camptothecin (CPT)-derivatives, CPT-11 (irinotecan; 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin) has already been registered in several countries as second-line treatment in colorectal carcinoma [17–22]. Cytotoxic effects have been thought to occur when a re-ligation enzyme reaction of nicked DNA strands is blocked by the inhibitor during DNA replication and transcription, converting the repairable single-strand breaks into lethal double-strand breaks [15, 23]. These inhibitors seem to have salient therapeutic values. For example, some of the CPT analogues, including Karenitecin-class Topo I inhibitors, are not a target of multiple drug resistance (MDR) proteins such as PGP170 and MRP [24]. Further, these drugs do not significantly affect cells in G_0 or G_1 [25], implying a minimum toxicity to normal tissues in a non-proliferating state *in vivo*. Despite these obvious advantages, drugs such as CPT-11 and topotecan, used as single agents, have not demonstrated a curative efficacy; the reported clinical response rates vary from 10 to 30% in colorectal cancers including 5-FU- and CDDP-refractory tumours [26].

To develop a protocol that can augment the efficacy of TS and Topo I inhibitors, we have attempted to elicit their synergistic actions based on the following basic biochemical properties: (1) Topo I inhibitors at clinically relevant concentrations are effective specifically during DNA replication in S phase [25]; (2) cancer cells exposed to ZD1694 undergo DNA strand breaks and S phase arrest due to a sustained apyrimidic state [27, 28]; (3) DNA replication forks, if arrested due to the absence of *de novo* thymidylate synthesis following ZD1694 treatment, can resume their movement when cells are supplied with exogenous thymidine (dThd) [27]. These findings led us to advance the following rationale: when 'ZD1694-primed' cells receive exogenous dThd along with Topo I inhibitors, DNA replication forks would be forced to move even under TS inhibition and collide with drug-stabilised cleavable complexes [15, 23], resulting in non-repairable lethal DNA double strand breaks. Since Topo I can be trapped at DNA nicks or gaps [29, 30], we also reasoned that DNA lesions induced by TS inhibition might provide *de novo* binding sites for Topo I, which would in turn increase the possibility for generating additional lethal DNA damage in the presence of Topo I inhibitors.

This paper describes a specific protocol constructed exclusively on the basis of the above rationale, which has proved to virtually abolish the clonogenicity of colorectal cancer cells *in vitro*. The effect achieved was due to a concerted interaction of TS and Topo I inhibitors which could synergistically induce a high level of DNA double-strand damage probably through several pathways. As a model Topo I inhibitor, we used Karenitecin BNP1100 (7-thiomethyl-camptothecin) (Figure 1), because this novel lipophilic C-7 modified CPT analogue has demonstrated superior antitumour cytotoxicities over CPT and SN-38 (7-ethyl-10-hydroxy-camptothecin) and is not a substrate for MRP and PGP170 [24].

MATERIALS AND METHODS

Drugs

ZD1694 ('Tomudex' or 'Raltitrexed': Zeneca Pharmaceutical Co., U.K.) was prepared as a stock solution in 0.1 M sodium bicarbonate, pH 8.3 [27] and stored at $-100°C$. A novel Topo I inhibitor, BNP1100 synthesised by BioNumerik Pharmaceuticals Inc. (San Antonio, Texas, U.S.A.) (Figure 1), was dissolved in spectrograde dimethylsulphoxide and stored in the dark at $-20°C$ without potency loss for at least 1 year. The drugs were diluted with culture medium immediately before use.

Cells

A human colon carcinoma cell line, HCT-8, was maintained as a monolayer with two passages per week at 37°C under dThd-deficient condition in RPMI 1640 medium supplemented with 10% dialysed horse serum [27]. The doubling time of HCT-8 cells during a logarithmically growing phase was approximately 18 ± 3 h [27]. To prepare the cells resting in G_0/G_1, cells were cultured for 24 h in serum-free medium after cell density reached semiconfluency. This method produced more than 95% of cells in G_0/G_1, as confirmed by flow cytometry and analysis of the rate of [^{3}H]-dThd incorporation into DNA (< 4% of untreated logarithmically growing population). For early S phase synchronisation, metaphase cells were prepared from log phase cultures by mitotic selection with colcemid [31, 32] and cultured for 12 h in the presence of 3 mM dThd for G_1/S arrest, as confirmed by flow cytometry. These cells were then allowed to progress through S phase for 2 h in dThd-free medium before the beginning of drug treatments. The plating efficiency of such populations was approximately 85% of untreated logarithmically growing cells.

*A protocol of sequential application with ZD*1694 *and BNP*1100

Since the level of DNA damage by ZD1684 is maximal when all the cells progress through S phase [27], it is crucial to allow TS inhibition to last for more than one cell-doubling time prior to treatment with a Topo I inhibitor. Accordingly, the basic protocol developed for HCT-8 cells using ZD1694 and BNP1100 was as follows: (1) cells were first treated for 2 h with 0.03–1 μM ZD1694; (2) at 24 h post-treatment, when DNA replication was arrested due to thymidylate depletion [27], cells were exposed for 24 h to 0.5–10.9 nM BNP1100 in the presence of 10 μM dThd.

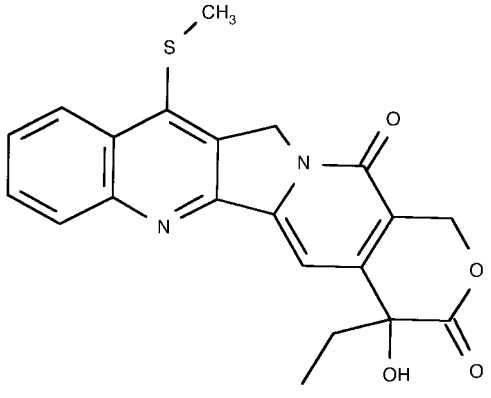

Figure 1. Structure of BNP1100 (7-thiomethyl-camptothecin).

Analysis of Topo I

As an enzyme source, non-histone protein nuclear fractions were prepared from HCT-8 cells according to the procedure established previously [32]. The conversion of supercoiled plasmid pHOT1 DNA into its relaxed form by serially diluted enzymes was carried out for 30 min at 37°C in a 20 μl reaction mixture (10 mM Tris–HCl, pH 7.9, 1 mM ethylene diamine tetra-acetic acid (EDTA), 150 mM NaCl, 0.1% bovine serum albumin, 0.1 mM spermidine, 5% glycerol), and the end products analysed by 1% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) [33].

Flow cytometric analysis

Cells were trypsinised, permealised with 0.37% NP-40, stained with propidium iodide (50 μg/ml in 0.1% sodium citrate, 0.02 g/ml RNase A, 0.37% NP-40) and analysed using a FACScan-FL2 fluorescence-activated cell sorter (Becton Dickenson, San Jose, California, U.S.A.) [27].

Analysis of DNA double-strand fragments and DNA–protein cross-links

Cells, prelabelled for 24 h with 0.2 μCi/ml of [^{3}H]-deoxycytidine, were treated with the drugs, embedded in 25 μl agarose plugs [33] and digested with proteinase K (1.2 mg/ml, 24 h), followed by incubation with RNase A (100 μg/ml, 2 h). After 2% agarose gel electrophoresis for 6 h in TAE buffer, the DNA fragments migrating through the gel were located by ethidium bromide staining, cut out and the radioactivity determined [33, 34]. To evaluate the enhancing effect of ZD1694 pretreatment on the level of DNA damage inducible by a subsequent treatment with BNP1100, we introduced an arbitrary enhancement index (EI) which was calculated by dividing the difference between the amounts of DNA damage with drug combinations and ZD1694 alone by the amount of DNA damage with BNP1100 alone. An EI of 1.0 or less was taken as indicating the absence of an enhancing effect. The relative amount of Topo I–DNA cleavable complexes was determined according to the method described by Zwelling and colleagues [35], except that BNP1100 replaced CPT as the cross-linking agent. By titration, 1 μM of BNP1100 was found sufficient to obtain the maximum values of cross-links.

