Preclinical profile of antitumor activity of a novel hydrophilic camptothecin, ST1968

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Abstract

ST1968 is a novel hydrophilic camptothecin (CPT) derivative of the 7-oxyiminomethyl series. Because ST1968 retained ability to form remarkably stable cleavable complexes, this study was done to investigate its preclinical profile of antitumor activity in a large panel of human tumor models, including irinotecan-resistant tumors. Although less potent than SN38 *in vitro*, i.v. administered ST1968 caused a marked tumor inhibition, superior to that of irinotecan, in most tested models. ST1968 exhibited an impressive activity against several tumors including models of ovarian and colon carcinoma in which a high rate of cures was observed. In the most responsive tumors, complete and persistent tumor regressions were achieved even with low suboptimal doses. Even tumors derived from intrinsically resistant cells exhibited a significant responsiveness. Histologic analysis of treated tumors supports a contribution of both proapoptotic and antiangiogenic effects to ST1968 antitumor efficacy. A study done in yeast cells transformed with CPT-resistant mutant forms of topoisomerase I documented that, in contrast to other tested CPT, ST1968 was active against yeasts expressing the mutant K720E enzyme. Based on its outstanding efficacy superior to that of irinotecan and of its good therapeutic index, ST1968 has been selected for clinical development. [Mol Cancer Ther 2008;7(7):2051–9]

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Introduction

The recognition that DNA topoisomerase I is a useful target for the design of effective antitumor agents has stimulated efforts focused on the identification of novel topoisomerase I inhibitors. A large number of camptothecin (CPT) derivatives and non-CPT topoisomerase I inhibitors are being developed in the attempt to improve the therapeutic/pharmacologic profile of the clinically available CPT analogues—topotecan and irinotecan (1–4). The design of novel CPT derivatives is aimed at overcoming the primary limitations of known CPT, including lactone instability, reversibility of the drug interaction in the cleavable complex, and drug resistance (5–9).

Recent evidence supports that lipophilicity confers potential advantages to CPT in terms of cytotoxic potency likely related to a rapid intracellular accumulation and lactone stability as documented by the efficacy of the 7-substituted lipophilic CPT derivatives of the 7-oxyiminomethyl series (10–14). However, our recent study documents that also hydrophilic 7-modified CPT analogues are able to form stable DNA-topoisomerase cleavable complexes (15). In particular, we have found that, among CPT derivatives of the 7-oxyiminomethyl series, two analogues characterized by the presence of a free amino group in the side chain at the 7-position exhibit persistent topoisomerase I-DNA cleavage complexes (15). Based on this observation, the present study was undertaken to explore the pharmacologic profile of ST1968, the most hydrophilic analogue of the series. The effects of ST1968 were evaluated in a large panel of human tumor cell lines and xenografts as well as against some mutant forms of human topoisomerase I in yeast.

Materials and Methods

Drugs

ST1968 and analogues were synthesized as described previously (12). For *in vivo* studies, ST1968 was dissolved in sodium lactate buffer (50 mmol/L) adjusted to pH 4.0 with addition of hydrochloric acid. Irinotecan (CPT11) and topotecan were dissolved in sterile distilled water. The drugs were administered i.v. very slowly in a volume of 10 mL/kg body weight. For *in vitro* studies, ST1968 and ST1976 were dissolved in sterile saline, whereas ST1481 was dissolved in DMSO.

Saccharomyces cerevisiae Yeast Strain and Yeast Spot Test

EKY3 *S. cerevisiae* strain (MATα, *ura*3-52, *his*3Δ200, *leu*2Δ1, *trp*1Δ63, and *top1*Δ:TRP1) was transformed with YCpGAL1, YCpGAL1-hTOP1, YCpGAL1-hTOP1G363C, YCpGAL1-hTOP1K720E, or YCpGAL1-hTOP1A653P. YCpGAL1-hTOP1G363C, YCpGAL1-hTOP1K720E, and YCpGAL1-hTOP1A653P plasmids express the mutant (G363C,

K720E, and A653P) form of the human DNA topoisomerase I under the control of galactose-inducible GAL1 promoter. Yeast cells transformed by treatment with lithium acetate (16) were maintained at 30°C in synthetic complete medium lacking uracil (uracil-) and supplemented with 2% glucose. For yeast spot test, cells were growth at 30°C in uracil- medium to an A_{595} of 0.3. Aliquots of serial 10-fold dilutions were spotted onto plate uracil- plus 2% galactose containing 45 μmol/L CPT analogues. Following 3 days of incubation at 30°C, images were acquired using the IMAGE MASTER VDS (Amersham Pharmacia Biotech).

