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Biochemical Pharmacology 65 (2003) 1281–1294
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Cellular bases of the antitumor activity of a 7-substituted camptothecin in hormone-refractory human prostate carcinoma models

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Received 3 September 2002; accepted 16 January 2003

Abstract

ST1481, a lead compound of a novel potent 7-substituted lipophilic camptothecin series, is able to overcome several mechanisms of drug resistance and was selected for clinical development. This study was designed to examine the antitumor activity of ST1481 in the treatment of preclinical models of human p53-defective hormone-refractory prostate carcinoma (DU145, PC3, and JCA-1) and to explore the cellular bases of the efficacy of camptothecins. A cellular pharmacology study (cytotoxicity, apoptosis, cellular drug accumulation, DNA damage, and cell cycle perturbation) was performed in DU145 and PC3 cells, characterized by a different cell cycle checkpoint status. The introduction of wild-type p53 in PC3 cells appreciably decreased the drug sensitivity. The 7-substituted camptothecins exhibited a high cytotoxic potency that paralleled their relative ability to induce DNA damage and a substantially increased cellular accumulation as compared to topotecan. The cytotoxic effect of camptothecins in DU145 cells was associated with arrest in S phase and early activation of apoptosis, whereas PC3 cells responded to drugs by a persistent block in G2 phase with a cytostatic effect and a late apoptosis. The efficiency of S phase checkpoint in DU145 cells was supported by a time-dependent decrease of DNA synthesis following treatment. In spite of an apparent cytostatic response and apoptosis resistance, the PC3 tumor was more responsive to *in vivo* treatment with camptothecins than the DU145 model. Indeed, the therapeutic outcome did not reflect the cell susceptibility to early activation of apoptosis. We suggest that cell death in PC3 cells is a delayed event consequent to persistent arrest in G2 and insufficient repair of DNA damage. ST1481 was appreciably more effective than topotecan in all tested tumors. In conclusion, the results indicated a relevant efficacy of camptothecins against human prostate carcinoma models, in spite of p53 alterations. Although p53 status could influence DNA damage and cell cycle checkpoints, p53 mutation was not a determinant of resistance. The results support that, in addition to the extent and persistence of topoisomerase I-mediated DNA damage, cell cycle checkpoints and DNA damage signaling pathways are critical determinants of tumor responsiveness to camptothecins. A role of cell cycle checkpoints activated by DNA damage in cell response is supported by the modulation of transcriptional profile.

Keywords: Camptothecins; Prostate carcinoma; Xenografts; Cytotoxicity; Antitumor activity; Apoptosis

1. Introduction

Hormone-refractory prostate cancer has long been recognized as a chemotherapy-resistant disease. The available antitumor agents can be effective in reducing the tumor burden and in providing some benefits in terms of quality of life. Thus, new drugs or regimens are being developed to improve the clinical outcome. The development of novel agents and an understanding of the cellular basis of resistance would contribute to improve the efficacy of the pharmacological treatment of hormone-refractory prostate cancer. Among the most widely studied drugs in the chemotherapy treatment of advanced hormone-refractory prostate cancer, appreciable results have been obtained with inhibitors of DNA topoisomerase II (including doxorubicin, mitoxantrone, and etoposide) [1]. Several biological features and alterations involving regulation of

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Abbreviations: BrdU, bromodeoxyuridine; PARP, poly(ADP-ribose)-polymerase; PI, propidium iodide; RPA, replication protein A; TUNEL, Tdt-mediated dUTP Nick-end labeling.

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doi:10.1016/S0006-2952(03)00079-0

apoptosis can contribute to the intrinsic resistance of prostate carcinoma [2,3]. Camptothecins, the clinically available inhibitors of DNA topoisomerase I, are cytotoxic agents with a wide spectrum of antitumor activity and are among the most promising drugs for the treatment of solid tumors [4,5]. In phase II clinical trials, the activity of camptothecins against hormone-refractory prostate cancer has been disappointing [6,7]. The clinically available camptothecins (e.g. irinotecan and topotecan) are water-soluble derivatives, but novel liphophilic camptothecins are undergoing preclinical and/or clinical evaluation, because they exhibit potential advantages, including intrinsic potency and favorable cellular accumulation and tissue distribution [8,9]. In addition, it is conceivable that an improvement of the efficacy may result from the use of analogs with more potent or persistent topoisomerase I inhibitory activity. We have recently reported that camptothecin analogs substituted in position 7 with lipophilic chains are endowed with a marked cytotoxic potency, likely related to a more potent and persistent stabilization of topoisomerase I-mediated DNA cleavage [9–12].

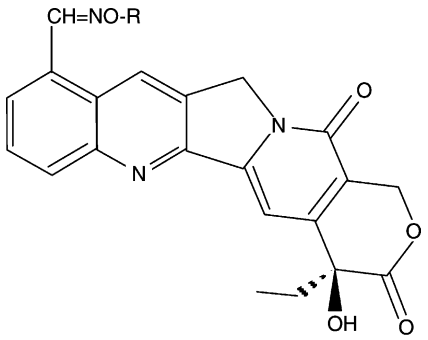
The aim of our study was to explore the therapeutic potential of camptothecin analogs against human prostate carcinoma models and to investigate the cellular basis of tumor response. Antitumor activity studies were performed in three human hormone-refractory prostate carcinoma models (DU145, PC3, and JCA-1) lacking p53 function and characterized by diverse alterations in apoptotic pathways [13,14]. For cellular pharmacology studies, we have selected two cell lines, DU145 and PC3, characterized by a different cell cycle checkpoint status and susceptibility to apoptosis [15]. The results indicate a marked efficacy of the novel analog ST1481. However, the two models exhibited a substantially different cellular response to camptothecins, likely depending on the cell cycle checkpoint status. The cell ability to undergo a rapid apoptotic response was not predictive of tumor responsiveness. Antitumor efficacy and cellular response support the preclinical interest of the novel 7-substituted lipophilic analog as a promising candidate for the treatment of hormone-refractory prostate cancer.

2. Materials and methods

2.1. Drugs and cell lines

The two camptothecin derivatives (ST1480 and ST1481) (Fig. 1) were obtained from Sigma-Tau. Analogs were dissolved in dimethylsulfoxide at 2 mg/mL. The dilution was in saline solution. Topotecan (Smith-Kline Beecham) was dissolved in sterile water.

The human androgen-independent DU145, PC3, and JCA-1 prostate carcinoma cell lines were maintained in RPMI 1640 medium (Bio-Whittaker) supplemented with 10% fetal calf serum (Life Technologies).



R = $CH_2C_6H_5$ ST1480

R = $C(CH_3)_3$ ST1481

Fig. 1. Chemical structure of camptothecins.

To obtain the PC3 cell line (PC3-SN3) expressing the p53 protein, PC3 cells were seeded in 5 cm diameter dishes and when they reached 70% confluency, they were incubated with serum-free medium containing 20 μL of lipofectin (Life Technologies) and 3 μg of plasmid DNA for 5 hr. The pCMV-SN3 plasmid was kindly supplied by Dr. Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). Medium was replaced and the individual clones were isolated after selection in the presence of 500 μg/mL G418 (Life Technologies). Transfectants, which were maintained in the presence of 500 μg/mL G418, exhibited a proliferation rate similar to parental cells. The expression of p53 was verified by flow cytometric analysis (FACScan; Becton Dickinson) with DO-7 monoclonal antibody (Dako).

In all assays, except for the colony growth assay, 24 hr after seeding cells were treated with different drug concentrations for 1 hr and then incubated in drug-free medium for different times.