Assays of growth inhibition and clonogenicity

After 400 cells/well in 96-well plates were cultured for 24 h, the drug treatment was initiated according to the proposed schedule as described above. Following 72 h culture in drug-free media, the growth rate was determined by the standard colorimetric sulphorhodamine B (SRB) assay [36]. For clonogenic assays, cells were drug treated according to the proposed schedule as described above, trypsinised and dispensed at 1000 cells/well (100 mm^2), each well containing 4 ml of fresh medium with 10 μM dThd. Colonies comprising more than around 60 cells, formed after 6 days in culture (equivalent to eight cell-doubling times), were stained with Giemsa and counted on a computerised video image analyser.

Isobole analysis

In the proposed sequential application of ZD1694 and BNP1100, the time interval between the two drug treatments was expected to play a crucial role in the induction of synergy. Previously, Steel and Peckham provided the logical basis so that the concept of 'envelope of additivity' could be applied to the two drugs that were used sequentially or even with one time interval [37]. Thus, it was possible to apply their method as a means of analysing the proposed sequential use of ZD1694 and BNP1100. The apparent IC_{50} and IC_{90} values for ZD1694 were 0.1 and 1 μM, respectively. The IC_{50} and IC_{90} values for BNP1100 were 3.7 and 10.9 nM, respectively. For a detailed plotting of experimental clonogenic values, two independent isobolograms based on both IC_{50} and IC_{90} values of drug combinations were constructed from the assays using two completely separate sets of quadruplicate plates. The obtained data were used to draw two isoeffect curves (modes I and II) by non-linear regression using a Sigmaplot computer program and the total area enclosed by these lines represented an 'envelope of additivity' [37]. When the IC_{50} or IC_{90} values of a given drug combination lay in the area outside of this envelope, the effect was interpreted as 'synergistic (superadditive)' or 'antagonistic (protective)' [37].

Analysis of cells surviving the drug treatment

Individual colonies formed after sequential treatment with ZD1694 and BNP1100, both given at IC_{90}, were isolated using cloning rings (PGC Scientific Corp. Frederick, Maryland, U.S.A.). To examine whether or not the survival of these colonies could be accounted for by a difference in their response to the schedules and drug doses used, cells prepared from each colony were seeded in 96-well plates in duplicates and then rechallenged by a serial dilution of either ZD1694 (0–5.0 μM) or BNP1100 (0–100 nM). After 72–120 h culture, the growth rate was colorimetrically determined using the standard SRB assay as described above [36]. Both IC_{50} and IC_{90} values were determined from the dose–response curves plotted. To examine whether the survival of cells after the initial sequential treatment with 1 μM ZD1694/10.9 nM BNP1100 was due to their stable drug-resistance property, 1.3×10^5 cells derived from the individual colonies formed were challenged by another round of sequential ZD1694/BNP1100 treatment and their clonogenic activities determined as described above. To also determine whether the initial survival was due to a fortuitous escape from the drug actions or newly acquired stable drug resistance, these cells were also cultured continuously for 14 days in the presence of either 1 μM ZD1694 or 10.9 nM BNP1100. To analyse chromosomes, standard cytological preparations were prepared, stained with Giemsa banding and observed with a Nikon microscope [38].

RESULTS

Topo I status in ZD1694-treated cells

It is known that many colorectal cancers express an elevated level of Topo I. This is an obvious advantage for the proposed protocol using their specific inhibitors. To elicit the concerted actions of ZD1694 and BNP1100 in line with our rationale, however, it is a prerequisite that cells subjected to a sustained thymidylate depletion for more than one cell-doubling time still contain an active enzyme form of Topo I. Using a serial enzyme dilution, we found that the specific activity of nuclear Topo I, as measured by the relaxation of supercoiled plasmid DNA, did not significantly differ between ZD1694-treated and control cells (Figure 2). This result confirmed that Topo I in HCT-8 cells under TS inhibition remained active, ensuring their susceptibility to Topo I inhibitors.

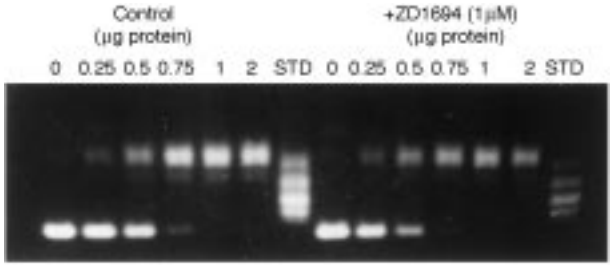

Figure 2. Topoisomerase I (Topo I) status under thymidylate synthase (TS) inhibition. Topo I enzyme assays were carried out using non-histone nuclear proteins of HCT-8 cells that were treated for 2 h with 1 μM ZD1694 and then cultured for 24 h drug-free. The end products of the enzyme reactions were analysed by 1% agarose gel electrophoresis. STD, standard as reference.

*Effects of ZD*1694 *and BNP*1100 *on cell growth and clonogenicity*

The useful range of drug concentrations for a proposed sequential application was initially determined by measuring the growth rate of HCT-8 cells with the SRB assay. The apparent IC_{90} values of ZD1694 and BNP1100, as single agents, were estimated to be 1.0 μM and 10.9 nM, respectively. When ZD1694 and BNP1100 were combined, the drug concentrations required to induce the comparable level of growth inhibition were significantly lower than those as single agents (Figure 3). For example, treatment with 0.2 μM ZD1694 followed by 2.5 nM BNP1100 induced more than 90% growth inhibition compared with only 75% inhibition by 0.2 μM ZD1694 alone or 20% inhibition by 2.5 nM BNP1100 alone. Although the SRB assays demonstrated a profile of gross cytotoxic effect (Figure 3), it remained unclear whether this was due to cytostatic or cell-killing activities [39, 40]. Using clonogenic assays, however, we were able to demonstrate that, over a range of doses equal to or below the respective IC_{90}, the protocol designed herein for ZD1694 and BNP1100 in fact reduced the viability of HCT-8 cells. Moreover, when applied at IC_{90}, these drugs virtually abolished the clonogenicity of HCT-8 cells (> 99.9%); the efficacy was 690-fold greater than that of ZD1694 alone and 585-fold greater than that of BNP1100 alone (Table 1).

To determine the mode of drug action in the proposed sequential application, we performed an isobologram analysis based on a pharmacological consideration proposed by Steel and Peckham [37]. A slight difference in the shape of the 'envelope of additivity' based on IC_{50} and IC_{90} values was due to the use of two completely separate sets of clonogenic assays for independent analyses (Figure 4). When plotted, both IC_{90} and IC_{50} values achieved by several ZD1694/BNP1100 combinations lay distant from the lines which corresponded to theoretically expected additive effects. These values were also mapped outside the envelope defined by two isoeffect curves (modes I and II) which encompassed additive effects calculated from the actual data of each drug [37, 40]. Strong synergism was also observed with 8 h treatment of BNP1100 (Table 1). Taken together, the results suggested that ZD1694 and BNP1100 over a wide range of drug concentrations below IC_{90} induced lethal effects synergistically.