Molecular Modeling

Three-dimensional molecular model of ST1968 was built on a Silicon Graphics O2 using the programs Insight II and Discover (Accelrys). Minimizations were done with the AMBER all-atom force field (17, 18) and the conjugate gradients algorithm. For atomic partial charges of the ligand atoms, Mulliken charges calculated on the minimized structure using the MOPAC program (18, 19) with the MNDO Hamiltonian were used. The structure of the ternary complex containing topoisomerase I, DNA, and topotecan was downloaded from the Protein Data Bank (PDB entry 1K4T). All the atoms were fixed according to AMBER atom type and hydrogens were added. The structure of ST1968 was appropriately overlapped with the structure of topotecan in the ternary complex, and the structure of topotecan was then deleted. The new complex was energy refined using the AMBER force field. During energy minimization, the structure of ST1968 was allowed to relax, whereas the structure of the protein-DNA complex was frozen.

Assessment of Cytotoxic Potency on Human Cancer Cell Lines

The panel of human tumor cell lines includes the non-small cell lung cancer H460, the colorectal adenocarcinoma HT29, SW620, HCT116, and LoVo, the ovarian carcinoma IGROV-1, A2780, and the corresponding doxorubicin-resistant subline (A2780/DX) and cisplatin-resistant subline (A2780/DDP), and the prostate carcinoma PC3, DU145, and the corresponding CPT-resistant subline (DU145RC1). Cells were cultured in RPMI 1640 containing 10% FCS. Cytotoxicity was assessed by growth inhibition assay after 2 h drug exposure, because this time allowed a maximum drug uptake by all tested cell lines (20). Twenty-four hours after seeding in well plates, cells were exposed to the drugs and harvested 72 h later and surviving cells were determined by MTT assay. For most cell lines, 72 h represent ~3 cell doublings. IC_{50} is defined as the drug concentration causing 50% cell growth inhibition determined by dose-response curves.

Apoptosis and Cell Cycle Analysis

Apoptosis was determined at 72 and 144 h after 1 h treatment, and cell cycle distribution was analyzed at 24 h. For the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Roche), cells were fixed in 4% paraformaldehyde and processed according to the manufacturer's instruction. The samples were analyzed on a FACScan (Becton Dickinson). For the Western blot analysis of apoptosis-related proteins, cells were lysed in hot sample buffer as described previously (21). After determination of protein concentration, whole-cell extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Filters were incubated with monoclonal antibody to poly(ADP-ribose) polymerase-1 (Oncogene Sciences) or with rabbit antibodies to cleaved caspase-3 (Cell Signaling Technology) or actin (Sigma) used as a control for loading.

For the cell cycle analysis, cells were washed, fixed in ice-cold 70% ethanol, and stored at −20°C. After rehydration in PBS, cells were stained with 10 μg/mL propidium iodide (Sigma) in PBS containing RNase A (66 units/mL; Sigma) for 18 h. The samples were processed by FACScan flow cytometry, and data were analyzed by ModFit software (Becton Dickinson). At least 40,000 cells were considered for DNA content.

Tumor Models and Evaluation of Antitumor Activity

All experiments were carried out using female athymic Swiss nude mice aged 7 to 10 weeks (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 Fondazione IRCCS Istituto Nazionale Tumori according to institutional guidelines.

Exponentially growing tumor cells (10^7 per mouse) were s.c. injected into the right flank of athymic nude mice. Tumor lines were achieved by serial s.c. passages of fragments (~2 × 2 × 6 mm) from growing tumors into healthy mice as described previously (22). Tumor growth was followed by biweekly measurements of tumor diameters with a Vernier caliper. Tumor volume (TV) was calculated according to the formula: TV (mm^3) = $d^2 \times D / 2$, where d and D are the shortest and longest diameters, respectively. Drugs were delivered i.v. every fourth day for four times (q4d × 4) starting when tumors were in the range of 50 to 100 mm^3. Experimental groups were eliminated when mean TV was ~1,500 mm^3.