2.2. Antitumor activity studies

Antitumor activity experiments were carried out using male athymic Swiss nude mice, 8–10 weeks of age (Charles River). Mice were maintained in laminar flow rooms, keeping temperature and humidity constant. Mice had free access to food and water. Experiments were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori of Milan. For experimental purposes, 10^7 cells from *in vitro* cultures were injected s.c. Randomized groups of five mice bearing bilateral s.c. tumors were used. The drugs were delivered by gavage in a volume of 10 mL/kg of body weight every fourth day for four times (q4d×4) at their maximum tolerated doses [9] starting when tumors were measurable (around 50 mm^3). Drug efficacy was evaluated on the basis of the following parameters: tumor volume inhibition (TVI), log-cell kill (LCK), and rate of complete regression (CR) [9]. For statistical analysis, tumor volumes were compared by Student's *t*-test.

2.3. Cell sensitivity studies

Cell sensitivity to drugs was measured by cell counting and colony growing assays. In the former assay, adherent cells were trypsinized and counted 72 hr after drug treatment by a cell counter (Coulter Electronics). The results are the mean ± standard deviation (SD) of at least three independent experiments. Cell diameter was evaluated by Coulter Multisizer (IL) immediately after trypsinization.

In the colony growing assay, 400 cells were seeded in 60-mm plates (Corning Costar) in triplicate and, when colonies were detectable (around 10 cells), they were counted and treated with the drugs for 1 hr. Then, samples were incubated in drug-free medium until colonies in control samples consisted of around 30 cells. Samples were stained with 1% crystal violet in methanol for 30 min, and then colonies consisting of at least 10 cells were counted [16]. The plating efficiency was around 40% in both cell lines.

2.4. Assessment of apoptosis

Apoptosis was detected at various times after treatment by morphological analysis of propidium iodide (PI)-stained cells and by Tdt-mediated dUTP Nick-end labeling (TUNEL) assay. In the former assay, floating and trypsinized cells were washed in PBS, counted, fixed in 70% ice-cold ethanol, and stained with a PI solution (Sigma) (10 μg/mL PI and 66 U/mL RNase A in PBS) for 18 hr. Cells were examined for nuclear morphology by a fluorescence microscope. At least 100 cells, in two different smears, were examined and the results were expressed as percentage of apoptotic cells (with condensed nuclei and/or fragmented chromatin) over the cell number of the whole population. In the TUNEL assay, cells (5×10^5) were fixed in 4% paraformaldehyde for 45 min at room temperature. After rinsing with PBS, cells were permeabilized in a solution of 0.1% Triton X-100 in 0.1% sodium citrate for 2 min in ice. Samples were then incubated in the TUNEL reaction mix (Boehringer Mannheim) for 1 hr at 37° in the dark and after rinsing with PBS, they were suspended in PBS and analyzed by FACScan flow cytometer equipped with an argon laser.

2.5. Cell cycle analysis

At various times after drug treatment, adherent cells were trypsinized, fixed, and stained with a PI solution as described earlier. At least, 10,000 cells were collected and analyzed by FACScan flow cytometry. Cell cycle distributions were calculated by LYSYS II software (Becton Dickinson).

For bromodeoxyuridine (BrdU) incorporation, treated and untreated cells were pulse labeled with BrdU (Sigma) (10 μM for 1 hr) at the timepoints to mark the cells undergoing DNA replication. Cells were then trypsinized and fixed in 70% ice-cold ethanol. Fixed cells were exposed to 1 N HCl for 30 min and to 0.1 M sodium tetraborate for 10 min. After 1 hr incubation in PBA (PBS containing 1% BSA and 0.2% Tween 20) containing BrdU antibody (Becton Dickinson), cells were re-incubated with an anti-mouse-FITC antibody (Sigma) solution, counterstained with a PI solution, and analyzed by FACScan flow cytometer. The green FITC-fluorescence, expressed on a logarithmic scale, indicated the BrdU incorporation and the PI-red fluorescence on a linear scale, was the index of DNA content.

2.6. Alkaline elution

DNA single-strand breaks were determined by the alkaline elution method [17]. Cellular DNA was labeled with 0.08 μCi/mL [2-^{14}C]thymidine (Amersham) for 30 hr. After further 18 hr in the absence of labeled thymidine, cells were exposed to the drugs for 1 hr and then processed immediately or incubated in drug-free medium up to 3 hr. A positive control of cells irradiated with 100 rad was used as a reference.

In the alkaline elution procedure for determination of double-strand breaks, the cells were lysed with SDS EDTA lysing solution, pH 9.6 (2% SDS, 25 mM Na_2EDTA, 100 mM glycine, and 0.5 mg/mL proteinase K), and the eluting solution was 20 mM H_4EDTA, 0.1% SDS, and 10% ammonium tetrapropylhydroxide in water, pH 9.6.

2.7. Caspase 3, poly(ADP-ribose)polymerase (PARP), and replication protein A (RPA) Western blot

Cell lysates (40 μg/lane) were fractionated by SDS–polyacrylamide gel electrophoresis and blotted on nitrocellulose sheets. Blots were preblocked for 1 hr at room temperature in PBS containing 5% (w/v) dried non-fat milk. Filters were incubated overnight at 4° with antibodies to caspase 3 (Pharmingen, Becton Dickinson), PARP (Oncogene Science), or RPA (NeoMarker). A mouse anti-actin antibody (Sigma) was used as control for loading because actin expression is not modulated by drug exposure in our experimental conditions. The membrane was then washed with PBS–Tween (0.1%) solution, and incubated for 1 hr with anti-rabbit (for caspase 3) or mouse (for PARP and RPA) horseradish-conjugated antibody (Amersham). At the end of incubation, the membrane was washed as described earlier, and the detection of the bands was performed with chemiluminescence using the ECL detection kit (Amersham).

2.8. Immunocytometric determination of proliferating cell nuclear antigen (PCNA) recruitment

Twenty-four hours after drug treatment, treated and control cells (1×10^6/sample) were harvested, washed in PBS, and lysed in cold hypotonic solution to release

soluble PCNA [18]. Briefly, cells were lysed in ice-cold 10 mM Tris–HCl buffer (pH 7.4) containing 2.5 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl-fluoride (PMSF). Then, cells were resuspended in cold PBS and fixed in ethanol to a final concentration of 70%. Cells were washed in PBS, incubated for 15 min at room temperature in PBA to prevent unspecific staining, and incubated for 1 hr in 100 μL of anti-PCNA monoclonal antibody (5 μg/mL) (Dako). After washing in PBA, samples were further incubated for 30 min in FITC-conjugated anti-mouse secondary antibody (Sigma) diluted 1:100. At the end of the reaction, cells were washed and resuspended in PBS containing 5 μg/mL PI and 66 U/mL RNase A (Sigma). Cells were analyzed with a flow cytometer (FACScan), as described earlier, and a total of 10^4 cells were measured for each sample.

2.9. Cellular accumulation studies

Cells, at a density of 6×10^5 and 5×10^5 cells/well for PC3 and DU145 cells, respectively, were exposed to the drug for 1 hr. Treated cells (3 wells/sample) were washed three times with PBS to remove the drug and immediately lysed or maintained 6 hr in drug-free medium. Then, cells were lysed and freeze-dried. ST1481 was extracted with 0.7 mL of a 0.1% acetic acid/acetonitrile solution 1:5 (w/w; pH 4.5) and then with 3 mL of chloroform. After centrifugation (5 min at 4500 *g*), the organic phase was evaporated to dryness and 0.15 mL acetonitrile was added for HPLC analysis. For extraction of topotecan, 0.5 mL of methanol was added to each cell pellet and centrifuged at 2000 *g* for 10 min at 4°. The clear topotecan-containing solution was then used for HPLC determination. Quantitative HPLC analysis was performed at room temperature using a C18 reverse-phase column (5 mm; 150 mm × 4.6 mm; Agilent). The flow rate was 1.0 mL/min. For ST1481, the eluent was a water/acetonitrile mixture 1:1 (w/w), and the drug was detected fluorometrically (excitation 340 nm, emission 510 nm). For topotecan, the eluent was a 10:90 (w/w) mixture of acetonitrile and aqueous 2% triethylamine acetate buffer (pH 5.5). Fluorimetric detection was performed at an excitation of 382 nm and an emission of 527 nm. Quantitative determination was performed by graphic interpolation of the reference curve obtained by plotting the peak areas vs. known amounts of the test drugs [12].