Table 1. *Effects of ZD*1694 *and BNP*1100 *on clonogenicity*

			Number of colonies formed*		
	BNP1100		ZD1694, 2 h pretreatment (μM)		
Cell stage	nM	Exposure time (h)	0	0.3	1.0†
	0		983	162	138
	2.5	24	661	11	19.3
	6.25	24	155	1.3	1.2
	10.9	24	117	1.7	0.2
Log phase‡					
	2.5	8	847	8.5	
	6.25†	8	667	5.3	
	10.9†	8	587	6.3	
	0		977	922	860
	2.5	24	991	896	825
G_0/G_1§	6.25	24	817	724	733
	10.9†	24	802	743	776

*Values (means based on more than two separate experiments) represent the number of colonies formed from a seed of 1000 cells/well at 6 days after sequential treatment with ZD1694 and BNP1100. †IC_{90} dose. ‡Cells under logarithmically growing phase. §Treated with ZD1694 and BNP1100 during G_0/G_1 arrest under serum deprivation (see Materials and Methods). The rate of ^{3}H-thymidine incorporation into DNA was less than 4% of logarithmically growing cell populations.

Effects of drug actions on non-proliferating stages and S phase

Considering the potential clinical applications, it is important to ask whether or not the cells in proliferating phases are

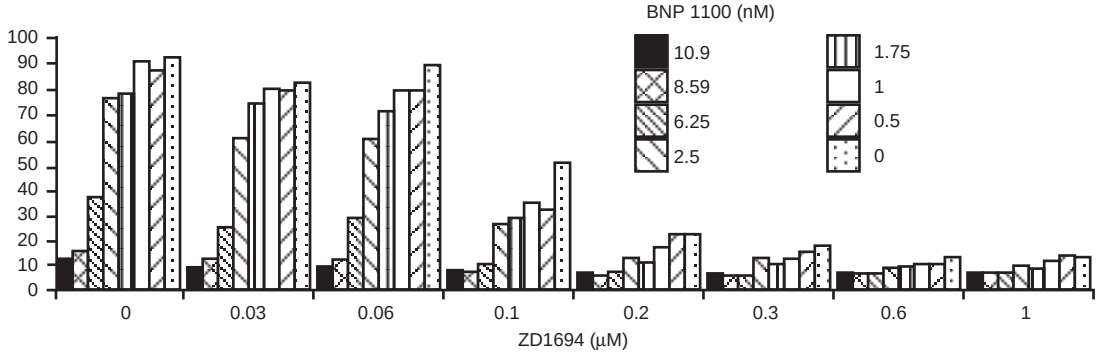

Figure 3. Cytotoxic effects by various concentrations of ZD1694 and BNP1100. After sequential treatment with ZD1694 and BNP1100, HCT-8 cells were cultured drug-free for 3 days and the growth rate was determined by sulphorhodamine B (SRB) methods (see Materials and Methods).

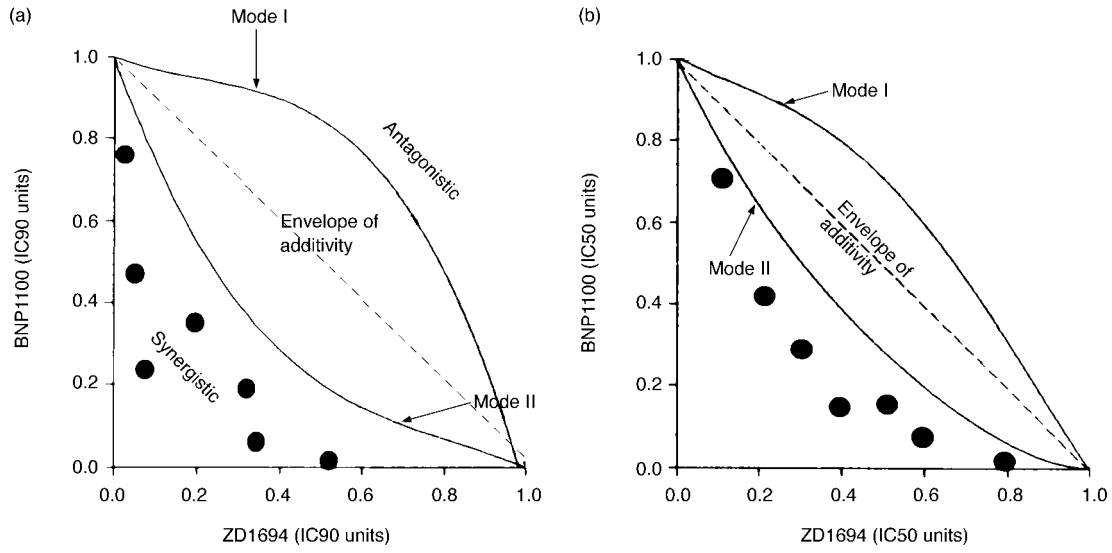

Figure 4. Mode of action by sequential application of ZD1694 and BNP1100. Isobolograms based on (a) IC_{90} and (B) IC_{50} values were constructed from two independent sets of experiments. Combinations of ZD1694 and BNP1100 corresponding to IC_{90} and IC_0 values (●) are plotted in the isobolograms in which a line (– – –) represents theoretically expected additive effects and the area of envelope enclosed by mode I and II lines represents the domain of additive effects, as defined by Steel and Peckham [37].

affected by the proposed protocol. To answer this question, HCT-8 cells in G_0/G_1 stage were challenged sequentially by ZD1694 and BNP1100. These non-proliferating cells showed only partial clonogenic suppression, suggesting that the drugs were preferentially effective on actively growing cells (Table 1).

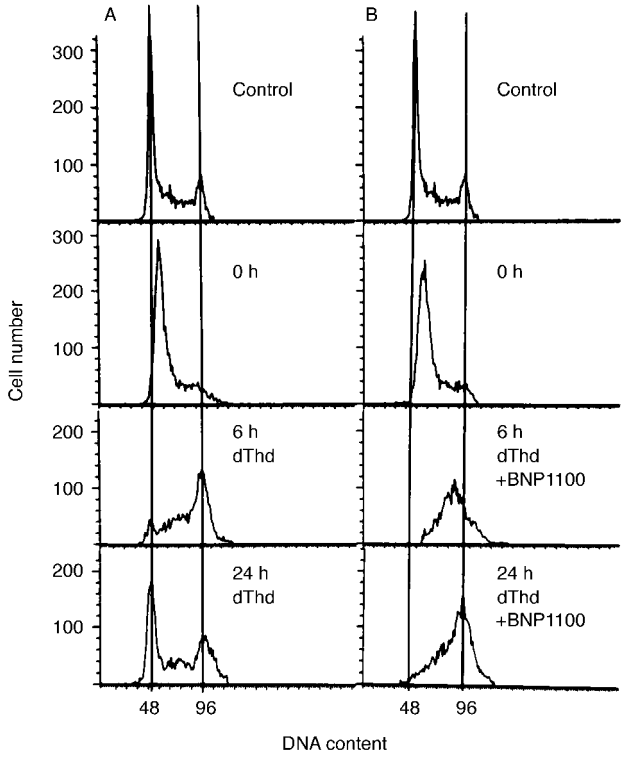

Figure 5. Terminal action points of ZD1694 and BNP1100. HCT-8 cells were treated for 2 h with 1 μM ZD1694. After 24 h culture in the drug-free medium, cells received (a) 10 μM thymidine alone or (b) thymidine plus 10.9 nM BNP1100 and cell cycle profiles at 0, 6 and 24 h later were determined using flow cytometry. The two vertical lines correspond to the DNA content in G_1 and G_2/M.

We have previously demonstrated that ZD1694 treatment can bring about dual effects on growing cells, i.e. S phase arrest and induction of primary DNA strand breaks at both single- and double-strand levels [27]. Therefore, it is crucial to determine which effect of a TS inhibitor provides a prerequisite condition for inducing synergy in concert with a Topo I inhibitor. When synchronised in S phase and then treated with BNP1100, the cells showed a decreased level of clonogenicity that was almost comparable to that of the asynchronous cell population similarly treated in the absence of ZD1694 pretreatment (data not shown). Thus, it seemed likely that the effect of S phase synchronisation *per se* played a small, if any, role in the observed synergy.