The efficacy of the drug treatment was assessed as follows:

(*a*) TV inhibition percentage (TVI%) in treated versus control mice calculated as TVI% = 100 - (mean TV treated / mean TV control × 100).

(*b*) complete response (CR) defined as tumor disappearance lasting for at least 10 days during or after treatment period.

(*c*) no evidence of disease (NED) at the end of experiment, at least 80 days after treatment end.

The toxicity of the drug treatment was determined as body weight loss and lethal toxicity. The highest body weight loss percentage induced by treatment is reported in the tables. Deaths occurring in treated mice before the death of the first control mouse were ascribed to toxic effects.

Histologic Examinations

When tumor lesions (A2780 and DU145) reached a volume of ~200 to 400 mm^3, 14 mice were treated with

ST1968 (30 mg/kg, q4d × 2 i.v.). Twenty-four hours after the second administration, mice were sacrificed by cervical dislocation. Tumor lesions were excised, split in two parts and fixed in zinc fixative for 24 h, dehydrated with ethanol, and paraffin embedded. Serial sections (4 μm thick) were stained with H&E or employed for immunohistochemistry. For the latter, endogenous peroxidase activity were blocked by incubation in 0.3% hydrogen peroxide and the nonspecific antibody binding was avoided by incubating with 10% normal rabbit or goat serum in TBS. Tumor sections were then incubated with anti-mouse PECAM/CD31 polyclonal antibody (BD PharMingen) at 2 μg/mL concentration for 1 h at room temperature. This step was followed by the hybridization with biotinylated rabbit anti-rat IgG followed by a streptavidin-horseradish peroxidase conjugate. Bound antibody was revealed using the substrate 3,3′-diaminobenzidine as chromogen. Sections were counterstained with hematoxylin. Images were captured by a Hamamatsu camera (Hamamatsu City) connected to a Nikon microscope. Blinded evaluation of vascularization (number of tumor vessels per mm^2) and mitotic index (mitotic cells per mm^2) at ×200 magnification was done by two observers, with an intervariability of <5%.

To investigate tumor apoptosis, rehydrated sections were stripped from proteins through incubation with 300 units/mL proteinase K (Sigma-Aldrich) for 15 min at 37°C, apoptotic nuclei were revealed by TUNEL, and the TUNEL labeling index (positive nuclei per mm^2) was calculated as reported previously (23).

Statistical Analysis

Student's *t* test (unpaired, two-tailed) was used for statistical comparison of TV. Results from the histochemistry studies concerning apoptosis, mitotic index, and vascularization were analyzed by the Mann-Whitney rank test. Analysis were done with GraphPad Prism (GraphPad Software).

Results

Cellular Pharmacology Studies

The antiproliferative effects of ST1968 were determined at 72 h, following 2 h drug exposure, on different cell lines of various tumor types (Table 1). Topotecan and SN38, the active metabolite of irinotecan, were used as reference drugs. In general, SN38 was more potent than ST1968 and topotecan. ST1968 was as potent as SN38 in reducing the proliferation of the ovarian A2780, which was the most sensitive cell line, but less active against the two cell lines with acquired resistance to doxorubicin or cisplatin. As observed for SN38, under the used conditions (short-term exposure), colon carcinoma cell lines were very sensitive to ST1968 (IC_{50}, range, 0.070-0.216 μmol/L). In contrast to what observed with SN38, prostate carcinoma cells exhibited a low sensitivity to both ST1968 and topotecan (IC_{50} >> 1 μmol/L). Against the DU145RC1, with acquired resistance to CPT, all the three compounds were less effective than against the parental DU145 cell line.

As expected based on the mechanism of action, cytotoxic concentrations of ST1968 caused a dose-dependent arrest in G_2-M phase of cell cycle in colon carcinoma cells HT29 and SW620, and the effect was already evident at 24 h after treatment (Fig. 1A). A time-dependent marked increase of the sub-G_1 fraction, more evident in SW620 cells, suggested induction of cell death. Indeed, the TUNEL assay supported a dose-dependent induction of apoptosis in both cell lines (Fig. 1B; data not shown). However, the onset of apoptosis was delayed in the HT29 cell system, which exhibited a large number of mitotic cells at 72 h (55 ± 5%) but a massive apoptosis only at 144 h (75 ± 10%). The different apoptotic response in the two cell lines was also documented by poly(ADP-ribose) polymerase-1 and caspase activation, which was delayed in HT29 cells (Fig. 1C).