2.10. cDNA array hybridization

PC3 and DU145 cells were seeded at a density of 4×10^6 cells/flask (75 cm^2). Twenty-four hours later, cells were incubated with culture medium containing 1 μM ST1481 or DMSO (at the same concentration as in ST1481-treated samples) for 1 hr. Cells were washed and left to grow in drug-free medium. After 6 hr, total RNA was extracted from cells using Trizol reagent (Life Technologies) according to the manufacturer's instructions. RNA was treated with DNase (Ambion Inc.) as specified by the manufacturer and was checked for quality by electrophoresis on denaturing formaldehyde/agarose gel, as well as by A_{260}/A_{280} ratios. Poly A+ RNA enrichment, ^{32}P-labeled cDNA probe preparation, and filter hybridization were carried out as recommended by Clontech. Nylon membranes (Atlas Human Array 1.2; Clontech) consist of 1176 human cDNA fragments, organized into broad functional groups. A complete list of the gene included on the membranes is available on the Clontech web site (http://www.clontech.com). The results of array hybridization were evaluated using phosphorimager (Molecular Dynamics) and analyzed with AtlasImage 2.0 software package (Clontech).

3. Results

3.1. Antitumor activity

In vivo studies were performed with ST1481 analog which exhibited more favorable solubility profile than ST1480. Topotecan was used as a reference compound for oral administration. Previous preclinical studies indicated a better efficacy of oral than i.v. administration of topotecan [19]. Under optimal treatment conditions (when protracted daily schedules are used), camptothecins may be curative against several human tumor xenografts growing in nude mice [20]. Use of a less effective, intermittent treatment schedule may be expected to single out the most effective agents. In the present study, for the purpose of comparison, the drugs were delivered orally according to a q4d×4 schedule [9]. PC3 and JCA-1 tumors were markedly responsive to both drugs, in terms of LCK and rate of complete responses (Table 1). In PC3 tumor ST1481 exhibited a superior efficacy at 3 mg/kg, a dose level which was not always tolerated in other models. As already reported [21], the maximum tolerated dose ranged between 2 and 3 mg/kg, depending on the tumor model. DU145 tumor was less responsive to camptothecins because no complete tumor responses were achieved by drug treatment. The reduced sensitivity of DU145 tumor was also documented by lower LCK values. ST1481 was significantly more effective than topotecan in inhibiting tumor growth.

3.2. Antiproliferative effects and drug-induced cell death

On the basis of *in vivo* studies, we have selected cellular pharmacology studies of DU145 and PC3 cells as representative of prostate tumors with a different sensitivity. The *in vitro* doubling times were similar for the two cell lines (~26 hr).

The antiproliferative activities of camptothecin analogs were determined at 72 hr, following 1 hr exposure to the

Table 1
Antitumor efficacy of topotecan and ST1481 against hormone-refractory human prostate carcinoma xenografts (s.c.) (treatment: per os, every fourth day for four times)

Tumor (D.T.)[a]	Drug	Dose (mg/kg)	TVI[b] (%)	LCK[c] (1 cm^3)	CR[d] (%)	BWL[e] (%)	Lethal toxicity[f]
JCA-1 (4.6)	Topotecan	15	95	1.5	30	15	0/5
		18	n.d.	n.d.	n.d.	n.d.	2/4
	ST1481	2	98	1.9	62.5	6	0/4
		3	n.d.	n.d.	n.d.	n.d.	3/5
PC3 (4.8)	Topotecan	15	95	2.1	60	12	0/5
	ST1481	2	95	2.1	30	6	0/5
		3	100	2.8	90	4	0/5
DU145 (7.6)	Topotecan	15	77	1.0	0	11	0/5
	ST1481	2	95**	1.7	0	10	0/4

n.d.: not determined.
[a] D.T.: tumor doubling time (days). Mice were tumor-implanted in both flanks.
[b] Tumor volume inhibition percent in treated over control mice, calculated after the end of treatment.
[c] Log-cell kill.
[d] Complete tumor response (i.e. no evidence of palpable tumors for at least 10 days). CR rate refers to all treated tumors in each group.
[e] Body weight loss percent induced by the treatment.
[f] Number of mice dead for toxicity over total number of mice.
** $P < 0.05$ vs. topotecan treatments (Student's *t*-test), assessed at the day of TVI percent measurement. The differences were still significant up to 52 days after the end of treatment.

drug. In contrast to *in vivo* response, the two cell lines exhibited a comparable sensitivity to the 7-substituted camptothecins (Table 2). The antiproliferative potency of the novel 7-substituted analogs was substantially higher than that of topotecan used as a reference compound. In order to investigate the mode of cell death of the prostatic carcinoma cells in response to the drug exposure, the amount of apoptotic cells was determined by fluorescence microscopy of PI-stained cells. In DU145 cells apoptosis was already detected at 48 hr after treatment (around 20%). The apoptosis level produced by equitoxic concentrations was 50–60% at 72 hr after treatment, whereas a low number of apoptotic cells were observed in treated PC3 cells (Fig. 2). These results were confirmed by cytofluorimetric analysis of TUNEL-positive cells (Fig. 2a and b). The 7-modified analogs were 10 times more potent than topotecan as apoptosis inducers. In PC3 cells treated with equitoxic drug concentrations (IC_{80}), the number of TUNEL-positive cells increased appreciably only at a prolonged time (up to 43% at 144 hr after exposure to ST1481), indicating a delayed apoptosis (Fig. 2, inset). The introduction of wild-type p53 in p53-defective PC3 cells (PC3-SN3) marginally decreased the drug antiproliferative effects and susceptibility to apoptosis of transfected cells (Table 2). This was not related to a change of proliferative rate.

PC3 cells treated with camptothecins appeared tightly adherent to the flasks with an increase of cell volume without evidence of floating cells in the medium. The Multisizer analysis indicated a cell diameter of 15.5 ± 2.1 μm in control cells and 25–30 μm in all samples treated with the IC_{80}. In order to explain the antiproliferative effect observed in PC3 cells at 72 hr after drug treatment, in spite of the low levels of apoptosis, the colony growth inhibition assay was performed which allowed to discriminate between cytotoxic and cytostatic effects. Drug treatments (1 hr) were performed when small colonies (around 10 cells) were present. Colonies were observed and counted before and after treatments to examine modifications both in the number of cells per colony and in the number of colonies. The number of colonies was determined 72 hr after treatment, when around 30 cells/colony were present in untreated samples. Fig. 3 shows the percentage of colonies with a size similar or larger than that observed at the time of treatment. A dose-dependent reduction in the number of colonies was observed in DU145 cells,

Table 2
Sensitivity and susceptibility to apoptosis of camptothecin-treated human prostate carcinoma cells

Drug	DU145		PC3		PC3-SN3	
	IC_{50}[a] (nM)	Apoptosis[b] (%)	IC_{50}[a] (nM)	Apoptosis[b] (%)	IC_{50}[a] (nM)	Apoptosis[b] (%)
Topotecan	200 ± 14	56 ± 13	600 ± 70	16 ± 8	790 ± 23	8 ± 5
ST1480	18 ± 3	55 ± 6	21 ± 3	18 ± 3	50 ± 6	6 ± 2
ST1481	37 ± 3	46 ± 13	43 ± 8	13 ± 5	70 ± 10	7 ± 6

[a] IC_{50} (drug concentration inhibiting cell growth by 50%) measured at 72 hr after 1 hr treatment.
[b] Levels of apoptosis at 72 hr after 1 hr exposure to the IC_{80} (drug concentration inhibiting cell growth by 80%).