Effects on cell cycle

To define the specific cellular responses to the combined use of ZD1694 and BNP1100, it is important to determine their terminal action point in the cell cycle. The representative results using the IC_{90} of both ZD1694 and BNP1100 are shown in Figure 5. In accordance with the previous study [27], cells treated with ZD1694 were arrested in S phase. Without a supply of dThd, this S phase arrest was sustained for more than 48 h (data not shown). However, these cells resumed DNA synthesis in the presence of exogenous dThd, many of which progressed even to the subsequent G_1, indicating that the ZD1694-induced S phase arrest was a transient effect. In the presence of dThd and BNP1100, the cells resumed DNA replication (Figure 5a), progressed through S phase and then were ultimately arrested in late S/G_2 phase as a terminal point (Figure 5b). These cells could not progress beyond this arrest point, as confirmed at 48 h after treatment (data not shown), indicating that cytotoxic effects were extensive.

DNA damage and DNA–protein cross-links induced by sequential drug treatments

Considering the known primary action of the drugs used, one would expect that the cytotoxic effects observed would most likely be related to lethal DNA damage. We examined

Table 2. DNA double-strand fragments induced by sequential application of ZD1694 and BNP1100 plus thymidine (dThd)

ZD1694 (μM)	BNP1100 (nM) 0	2.5	6.25	10.9
	% DNA damage* (Enhancement index†)			
0	0	0.24 ± 0.05	0.38 ± 0.8	0.67 ± 1.2
0.3	3.5 ± 0.9	7.1 ± 1.4 (15.0)	8.6 ± 1.8 (13.4)	12.1 ± 2.0 (12.8)
1.0	5.3 ± 1.0	8.8 ± 0.6 (14.6)	10.0 ± 1.3 (12.4)	13.4 ± 1.8 (12.1)
1.0‡	5.5 ± 0.3	N.A.	5.7 ± 0.4 (0.52)	5.6 ± 0.3 (0.15)

N.A., not available. *Mean ± standard deviation based on more than four experiments. †EI = % DNA damage with drug combination – % DNA damage by ZD1694 alone/% DNA damage by BNP1100 alone (see Materials and Methods). ‡Cells pretreated with ZD1694 were exposed for 24 h to BNP1100 in the absence of dThd.

whether or not the observed synergy on cell lethality was quantitatively related to the level of DNA damage. The maximum amount of DNA double-strand fragments produced by ZD1694 alone and BNP1100 alone was approximately 5% and 0.7%, respectively. However, sequential application with 1.0 μM ZD1694 and 10.9 nM BNP1100 was found to induce up to 13% DNA fragmentation (Table 2). Judging from the EI (Table 2), it was obvious that pretreatment with ZD1694 increased the DNA-damaging capability of BNP1100 by up to 15-fold. Although the method used analysed only the DNA double-strand fragments smaller than 1000 kbp [34], the findings clearly demonstrated that the two drugs worked synergistically. When the cells were exposed to BNP1100 in the absence of dThd, there was essentially no increase in the amount of DNA fragments over those produced by ZD1694 alone (Table 2), implying that the resumption of DNA replication using a salvage pathway via thymidine kinase was indeed necessary for DNA-bound Topo I to induce additional DNA damage in the presence of BNP1100.

It was of special interest to determine what kind of mechanisms underlay the amplification of DNA damage by BNP1100 in the HCT-8 cells pretreated with ZD1694. The rationale predicted that DNA lesions or gaps created under thymidylate depletion [27, 28] might become Topo I binding sites [30] which would, in turn, provide additional targets for the action of Topo I inhibitors. To ascertain if this was the case, the level of DNA-bound Topo I in ZD1694-treated cells was quantified (Table 3). At 24 h after drug treatment, the number of Topo I-bound DNA sites increased up to approximately 4-fold in a dose-dependent manner. Thus, this was consistent with the above DNA damage analysis that the pretreatment with ZD1694 enhanced the action of BNP1100 (Table 2).

Table 3. DNA–topoisomerase I (Topo I) complexes in HCT-8 cells 24 h after treatment with ZD1694

Treatment with ZD1694 (μM)	No. of experiments	% Topo I–DNA cross-links*	Fold over control
0	4	0.93 ± 0.11	1
0.1	4	1.27 ± 0.22	1.4
0.3	5	3.62 ± 0.26	3.9
1.0	5	3.58 ± 0.33	3.8

*Mean ± standard deviations. The values were normalised by subtracting the background level of 0.22 to 0.95%. The amounts of Topo I–DNA cross-links in HCT-8 cells, 24 h after a 2 h treatment with 0.1, 0.3 and 1.0 μM ZD1694, were determined by incubating for 30 min with 1 μM of BNP1100 as a stabilising agent, followed by brief sonication and KCl/sodium dodecyl sulphate precipitation (see Materials and Methods).

Property of surviving colonies

Considering its potential future translation into a clinical regimen, it was important to determine whether or not the present protocol might have induced undesirable genetic changes in cancer cells which survived the sequential treatment with 1 μM ZD1694/10.9 nM BNP1100. A primary concern was the possibility that cancer cells treated with the protocol might have acquired drug resistance. To test this possibility, 26 colonies individually isolated from a total of 1.3×10^5 HCT-8 cells after the treatment were subjected to cytotoxicity assays. The dose response to ZD1694 or BNP1100 (data not shown) as well as the IC_{50} and IC_{90} values (Table 4) displayed by these clones were essentially identical to those of untreated HCT-8 cells. These cells were also cultured continuously for 14 days in the presence of 1 μM ZD1694 or 10.9 nM BNP1100, but no colony was found to form. These results strongly indicate that the protocol used did not confer drug resistance upon the treated cancer cells. To confirm this point, each of the initial 26 colonies was subjected to another round of the sequential treatment with 1 μM ZD1694/10.9 nM BNP1100, and their clonogenic potential determined. The number of colonies formed was not significantly different from that demonstrated by HCT-8 cells that had not received treatment with the proposed protocol (Table 4).

Topo I inhibitors have been demonstrated to possess the potential to induce non-homologous chromosome recombination [41, 42]. Considering its obvious potential as a precursor of viable translocation which, by position effect of vital genes, may produce genetically altered cancer cells, it is possible that the presence of non-homologous chromosome recombination in the cells surviving the protocol may well pose an imminent risk in clinical situations. To analyse whether such undesirable recombination was induced and remained as a stable chromosome translocation, we determined the karyotype of each of 26 colonies. The modal number of chromosomes in the untreated HCT-8 cell line was 45. Although this cell line is known to be established from ileocaecal adenocarcinoma in a male patient, these cells lacked a Y chromosome and contained a single translocation, t(3;12)(q12;q13). Both G- and Q-banding analyses showed that none of the surviving colonies isolated from the drug-treated cells possessed additional chromosome rearrangement or alteration (data not shown).