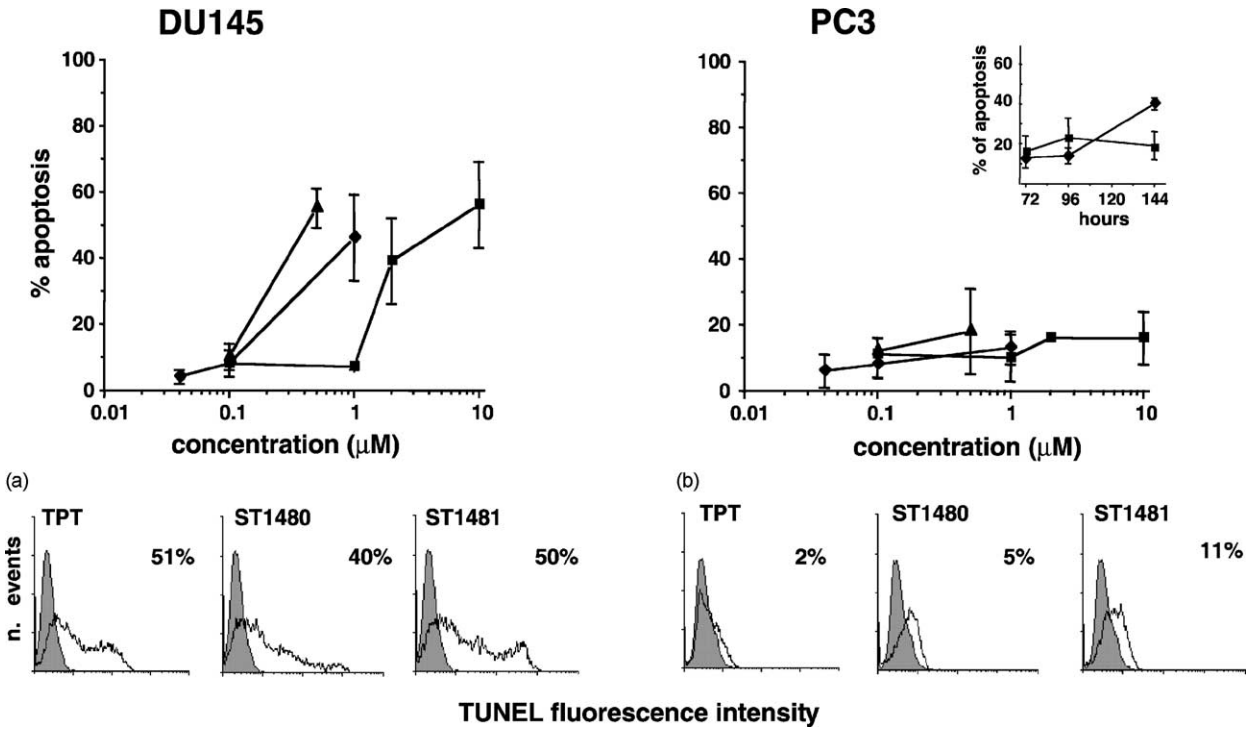


Fig. 2. Level of drug-induced apoptosis in DU145 and PC3 cells. Apoptosis was determined by morphological analysis of PI-stained cells 72 hr after 1 hr treatment with a range of concentrations for each drug. Inset: level of apoptosis in PC3 cells at different times after treatment with the IC_{80}. The results are the mean ± SD of three independent experiments. (■) Topotecan (TPT); (▲) ST1480; (◆) ST1481. (a) and (b) Apoptosis determined by TUNEL assay using FACS analysis in DU145 and PC3 cells, respectively, at 72 hr after 1 hr treatment with the IC_{80}. Gray profiles: control untreated cells; white profiles: cells exposed to the IC_{80} of the different drugs (TPT, 10 μM; ST1480, 0.5 μM; ST1481, 1 μM). The percentages of TUNEL-positive cells obtained by LYSYS II software program are indicated. The results are from one representative of two independent experiments.

whereas at the tested concentrations, no reduction of the amount of colonies was observed in PC3 cells. Moreover, in the PC3 cell line the number of cells per colony was similar before and after treatment, thus suggesting a cytostatic effect. An appreciable decrease in the number of colonies required a prolonged time (not shown).

Caspase activation is a key determinant in apoptotic response to camptothecin treatment [22]. CPP32 (caspase 3)

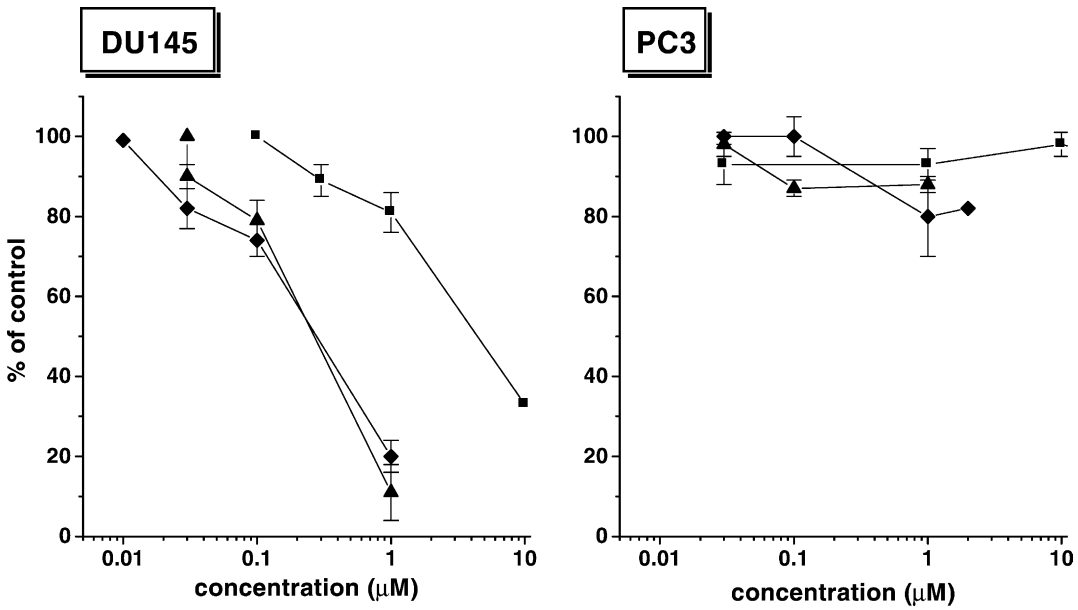


Fig. 3. Colony growing inhibition. Five days after seeding, when around 10 cells/colony were present, cells were treated with the drug for 1 hr and colonies containing at least 10 cells were counted 72 hr later. The results are the mean ± SD of two independent experiments with three replications for each concentration point. In control samples around 180 colonies were counted. (■) Topotecan; (▲) ST1480; (◆) ST1481.