DISCUSSION

The primary goal of this study was to formulate a novel protocol using clinically achievable schedules and drug doses, which could elicit highly lethal drug synergisms against cancer cells. A strategy tailored herein for the two most promising agents chosen from TS and Topo I inhibitors appears to meet this goal, at least *in vitro*. ZD1694 is an ideal TS inhibitor not only because the maximum effect requires only a brief treatment [27, 28], but also because this antifolate drug has demonstrated efficacy equivalent to that of 5-FU as well as a better quality of life during treatment in clinical trials

Table 4. Drug response and clonogenicity of cells surviving a ZD1694/BNP1100 combination protocol

Cells	Doubling time (h)	Response to drug treatment*				No. of colonies formed after sequential treatment with ZD1694/BNP1100† (per 1.3×10^5 seeds)
		ZD1694		BNP1100		
		IC_{50} (μM)	IC_{90} (μM)	IC_{50} (nM)	IC_{90} (nM)	
Untreated	18 ± 3	0.1 ± 0.02	1.0 ± 0.2	3.7 ± 0.3	10.9 ± 2.3	24 ± 4
Surviving colonies‡	25 ± 4	0.1 ± 0.01	1.1 ± 0.2	4.0 ± 0.4	11.5 ± 3.0	28 ± 4

The differences in all parameters (except for doubling time) between untreated HCT-8 cells and surviving colonies are expressed as mean ± S.D. (standard deviation). $P > 0.5$. *Cells from untreated HCT-8 cells and each of 26 colonies which formed following sequential treatment with 1 μM ZD1694/10.9 nM BNP1100 (for details, see Results) were seeded into 96-well plates and challenged with a serial dilution of ZD1694 or BNP1100. After 72–120 h culture, IC_{50} and IC_{90} values were determined using a sulphorhodamine B (SRB) assay (see Materials and Methods). †Clonogenicity was determined by seeding, per T75 flask, 1.3×10^5 cells sequentially treated with 1 μM ZD1694/10.9 nM BNP1100, followed by 5–7 day culture in the drug-free medium. ‡When continuously exposed for 14 days in the presence of ZD1694 or BNP1100 at IC_{90}, no cells derived from these colonies were found to grow.

[13, 43]. In this study, Karenitecin BNP1100 was used as a model Topo I inhibitor because it displayed a higher plasma stability and cytotoxicity than SN-38 and CPT [24]. A Karenitecin, BNP1350 (20S)-7-(2-trimethylsilyl)ethyl camptothecin), structurally similar to BNP1100, has most recently received Food and Drug Administration (FDA) approval for phase I trials at the University of Chicago and Roswell Park Cancer Institute. In view of the fact that at least several newly developed TS and Topo I inhibitors including, but not limited to, irinotecan (CPT-11 and its active metabolite SN-38) and (1*S*,9*S*)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4 - methyl-1*H*,12*H*-benzol[de]pyranol[3′,1′:6,7]indolizinol[1, 2-*b*]quinoline-10,13(9*H*,15*H*)-dione monomethanesulphonate dihydrate (DX-8951f) [44], possess favourable pharmacological properties similar to those of ZD1694 and Karenitecins [2, 9, 24], the rationale used herein may warrant a broad application [45]. In fact, our preliminary studies unequivocally demonstrate that, when combined with ZD1694 *in vitro*, both SN-38 and BNP1350 are as equally effective as BNP1100 [45]. It is also important to note that the protocol using a clinically relevant schedule and drug doses exerts synergistically lethal activities against several cancer cell lines including three colorectal cancer cells, HCT-8, HCT-116 and SW480, irrespective of their p53 status and MDR expression [45]. Thus, it appears that, inasmuch as their application follows a proposed sequence, the synergistic interaction between ZD1694 and Topo I inhibitors is a universal effect at least *in vitro*.

Of special importance is the finding that, with the proposed schedule, the clinically relevant concentrations of ZD1694 and BNP1100 were sufficient to virtually abolish the clonogenicity of colorectal cancer cells (Table 1). Judging from the findings on DNA damage and DNA–Topo I cross-links (Tables 2 and 3) and also the known properties of the drugs, the enhanced activity against cancer cells (Tables 1 and 2; Figure 4) might have been due to at least four logically permissible pathways (Figure 6). (1) DNA damage induced by ZD1694; (2) DNA damage induced by BNP1100; (3) BNP1100-mediated conversion of ZD1694-induced non-lethal single-strand lesions into lethal DNA double-strand breaks; and (4) BNP1100-mediated recombination of non-homologous chromosomal DNA. Obviously, the actions of TS and Topo I inhibitors, well-defined in many previous studies [8–10, 15, 16, 23–25, 27, 28, 30], would verify pathways 1 and 2.

The analyses of DNA damage (Table 2) and DNA–Topo I cross-links (Table 3) strongly suggest that pretreatment with a TS inhibitor can significantly enhance the DNA-damaging capability of Topo I inhibitors. While an exact mechanism(s) responsible for the increased Topo I binding to DNA after ZD1694 treatment remains to be pursued, it seems worth-

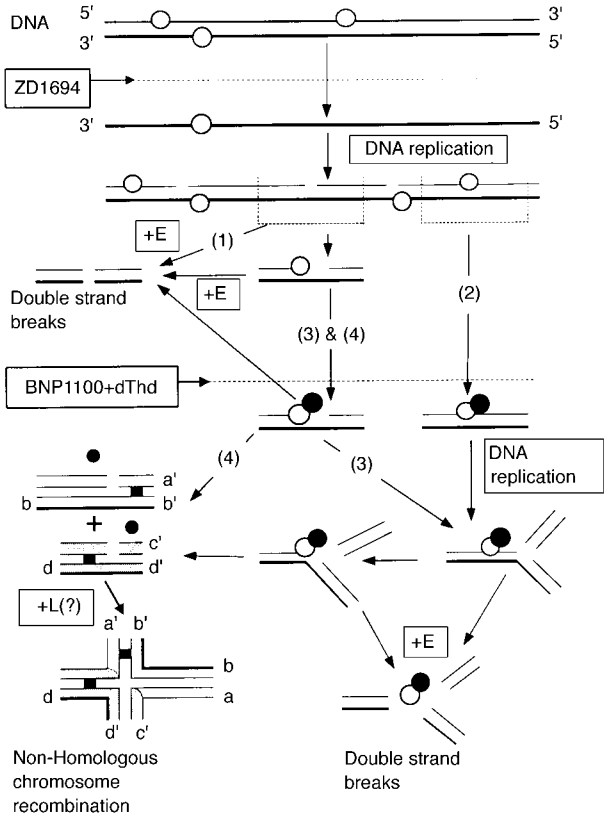

Figure 6. Possible pathways leading to lethal DNA damage. (+E) Endonucleases produce DNA double-strand breaks at ZD1694-induced single-strand lesions [27] as well as at single-strand gaps induced by drug-stabilised cleavable complexes in the downstream of pathway 2. (+L) Although non-homologous chromosome recombination induced by BNP1100, SN-38 and camptothecin is most probably due to the re-ligation reaction of the topoisomerase (Topo I) enzyme itself [41] [46], a possibility cannot be ruled out that a ligase is independently involved. ○, Topo I; ●, Topo I inhibitor (for details, see Discussion). (1) DNA damage induced by ZD1694; (2) DNA damage induced by BNP1100; (3) BNP1100-mediated conversion of ZD1694-induced non-lethal single-strand lesions into lethal DNA double-strand breaks; and (4) BNP1100-mediated recombination of non-homologous chromosomal DNA.