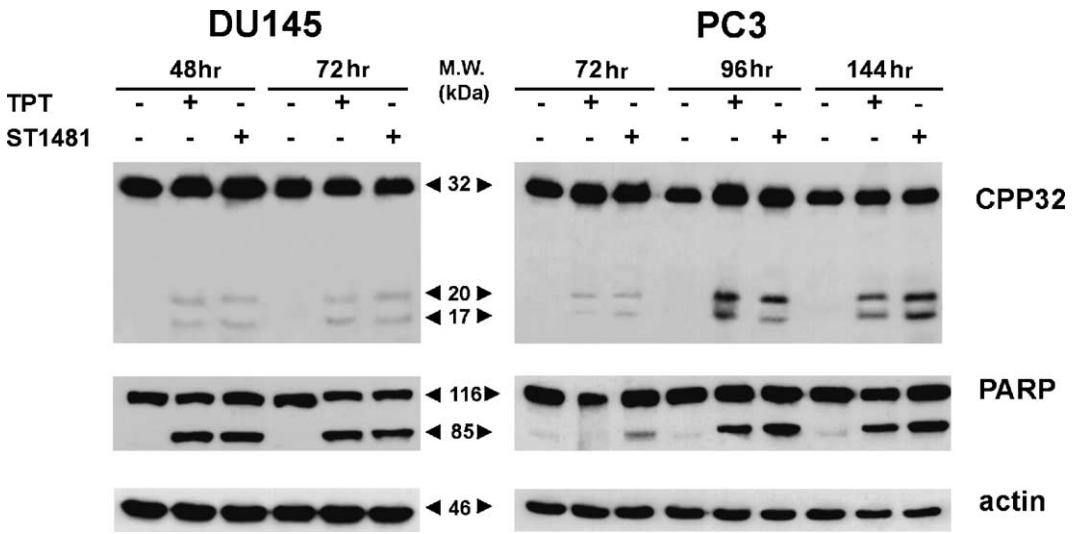


Fig. 4. Western blot analysis of CPP32, PARP, and their cleavage products. DU145 and PC3 cells were exposed for 1 hr to 10 or 1 μM of TPT or ST1481, respectively. Actin is shown as control of the loading.

upon activation is converted to p20 and p17 subunits. As shown in Fig. 4, the active forms of CPP32 were detected in DU145 cells at 48 and 72 hr after treatments, whereas in PC3 cells cleavage subunits were detectable only after 72 hr and their levels increased with time. Again, a concomitant time-dependent cleavage of PARP, one of the caspase substrates in apoptosis, was observed in the two cell lines. Thus, the different time course of caspase activation in the two cell lines did not reflect a different growth rate, that is comparable in the two cell lines, but was consistent with the different onset of apoptosis. In PC3 cells, the effects of ST1481 were more marked than those of topotecan accordingly with the observed level of apoptosis (Fig. 2).

3.3. Cellular drug accumulation and retention

Cellular drug content was determined after 1 hr exposure to the drug at a concentration reflecting approximately the relative cytotoxic potency (i.e. 2 and 20 μg/mL for ST1481 and topotecan, respectively) (Table 3). Under these conditions, a comparable cellular content of topotecan and ST1481 was found in DU145 cells. Drug uptake by PC3 cells was substantially higher than that found in DU145 cells, in spite of comparable antiproliferative effects. Retention of ST1481 was markedly higher than that of topotecan. The cellular pharmacokinetics behavior of ST1481 is consistent with its lipophilic nature and an increased stability of ST1481 interaction with the intracellular target [9].

Table 3
Intracellular accumulation and retention of topotecan and ST1481 in prostate carcinoma cells

Time (hr)	Cellular content (ng/mg protein)		
	DU145		PC3
	Topotecan	ST1481	ST1481
1[a]	26.4 ± 6.7	21.24 ± 0.5	44.85 ± 4.38
6[b]	0.11 ± 0.1	1.32 ± 0.1	3.23 ± 0.84

[a] Cells were exposed for 1 hr to the drug and immediately processed for analysis. Values are the mean ± SD of quadruplicate determinations.
[b] Drug retention following 1 hr exposure and 6 hr incubation in drug-free medium.

3.4. DNA damage and repair

DNA single-strand breaks were determined after 1 hr treatment (Fig. 5). Using a range of drug concentrations between the IC_{50} and the IC_{90}, the two cell lines exhibited a somewhat comparable extent of DNA damage, in spite of a different cellular drug content. In both DU145 and PC3 cells, equitoxic concentrations of topotecan, ST1480, and ST1481 induced a similar extent of DNA damage (Fig. 5A). The two analogs were 10 times more potent than topotecan in inducing DNA damage, since a similar level of DNA breaks were obtained at 10-fold lower concentrations; again, the increased potency of the novel camptothecins is consistent with other cellular effects.

The recovery from DNA damage induced by the camptothecin derivatives was investigated up to 3 hr after drug removal (Fig. 5B). The experiments were performed with equimolar drug concentrations. The results show that, both in DU145 and PC3 cell lines, most of DNA single-strand breaks were already reversed 1 hr after drug removal. A slower recovery of the DNA damage induced by ST1481 was observed as compared to topotecan.

The primary topoisomerase I-mediated DNA lesions are single-strand breaks, but a time-dependent formation of DNA double-strand breaks are expected as a consequence of collision between the replication fork and topoisomerase I–DNA cleavage complex. The production

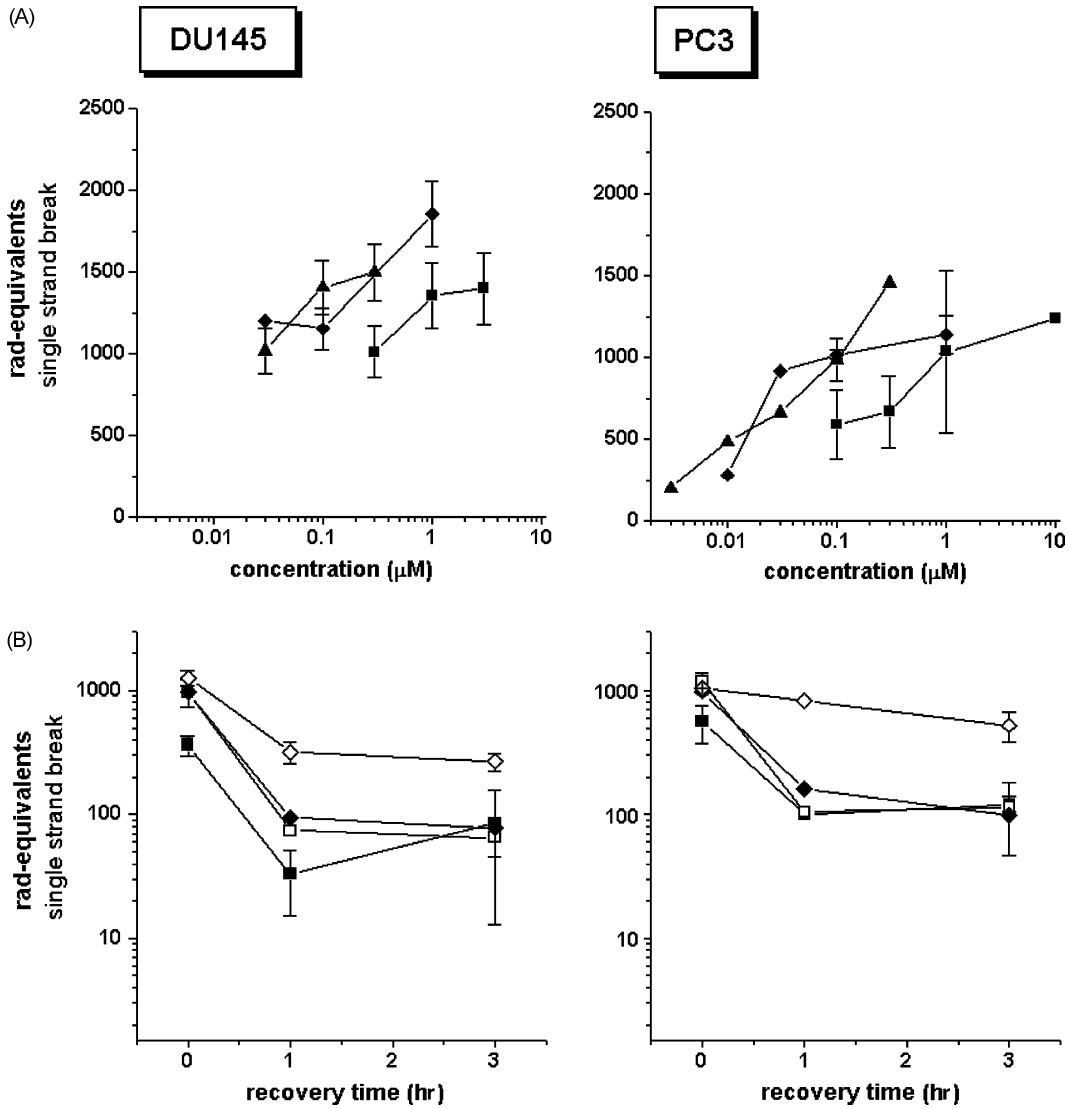


Fig. 5. DNA single-strand break induction (A) and recovery (B) after drug treatment. Cells were exposed to increasing drug concentrations for 1 hr and (A) lysed for measurement of single-strand breaks or (B) incubated with drug-free medium for the indicated times and then lysed. Lysis was performed in the presence of proteinase K. See Section 2 for details. The results, expressed as rad-equivalent, are the mean ± SD of two independent experiments. (A) (■) Topotecan; (▲) ST1480; (◆) ST1481. (B) (■) Topotecan, 0.1 μM; (□) Topotecan, 1 μM; (◆) ST1481, 0.1 μM; (◇) ST1481, 1 μM.

of replication-encounter lesions depends on the stability and persistence of the ternary enzyme–DNA–drug complex. Shortly after drug exposure (i.e. 6 hr incubation in drug-free medium) an appreciable extent of DNA double-strand breaks was detected only in DU145 cells treated with ST1481 (Fig. 6). These lesions could not be ascribed to an early apoptotic cleavage because apoptosis induction by the drug was not evident up to 48 hr (data not shown).

3.5. Effects on cell cycle progression and DNA synthesis

Cell cycle perturbations were examined at various times after treatment with equitoxic doses of each compound (Fig. 7). Already at 24 hr after treatment around 50% of DU145 cells were found in S phase. At 72 hr after treatment, the percentage of S phase cells decreased to 35%. At the same time, the percentage of untreated cells in S phase was less than 15%. In PC3 cells topotecan induced a transient arrest in G2/M phase, whereas a persistent (up to 96 hr) accumulation was observed in cells treated with ST1480 or ST1481.

In DU145 cells, which exhibited accumulation in S phase following drug exposure, topotecan caused a time-dependent reduction of BrdU incorporation, suggesting slow DNA replication in treated cells. This effect was more evident in ST1481-treated cells. In PC3 cells BrdU incorporation was found only at 24 hr in S and G2 phases of treated cells (Fig. 8). On the contrary, at 48 hr PC3 cells arrested in G2 did not show appreciable BrdU incorporation. The behavior is consistent with a fast progression of PC3 cells through S phase and supports a cytostatic effect of the drugs.

The pattern of the PCNA-insoluble form distribution in cell cycle was examined 24 hr following drug treatment

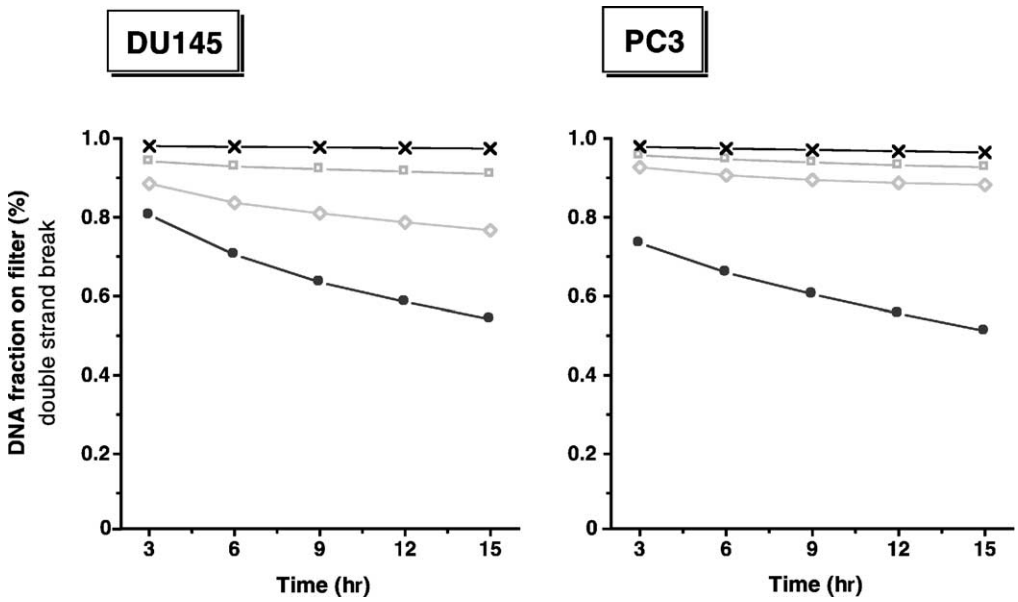


Fig. 6. DNA double-strand breaks induced by camptothecin derivatives in DU145 and PC3 cells. Cells were treated for 1 hr with the equitoxic and equimolar concentrations (1 μM). Alkaline elution was performed, in the presence of proteinase K, 6 hr after drug removal. The results are the mean ± SD of two independent experiments. (×) Untreated control; (●) 3000 rad; (□) topotecan; (◇) ST1481.

(Fig. 9). Whereas high levels of PCNA were present in S phase in control DU145 cells, treated cells did not exhibit positivity for PCNA. The low levels of PCNA in S phase-arrested DU145 cells following treatment are likely due to the disassembly of PCNA from DNA replication sites as a consequence of DNA breaks and replication fork arrest. A similar phenomenon has already been described in UV-irradiated cells at later times after DNA damage [23]. This finding is consistent with a low BrdU incorporation. On the contrary, an increase in PCNA immunofluorescence was observed only in G2-arrested PC3 cells, suggesting DNA repair synthesis.

RPA phosphorylation was observed in both cell lines, suggesting that drug-induced DNA damage was recognized during the signaling process in spite of the different perturbation of cell cycle (Fig. 10), since hyperphosphorylation of this protein is a step of the pathway activated by camptothecin-induced DNA lesions [24].

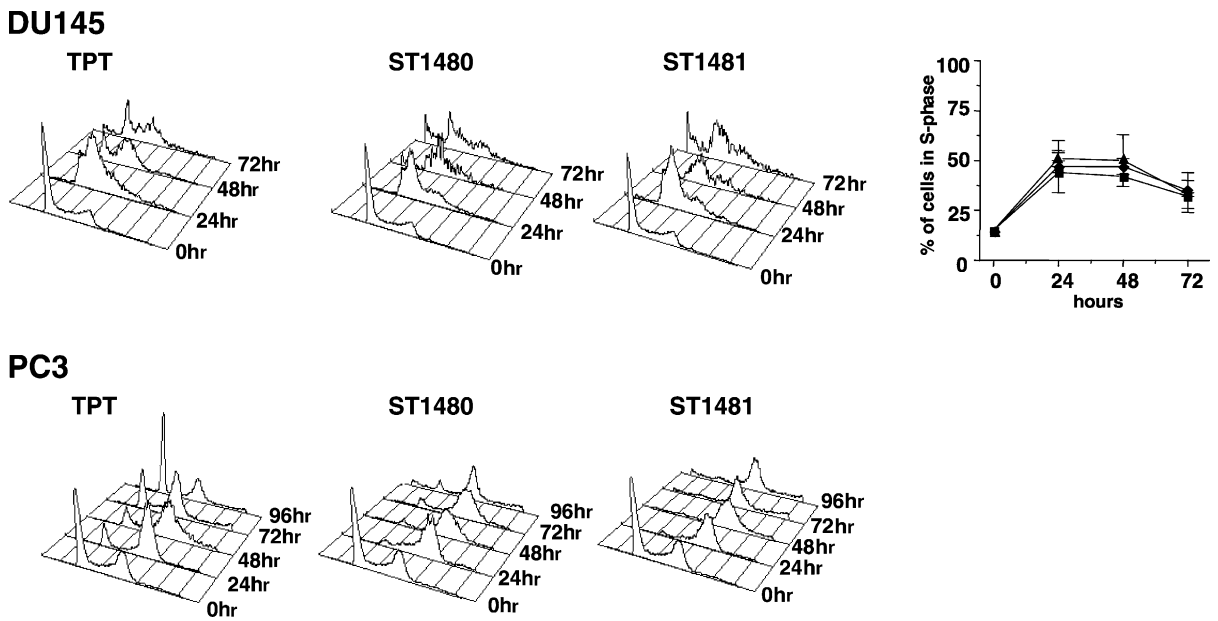


Fig. 7. Cell cycle distribution of DU145 and PC3 cells at various times after drug treatment. Cells were treated for 1 hr with the IC_{80} of each drug and FACS analysis was performed on PI-stained cells. Percentage of DU145 cells in S phase was calculated by LYSYS II software of DNA profiles. (■) Topotecan (TPT); (□) ST1480; (◆) ST1481. Results are from one representative experiment.