while to contemplate the following possibilities. First, TS inhibition might have stimulated the transport of free enzymes into a nucleus and their non-specific binding to DNA. The imbalance of pyrimidine pools or mis-incorporation of dUMP into DNA under TS inhibition might have contributed to such abnormal enzyme binding. Second, the apyrimidic DNA lesions formed under TS inhibition might have served as *de novo* Topo I binding sites. Third, the DNA strand lesions created under TS inhibition might have trapped the DNA-bound enzymes without their turnover. While the first scenario remains to be proved, the reality of the second and third possibilities may be supported by the following findings: Topo I has been shown to attach preferentially to the 3′-terminus of gaps and deletion sites adjacent to certain nucleotide sequences on DNA strands [46]. Furthermore, recent studies have demonstrated that DNA single-strand nicks or gaps created by chemotherapeutic drugs can trap Topo I [29, 30]. Consistent with the present study, Guichard and colleagues also found that the formation of Topo I–DNA complexes in HT-29 colorectal cancer cells was 1.4 times greater after treatment with another TS inhibitor, 5-FU [47]. Whatever the mechanism for the increased Topo I binding to DNA may be, it appears, as postulated as pathway 3 (Figure 6), that the observed synergy is due, at least in part, to the increase in the number of targets accessible by Topo I inhibitors. Pathway 4 (Figure 6) is related to the specific ability of Topo I inhibitors that can induce non-homologous chromosome recombination [15, 30, 41, 42, 46]. The possible role of pathway 4 as a cause of cytotoxicity has been suggested by the previous finding that cells carrying such an aberrant chromosomal recombination induced by BNP1100, CPT or SN-38 undergo a permanent G_1 arrest and lose their proliferating activity [42]. In view of the previous findings that Topo I bound proximal to drug-induced strand break points can induce non-homologous DNA recombination [30, 41], it may be possible to assume that the Topo I inhibitor-mediated recombination would readily occur at a chromosomal level as well [29, 30, 41]. Whether a re-ligation activity of Topo I itself is responsible or a separate ligase(s) is necessary for such anomalous structural rearrangement of chromosomes remains to be studied (Figure 6).

The synergistic interaction between TS and Topo I inhibitors, described here, was demonstrated only in *in vitro* experiments, even though the proposed protocol was uniformly active against all of the cell lines tested [45]. Although we used BNP1100 as a model Topo I inhibitor, this novel drug still awaits FDA approval for clinical trials. Thus, it remains uncertain whether the proposed protocol can translate into a clinical regimen(s). Nevertheless, a strategy proposed herein may provide significant clinical implications. First, although MDR factors and $p53^{mutant}$ are expressed in more than 50% of colorectal cancers [48], BNP1100, BNP1350 and SN-38 are all active against the cells expressing these factors [24, 27, 42, 45]. Second, both TS and Topo I inhibitors are S phase-specific cytotoxic agents [15, 23–25, 49–51], and cells in non-growing states are minimally affected by the ZD1694/BNP1100 protocol (Table 1). Accordingly, it may not be unreasonble to expect that, when this protocol with appropriate doses and schedules is applied to cancer patients, normal tissues resting in G_0 should only be minimally affected. Third, the drug concentrations found here to be synergistic are within a range readily achievable in the plasma of cancer patients in clinical trials. For example, at the tolerable bolus dose of 3.0–3.5 mg/m^2, the plasma level of ZD1694 in cancer patients can reach approximately 2 μM with $t_{1/2}$ of 0.8–3 h [8]. A steady-state plasma level of more than 10 nM has been readily achieved with CPT-11, SN-38 and 9-amino-CPT [52–54]. Accordingly, it is likely that, if the expected plasma levels are achieved, these drugs will significantly affect the viability of cancer cells in a clinical situation as well. In recent clinical trials, CPT-11 has so far proved to be a highly promising Topo I inhibitor active against several cancer types [16–22, 26]. Since SN-38 is as synergistically active as BNP1100 when used with ZD1694 [45], its prodrug CPT-11 may well be a reasonable choice for the initial clinical trials of the proposed protocol. Although human plasma contains less than 0.1 μM of dThd under non-pathological conditions [55], several clinical studies have previously demonstrated that it is possible to bring up the steady-state plasma level to 1–10 μM by infusion; this has proved to rescue effectively cancer patients from methotrexate-induced side-effects [56]. Yet, one might be concerned about the possibility that when cancer patients undergo a therapy based on this protocol, the administration of exogenous dThd while treating with Topo I inhibitors may compromise the efficacy of ZD1694. However, once the action of the Topo I inhibitors commences on the DNA-bound enzymes, a continuous TS inhibition would no longer be necessary (Figure 6). With simultaneous administration of exogenous dThd during Topo I inhibitor treatments, patients would benefit by circumventing the overlapping toxicity of TS and Topo I inhibitors [50]. Furthermore, since a short exposure (8 h) to BNP1100 is as effective as 24 h exposure (Table 1), a protracted treatment with a Topo I inhibitor may not be necessary in the clinical situation as well. Fourth, since, unlike the case with Topo II-specific inhibitors [57], no secondary leukaemia among Topo I inhibitor-treated patients has been reported so far, the combined use of TS and Topo I inhibitors may have a minimum risk of acute mutagenic activity. However, it should be stressed that the follow-up of patients treated with Topo I inhibitors, as of December 1998, is still too short to justify this assumption. The analysis of the surviving colonies (Tables 1 and 4) leads us to infer that the frequency of viable genetic changes, if induced in chromosomes or genes responsible for resistance to the used drugs, would probably be smaller than 8×10^{-6}, a value in the range of spontaneous mutation *in vitro*. This inference, however, does not unconditionally warrant the absence of genetic changes. It is possible that, if analysis was extended to a much greater number of surviving colonies, certain drug-induced genetic changes might have been detected. Further, it should be emphasised that if analysis was extended to other genetic parameters including point mutation, we might have detected these changes. Thus, we are not in a position as yet to suggest conclusively that the proposed protocol can be applied to the same patient repeatedly, without an undue fear of potentially adverse mutation. Finally, in consideration of the unexpected problems which, unlike the case with the *in vitro* system, may arise in the clinical situation, it would be necessary to evaluate rigorously the proposed protocol using cancer xenografts transplanted in nude and severe combined immuno deficient (SCID) mice.

There have previously been attempts to augment the efficacy of TS and Topo I inhibitors by combining them with other agents. An increasing number of phase I and II studies have reported the remarkable antitumour activity achieved by

the CPT-11/5-FU combination [58]. Reviewing this progress, Bulusu has most recently detailed multiple questions which have inevitably arisen with respect to the perspective of such combination schemes, as well as the need for clarification of underlying mechanisms [59]. The response rates reported in these clinical studies seem to vary extensively. This may be partly due to the fact that CPT-11 has a large interpatient variability in its conversion to SN-38 and SN-38 glucuronide [20–22]. Besides, 5-FU has a considerably less DNA damage-inducing ability compared with 5-fluoro-5′-deoxyuridine (FdUrd) and ZD1694 [60]. Further, the amount of Topo I–DNA complexes induced by ZD1694 (Table 3) is apparently higher than that induced by 5-FU [46]. Thus, it remains to be determined whether or not 5-FU presents the foremost TS inhibitor to achieve the strongest synergistic action in concert with Topo I inhibitors. Several phase I–II trials studied the combination of CPT-11 with paclitaxel, docetaxel, CDDP, carboplatin, oxaloplatin, cyclophosphamide, etoposide, γ-irradiation and mitomycin C [61], but the reproducibility of therapeutic improvement has been questioned [1]. Most recently, Pressacco and colleagues [62] demonstrated that 5-iodo-2′-deoxyuridine (IdUrd) incorporation into DNA in two colon cancer cell lines can be significantly enhanced by inhibiting TS with ZD1694, resulting in a strong synergistic cytotoxic effect *in vitro*. Among many experimental approaches known today, their protocol is based on one of the most promising mechanism-based rationale and, therefore, it is of special interest to await the results of the clinical trials.

After completion of this work, we have become aware of the report by Aschele and associates [63] who demonstrated schedule-dependent synergism between ZD1694 and SN-38. Their results suggested that the sequence of SN-38 followed by ZD1694 produced a better synergy than the reverse sequence, even though the underlying mechanisms for synergistic actions were unknown. Although the conditions used in that study did not involve the dThd-induced collision of DNA replication forks with cleavable complexes [63], their findings and our results essentially concurred in that, when applied sequentially with a certain interval, ZD1694 and a Topo I inhibitor worked synergistically against colorectal cancer cells. At present, however, we do not know which schedule is more effective, SN-38 followed by ZD1694 or ZD1694 followed by SN-38 (or any Topo I inhibitor) in the presence of dThd. Further experiments under comparable stringent conditions would undoubtedly be necessary to clarify this point.