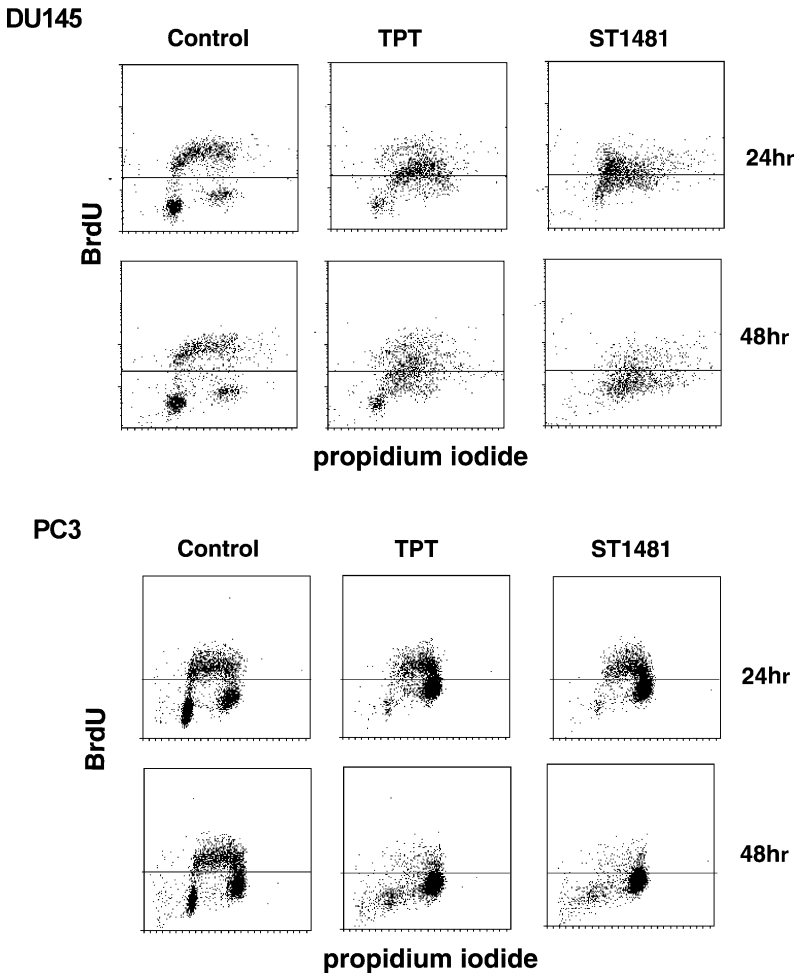


Fig. 8. Effects of camptothecin derivatives on BrdU incorporation. Cells, treated with the IC_{80}, were exposed to BrdU 1 hr before the indicated time and then trypsinized. Fixed cells were incubated with the anti-BrdU antibody and then with an anti-mouse-FITC antibody and counterstained with PI. Fluorescence was determined by FACScan analysis. PI staining (DNA content) is plotted on the abscissa and FITC-fluorescence (BrdU incorporation) is plotted on the ordinate axis. The profiles from one representative experiment are shown. TPT, topotecan.

3.6. cDNA array analysis

In the analysis of gene expression profiles, we have selected those genes whose expression resulted at least 3-fold up- or down-regulated after ST1481 treatment. By comparing treated vs. control cells, we have found that in PC3 cells a number of genes were consistently down-regulated at 6 hr after 1 hr exposure. Table 4 shows

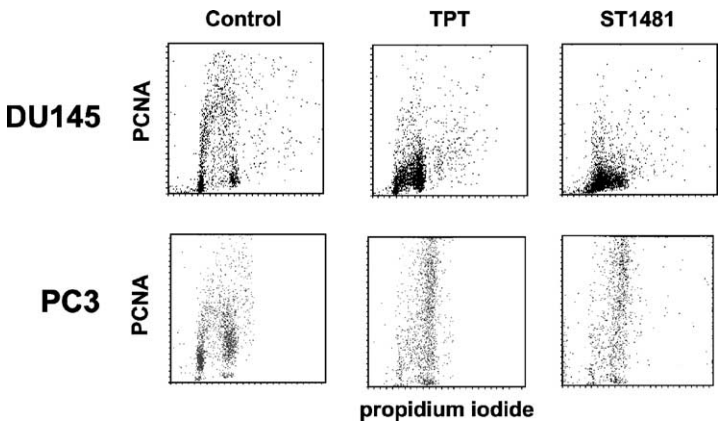


Fig. 9. Flow cytometric analysis of cell cycle distribution of insoluble form of PCNA. Biparametric dot plots of PCNA immunofluorescence vs. DNA content are shown. Twenty-four hours after 1 hr drug exposure to the IC_{80} cells were harvested, lysed, fixed, and processed for PCNA antibody detection and counterstained with PI. Dot plots of a typical experiment are shown. TPT, topotecan.

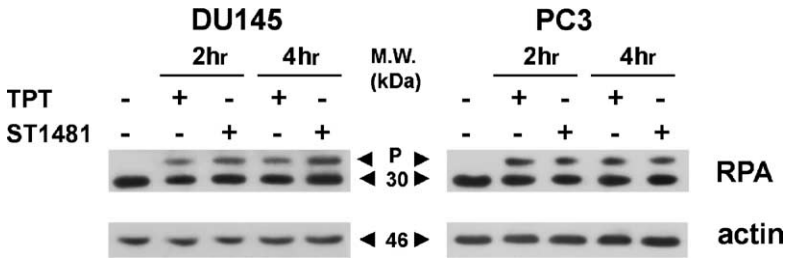


Fig. 10. Western blot analysis of RPA and its phosphorylated form. Cells were exposed to topotecan (TPT) or ST1481 at 10 or 1 μM, respectively, for 1 hr and harvested 2 and 4 hr after treatment. Actin is shown as control of the loading. A representative experiment is shown.

Table 4
Modulation of gene expression in PC3 and DU145 cells at 6 hr after 1 hr exposure to 1 μM ST1481

Gene code		Fold change	Protein
PC3			
DNA damage/stress response			
M18112; J03473	Down	5.8	PARP
J04111	Up	3.5	AP-1
M62399	Up	7.0	NF-κB
DNA replication and repair			
M63488	Down	3.7	RPA70
L07493	Down	3.9	Replication protein A (14-kDa subunit)
M31899	Down	3.9	ERCC3
AY092780	Down	19.0	ERCC2
Apoptosis			
U13737	Down	3.6	Caspase 3
U13021 + U13022	Down	3.8	Caspase 2
U66879	Down	4.0	BAD
DU145			
DNA damage/stress response			
AF078078	Up	31.9	GADD45 gamma
M60974	Up	5.1	GADD45
M62399	Up	3.3	NF-κB
Cell cycle			
X51688	Down	5.3	G2/mitotic-specific cyclin A
M25753	Down	2.1	G2/mitotic-specific cyclin B1
DNA replication and repair			
AF067855	Up	3.1	Geminin
D38073	Up	3.4	MCM3 DNA replication licensing factor
M36089	Up	7.4	XRCC1
Apoptosis			
U76638	Up	9.0	BRCA1-associated ring domain protein (BARD1)

the genes implicated in DNA damage/stress response, DNA repair and replication, and apoptosis. Among the genes identified with this analysis, only NF-κB and AP-1 mRNA were found to be up-regulated in PC3. A completely different pattern of modulation of gene expression was found in DU145 cells. With the exception of genes involved in cell cycle control (i.e. cyclin A and cyclin B1), several transcripts implicated in DNA damage response and DNA replication or repair were found to be up-regulated. Again, this analysis was consistent with a different cellular response of the two cell lines to camptothecin treatment.

4. Discussion

The prostate carcinoma models used in our study are characterized by a loss of p53 function, as a consequence of mutation (DU145 and JCA-1) or lack of expression (PC3). Alterations of p53 have been implicated in apoptosis resistance and, therefore, in a low responsiveness to conventional DNA-damaging agents, but conflicting results have been reported on the role of p53 in response to camptothecins and may reflect a different biological context [25–27]. The present study as well as previous observations in ovarian carcinoma cells with a different p53

status [21] support that alterations involving p53 are not critical determinants of cell sensitivity to camptothecins and this behavior may account for the chemosensitivity of p53-defective prostate carcinoma cells to these agents. The results presented in this study indicated that the lipophilic analog ST1481 was appreciably more effective than topotecan, at least, in terms of rate of complete responses. Several favorable features, including high intracellular drug accumulation and stability of drug–target interaction, could account for the improved efficacy of ST1481 [11] and could represent potential advantages for clinical use.

A surprising observation in this study was a reduced *in vivo* responsiveness of DU145 tumor as compared to PC3 tumor in spite of cell susceptibility to drug-induced apoptosis observed *in vitro*. The different *in vivo* doubling time of PC3 and DU145 tumors (Table 1) and the different cellular pharmacokinetics (Table 3) could contribute to the observed tumor response. However, the impressive *in vivo* responsiveness of PC3 tumor exhibiting a high rate of complete response (60–90% of treated tumors) was somewhat unexpected on the basis of an apparent cytostatic effect and low level of drug-induced apoptosis at cellular level. The cytostatic effect was associated with a G2/M arrest, which was persistent in PC3 cells treated with ST1481 analog. PC3 and DU145 cells displayed drastically different cell cycle perturbations following exposure to camptothecins. In contrast to PC3 cells, which responded to camptothecins by an accumulation in the G2/M phase, drug treatment of DU145 cells led to an arrest in S phase which likely reflects the efficiency of the S phase checkpoint in response to damaged DNA. This interpretation is supported by low rate of DNA synthesis of S phase-arrested cells, as indicated by the time-dependent reduction of BrdU incorporation and the low number of PCNA-positive cells. DNA lesions generated by stabilization of topoisomerase I-containing cleavage complex could be recognized in S phase, thus resulting in apoptotic signals. The early formation of a low but appreciable extent of double-strand breaks and the recognition of these lethal DNA lesions could account for the earlier apoptotic response in DU145 cells. The manner in which the presence of damaged DNA is signaled to control systems is likely different in the two cell systems, since cell death is a delayed event in PC3 cells. A role of cell cycle control at checkpoints activated by DNA damage in the pattern of cellular response of these prostate carcinoma cell lines to camptothecins is also supported by modulation of gene expression detected by cDNA expression arrays analysis performed 6 hr after 1 hr exposure to 1 μM ST1481. In particular, the marked down-regulation of several transcripts involved in DNA damage stress response and DNA repair (e.g. PARP, ERCC2, and ERCC3) in PC3 cells is consistent with a defective S phase checkpoint. Apparently, PC3 cells were able to proceed through S phase, but DNA damage activated the G2 checkpoint which arrested further progression. In contrast, DU145 cells displayed a down-regulation of genes involved in the cell cycle control (cyclin A and cyclin B1) and up-regulation of genes implicated in DNA repair and replication (e.g. geminin, XRCC1, GADD45, MCM3). Specifically, geminin is an inhibitor of DNA replication and could play a role in DNA damage checkpoint since it is known to be present in S phase [28–30]. XRCC1 protein involved in PARP-related DNA repair process could play a relevant role in camptothecin resistance [31]. If our interpretation is correct, it is conceivable that defects in checkpoint pathways are critical determinants of the mode of cell death of prostate carcinoma cells in response to camptothecins. A different pattern of response of these cells following treatment with paclitaxel has been reported [15]. The molecular basis of the different behavior of these cell lines remains unclear. DU145 cells are characterized by p53 mutation and PC3 cells are p53 null [32]. These alterations are consistent with the inability of camptothecins to cause arrest of cells in G1. However, a role for p53 in activation of DNA damage checkpoint, in spite of mutation, could not be ruled out in DU145 cells. A defective S phase checkpoint in PC3 cells was associated with a persistent arrest in G2 and an apparent cytostatic effect. Since camptothecins were more effective *in vivo* against PC3 than DU145 tumor, it is likely that defects in cell cycle control and in DNA damage recognition and repair, resulting in G2 accumulation, are responsible for a delayed cell death. This interpretation is also supported by evidence that the apoptosis levels and the cell-specific perturbations of cell cycle progression were more marked and persistent for the two potent 7-substituted camptothecin analogs as compared to topotecan. Our results support that S and G2 arrests play a different role in the cytotoxic mechanism of camptothecins. This interpretation is consistent with the recent observation that loss of ATR-dependent checkpoint function, involved in response to DNA damage during S phase, sensitizes cells to camptothecins [33]. However, it is likely that the relevance of such mechanisms in determining chemosensitivity is dependent on the biological context which might favor or inhibit protective actions (e.g. stimulation of DNA repair or sensitization to apoptosis). Thus, on the basis of the complexity of determinants involved in cytotoxic/antitumor response, the influence of cell cycle perturbations, mode of cell death, as well as p53 status on the chemosensitivity to camptothecins could not be easily predicted.

In conclusion, the novel analog ST1481 was consistently more effective than topotecan in all tested tumors, and may be considered for the clinical evaluation in the treatment of prostate carcinoma. The improvement of drug efficacy is likely related to the favorable pharmacological profile [9]. p53 mutation was not a determinant of resistance, but p53 status could influence cell cycle checkpoints which may have a role in cytotoxic response. The *in vivo* efficacy of the tested camptothecins was not predicted by the cellular response. In fact, in spite of the susceptibility of DU145

cells to undergo drug-induced apoptosis, the responsiveness of DU145 xenotransplants to *in vivo* treatment was substantially lower than that of PC3 tumor model which responded *in vitro* to drug treatment with an apparent cytostatic effect. A tentative explanation of the lack of predictive value of *in vitro*-induced apoptosis is that only a fraction of DU145 cells activate a pathway resulting in cell death, but a substantial number of cells arrested in S phase are able to recognize and repair DNA damage. Conversely, it is conceivable that cell death in PC3 tumor following treatment is a delayed event consequent to persistent arrest in G2 phase and insufficient repair of DNA damage. We suggest that, in addition to the extent and persistence of topoisomerase I-mediated DNA damage, cell cycle checkpoint pathway regulation and signaling pathways are critical determinants of tumor responsiveness to camptothecins.

Acknowledgments

Grant sponsors: Associazione Italiana per la Ricerca sul Cancro (Milan), by the Ministero della Sanità (Rome), and by the Consiglio Nazionale delle Ricerche (Rome), Italy.

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