1. Cohen AM, Minsky BD, Schilsky RL. Cancer of colon. In DeVita VT, Hellman S, Rosenberg SA, eds. *Cancer: Principles & Practice of Oncology*, 5th edn. Philadelphia and New York, Lippincott-Raven, 1144–1234.
2. Rustum YM, Harstirick A, Cao S, *et al*. Thymidylate synthase inhibitors in cancer therapy: direct and indirect inhibitors. *J Clin Oncol* 1997, **15**, 389–400.
3. Leichman CG, Fleming TR, Muggia FM, *et al*. Phase II study of fluorouracil and its modulation in advanced colorectal cancer: a Southwest Oncology Study. *J Clin Oncol* 1995, **13**, 1303–1311.
4. Cadman E, Heimer R, Davis L. Enhanced 5-fluorouracil nucleotide formation after methotrexate administration: explanation for drug synergism. *Science* 1970, **205**, 1135–1137.
5. Lokich J, Cantrell J, Ahlgren J, Phillips J for the Mid-Atlantic Oncology Program (MAOP). A phase III trial of protracted infusional 5FU (PIF) versus PIF plus weekly bolus cisplatin (CDDP) in advanced measurable colon cancer (MAOP protocol 5286). *Proc Am Soc Clin Oncol* 1988, **8**, 104.
6. Elias L, Crissman HA. Interferon effects upon the adenocarcinoma 37 and HL-60 cell lines: antiproliferative responses and synergistic interactions with halogenated pyrimidine antimetabolites. *Cancer Res* 1988, **48**, 4868–4873.
7. Loehrer PJ, Turner S, Kubilis P, *et al*. A prospective randomized trial of fluorouracil versus fluorouracil plus cisplatin in the treatment of metastatic colorectal cancer: a Hoosier Oncology Group Trial. *J Clin Oncol* 1988, **6**, 642–648.
8. Duch DS, Banks S, Dev IK, *et al*. Biochemical and cellular pharmacology of 1843U89, a novel benzoquinazoline inhibitor of thymidylate synthase. *Cancer Res* 1993, **53**, 810–818.
9. Harrap KR. Initiatives with platinum- and quinazoline-based antitumor molecules. *Cancer Res* 1995, **55**, 2761–2768.
10. Zalcberg JR, Cunningham D, Van Cutsem E, *et al*. ZD1694: a novel thymidylate synthase inhibitor with substantial activity in the treatment of patients with advanced colorectal cancer. *J Clin Oncol* 1996, **14**, 716–721.
11. Clarke SJ, Hanwell J, Boer M, *et al*. Phase I trial of ZD1694, a new folate-based thymidylate synthase inhibitor, in patients with solid tumors. *J Clin Oncol* 1996, **14**, 1495–1503.
12. Harper P and study group. Advanced colorectal cancer: results from the latest raltitrexed 'Tomudex' comparative study. *Proc Am Soc Clin Oncol* 1997, **16**, 228a.
13. Herrmann R. Thymidylate synthase inhibition, a dead end? *Eur J Cancer* 1995, **31A**, 1919–1920.
14. Zeneca. Tomudex (raltitrexed). Clinical literature compiled by Zeneca Pharmaceuticals, 1998.
15. Liu LF, Duann P, Lin CT, D'Arpa P, Wu J. Mechanism of action of camptothecin. *Ann NY Acad Sci* 1996, **803**, 44–73.
16. Willson JKV. Topoisomerase-I inhibitors in the management of colon cancer. *Ann N Y Acad Sci* 1996, **803**, 256–263.
17. Rothenberg ML, Eckardt JR, Burris HA III, *et al.* Irinotecan (CPT-11) as second-line therapy for patients with 5FU-refractory colorectal cancer. *Proc Am Soc Clin Oncol* 1994, **13**, 198 (abstr. no. 578).
18. Armand JB, Ducreux M, Mahjoubi M, *et al*. CPT-11 (irinotecan) in the treatment of colorectal cancer. *Eur J Cancer* 1995, **31A**, 1283–1287.
19. O'Reilly S, Rowinsky EC. The clinical status of irinotecan (CPT-11), a novel water soluble camptothecin analogue. *Crit Rev Oncol Hematol* 1996, **24**, 47–70.
20. Van Hoff DD, Rothenberg ML, Pitot HC, *et al*. Irinotecan (CPT-11) therapy for patients with previously treated metastatic colorectal cancer (CRC): overall results of FDA-reviewed pivotal US clinical trials. *Proc Am Soc Clin Oncol* 1997, **16**, 228a.
21. Pazdur R. Irinotecan: toward clinical end points in drug development (Review). *Oncology* 1998, **6**(Suppl.), 13–22.
22. Eckhardt SG. Irinotecan: a review of the initial phase I trials (Review). *Oncology* 1998, **6**(Suppl.), 31–38.
23. Squires S, Ryan AJ, Strutt HL, Johnson RT. Hypersensitivity of Cockayne's syndrome cells to camptothecin is associated with the generation of abnormally high levels of double strand breaks in nascent DNA. *Cancer Res* 1993, **53**, 2012–2019.
24. Hausheer F, Haridas K, Zhao M, *et al*. Karenitecins: a novel, potent class of oral highly lipophilic topo I inhibitors. *Proc Am Assoc Cancer Res* 1997, **38**, 227.
25. Houghton PJ, Stewart CF, Zamboni WC, *et al*. Schedule-dependent efficacy of camptothecins in models of human cancer. *Ann N Y Acad Sci* 1996, **803**, 188–201.
26. Shimada Y, Yoshino M, Wakui A, *et al.* and the CPT-11 gastrointestinal cancer study group. Phase II study of CPT-11, a new camptothecin derivative, in metastatic colorectal cancer. *J Clin Oncol* 1993, **11**, 909–913.
27. Matsui S, Arredondo MA, Wrzosek C, Rustum YM. DNA damage and p53 induction do not cause ZD1694-induced cell cycle arrest in human colon carcinoma cells. *Cancer Res* 1996, **56**, 4715–4723.
28. Lu K, Yin M, McGuire JJ, Bonmassar E, Rustum YM. Mechanisms of resistance to N-[5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl]-L-glutamic acid (ZD1694), a folate-based thymidylate synthase inhibitor, in the HCT-8 human ileocecal adenocarcinoma cell line. *Biochem Pharmacol* 1995, **50**, 391–398.
29. Pourquier P, Ueng LM, Kohlhagen G, *et al*. Effects of uracil incorporation, DNA mismatches, and abasic sites on cleavage and religation activities of mammalian topoisomerase I. *J Biol Chem* 1997, **272**, 7792–7796.

30. Pourquier P, Pilon AA, Kohlhagen G, Mazumder A, Sharma A, Pommier Y. Trapping of mammlian topoisomerase I and recombinations induced by damaged DNA containing nicks or gaps. *J Biol Chem* 1997, **272**, 26441–26447.
31. Matsui S, Weinfeld H, Sandberg AA. Quantitative conservation of chromatin-bound RNA polymerase I and II in mitosis: implications for chromosome structure. *J Cell Biol* 1979, **80**, 451–464.
32. Matsui S, Seon BK, Sandberg AA. Disappearance of a structural chromatin protein A24 in mitosis: implications for molecular basis of chromatin condensation. *Proc Natl Acad Sci* 1979, **76**, 6386–6390.
33. Finney M. *Current Protocols in Molecular Biology*. Abusubel FM, Brent R, Kingston RE, *et al.* eds. New York, Wiley, 1989, 11–15.
34. Voigt W, Matsui S, Burhans WC, Minderman H, Rustum Y. Topoisomerase-I inhibitor SN-38 can induce DNA damage and chromosomal aberrations independent from DNA synthesis. *Anticancer J* 1999, **18**, 3499–3506.
35. Zwelling LA, Hinds M, Chan D, *et al.* Characterization of an amsacrine-resistant line of human leukemia cells. *J Biol Chem* 1989, **264**, 16411–16420.
36. Skehan P, Storeng R, Scudiero D, *et al.* New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990, **82**, 1107–1112.
37. Steel GG, Peckham MJ. Exploitable mechanisms in combined radiotherapy–chemotherapy: the concept of additivity. *Int J Radiat Oncol Biol Phys* 1979, **5**, 85–91.
38. Wake N, Tanaka K, Chapman V, Matsui S, Sandberg AA. Chromosomes and cellular origin of choriocarcinoma. *Cancer Res* 1981, **41**, 3137–3143.
39. Boyd MR. The NCI *in vitro* anticancer drug discovery screen, concept, implementation, and operation, 1985–1995. In Teicher B, ed. *Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials and Approval*. Humana Press, 1997, 23–42.
40. Tsai C, Gazdar AF, Venzon DJ, *et al.* Lack of *in vitro* synergy between etoposide and cis-diamminedichloroplatinum (II). *Cancer Res* 1989, **49**, 2390–2397.
41. Pommier Y, Jenkins J, Kohlhagen G, Leteurtre F. DNA recombinase activity of eukaryotic DNA topoisomerase I; effects of camptothecin and other inhibitors. *Mutation Res* 1995, **337**, 135–145.
42. Matsui S, Endo W, Wrzosek C, *et al.* New DNA topoisomerase I inhibitor (TI), BNPI 1100, induces cytotoxicity through a dual mechanism in HCT-8 human ileocecal carcinoma cells. *Proc Am Assoc Cancer Res* 1997, **38**, 17.
43. Cunningham D. Mature results from three large controlled studies with raltitrexed ("Tomudex"). *Br J Cancer* 1998, 77(Suppl.), 15–21.
44. Kumazawa E, Tohgo A. Anti-tumor activity of DX-8951f: a new camptothecin derivative. *Exp Opin Invest Drugs* 1998, **7**, 625–632.
45. Matsui S, Endo W, Wrzosek C, *et al.* Highly synergistic cytocidal effects achieved by a mechanism-based ZD1694/DNA topoisomerase I inhibitor sequential combination. *Proc Am Soc Clin Oncol* 1998, **17**, 203a.
46. Bullock P, Champoux JJ, Botchan M. Association of crossover points with Topoisomerase I cleavage sites: a model for nonhomologous recombination. *Science* 1985, **230**, 954–958.
47. Guichard S, Hennebelle I, Bugat R, Canal P. Cellular interactions of 5-fluorouracil and the camptothecin analogue CPT-11 (Irinotecan) in a human colorectal carcinoma cell line. *Biochem Pharmacol* 1998, **55**, 667–676.
48. Toth K, Vaughan MM, Schwartz G, *et al.* Expression of the MRP and MDR1 multidrug resistance gene products in 160 untreated human carcinomas by immunohistochemical methods in formalin–paraffin sections. *Int J Pathol* 1998, **6**, 145–154.
49. Goldwasser F, Shimizu T, Jackman J, *et al.* Correlations between S and G2 arrest and the cytotoxicity of camptothecin in human colon carcinoma cells. *Cancer Res* 1996, **56**, 4430–4437.
50. Touroutoglou N, Pazdur R. Thymidylate synthase inhibitors. *Clin Cancer Res* 1996, **2**, 227–243.
51. Nitiss JL, Rose A, Sykes KC, *et al.* Using yeast to understand drugs that target Topoisomerases. *Annals N Y Acad Sci* 1996, **803**, 32–43.
52. Herben VVM, Ten Bokkel Huinink WW, Schot M, *et al.* Continuous infusion of low-dose topotecan (TOP): a clinical pharmacologic phase II study in patients with small cell lung cancer (SCLC). *Proc Am Soc Clin Oncol* 1997, **16**, 238.
53. Van Groeningen CJ, Van der Vijgh WJF, Giaccone G, *et al.* Phase I clinical and pharmacokinetics study of 5 day CPT-11 hepatic arterial infusion (HAI) chemotherapy. *Proc Am Soc Clin Oncol* 1997, **16**, 219.
54. Mani S, Iyer L, Janisch L, *et al.* Phase I clinical and pharmacokinetic (PK) study of oral 9-aminocamptothecin (9-AC). *Proc Am Soc Clin Oncol* 1997, **16**, 201a.
55. Rubini JR, Cronkite EP, Bond VP, Fleisher TM. The metabolism and fate of tritiated thymidine in man. *J Clin Invest* 1960, **39**, 909–918.
56. Ensminger WD, Frei III E. The prevention of methotrexate toxicity by thymidine infusions in humans. *Cancer Res* 1977, **37**, 1857–1863.
57. Winik NJ, McKenna RW, Shuster JJ, *et al.* Secondary acute myeloid leukemia in children with acute lymphoblastic leukemia treated with etoposide. *J Clin Oncol* 1993, **11**, 209–217.
58. Fonseca R, Goldberg RM, Erlichman C, Kaufmann SH, Sloan JA, Miller LL. Phase I study of CPT-11/5-FU/leucovorin. *Proc Am Assoc Cancer Res* 1997, **38**, 76.
59. Bulusu VR. Irinotecan and 5-fluorouracil in colorectal cancer: time for a pause? *Eur J Cancer* 1998, **34**, 286–289.
60. Yin MB, Rustum YM. Comparative DNA strand breakage induced in FUra and FdUrd in human ileocecal adenocarcinoma (HCT-8) cells: relevance to cell growth inhibition. *Cancer Commun* 1991, **3**, 45–51.
61. Fukuda M, Nishio K, Kanazawa F, *et al.* Synergism between cisplatin and Topoisomerase I inhibitors, NB-506 and SN-38 in human small cell lung cancer cells. *Cancer Res* 1996, **56**, 789–793.
62. Pressacco J, Hedley DW, Erlichman C. ICI D1694 and idoxuridine: a synergistic antitumor combination. *Cancer Res* 1994, **54**, 3772–3778.
63. Aschele C, Baldo C, Sobrero AF, Debernardis D, Bornmann WG, Bertino JR. Schedule-dependent synergism betwen raltitrexed and irinotecan in human colon cancer cells *in vitro*. *Clin Cancer Res* 1998, **4**, 1323–1330.

Acknowledgements—We would like to thank Mrs Elva Winslow for maintaining the cancer cell lines. We are also deeply indebted to Drs Charles Wenner, Philip Hohmann, Ralph Bernacki, Peter Aplan and William Burhans of our institute for a critical reading of this work, discussion and advice. We also wish to express a sincere appreciation to Dr Ann L. Jackman, Institute of Cancer Research, Sutton, U.K. for a valuable discussion at the Annual Meetings of the American Association for Cancer Research, New Orleans, Louisiana, 28 March–1 April 1998. This study was supported in part by BioNumerik Pharmaceutical Co. (KH, PS, FHH).

Subramanian and colleagues (*Cancer Res* 1998, **58**, 976–984) most recently demonstrated that DNA damage induced by ultraviolet stimulates topoisomerase I-DNA complex formation. Thus, irrespective of the type of DNA lesions, the increase of enzyme-DNA complex is a universal phenomenon which may justify the use of Topo I inhibitors as a second treatment.