Table 1. Antiproliferative activity of ST1968, SN38, and topotecan on different tumor cell lines

Tumor type	Cell line	IC_{50} (μmol/L)*		
		ST1968	SN38 (R)	Topotecan (R)
Ovarian carcinoma	A2780	0.041 ± 0.007	0.041 ± 0.0078 (1)	0.17 ± 0.06 (4.1)
	A2780/DX	0.45 ± 0.012	0.0195 ± 0.001 (0.04)	0.315 ± 0.01 (0.7)
	A2780/DDP	0.355 ± 0.071	0.036 ± 0.0007 (0.1)	0.063 ± 0.024 (0.2)
	IGROV-1	0.673 ± 0.117	0.292 ± 0.051 (0.4)	2.613 ± 0.660 (3.9)
Colon carcinoma	HT29	0.133 ± 0.035	0.390 ± 0.014 (2.9)	0.092 ± 0.035 (0.7)
	SW620	0.07 ± 0.02	N.D.	N.D.
	HCT116	0.105 ± 0.027	0.027 ± 0.007 (0.25)	0.371 ± 0.075 (3.5)
	LoVo	0.216 ± 0.061	0.050 ± 0.015 (0.2)	0.400 ± 0.109 (1.85)
Lung carcinoma	H460	0.43 ± 0.09	0.21 ± 0.01 (0.5)	1.03 ± 0.03 (2.4)
Prostate carcinoma	DU145	1.149 ± 0.394	0.269 ± 0.083 (0.2)	4.082 ± 1.37 (3.55)
	DU145RC1	4.711 ± 0.627	0.749 ± 0.079 (0.15)	3.979 ± 0.331 (5.3)
	PC3	6.253 ± 2.200	1.310 ± 0.29 (0.2)	8.88 ± 2.49 (6.8)

Abbreviation: R, ratio of IC_{50} over that of ST1968.
*IC_{50}, drug concentration required for 50% reduction of cell viability compared with untreated controls after 2 h exposure to the drug. Mean ± SD of at least three experiments.

Antitumor Activity Studies

The antitumor activity of ST1968, administered i.v. with an intermittent q4d × 4 schedule, was studied in a large panel of 21 human tumor xenografts of various tumor types (Table 2), including those investigated in *in vitro* studies. Irinotecan was used as a reference drug in the range of optimal doses (50-60 mg/kg) in most experiments, whereas topotecan (10-15 mg/kg) was used only in few models, including ovarian carcinoma. In previous studies aimed at identifying the maximum tolerated dose of i.v. treatment with irinotecan, we have found that doses higher than 60 mg/kg caused an unacceptable rate of immediate deaths. This acute effect, not related to the drug cytotoxicity, has been already described by other authors (24). The tolerability of bolus i.v. injection is likely related to the mouse strain (25). Because, under our conditions, 60 mg/kg irinotecan and 35 mg/kg ST1968 still caused a variable number of immediate deaths (~10%), in most experiments, we used 50 and 30 mg/kg for irinotecan and ST1968, respectively. These dose levels (~85% of the respective maximum tolerated dose) were well tolerated for both compounds with minimal delayed deaths.

In the majority of tested tumor models, the i.v. administration of ST1968 caused a dramatic inhibition of tumor growth, including complete regression. It is noteworthy that, in the most responsive tumors (e.g., ovarian carcinoma A2780 and its resistant sublines; colon carcinoma SW620; small cell lung cancer H69, larynx carcinoma Hep2), a complete tumor regression was achieved even with low suboptimal doses (15-17.5 mg/kg). In the range of well-tolerated doses, the antitumor effect was persistent with no evidence of tumor regrowth (NED) at the end of the experiments. In the same tumor models, the rate of CR and NED observed in mice treated with irinotecan or topotecan was definitely lower. In other tumor models, the effect of ST1968 was less impressive, with a lower rate of CR. Against the ovarian carcinoma IGROV-1, the non-small cell lung cancer H460 and A549, the colon carcinoma LoVo, the mesothelioma MESO, the pancreas carcinoma MiaPaCa2, and the bladder carcinoma HT1736, the efficacy of ST1968 was significantly superior to that of irinotecan (Table 2). In particular, ST1968 was active against some intrinsically resistant tumors (IGROV-1, A549, and HT1736), in which irinotecan was unable to inhibit tumor growth by >50%. ST1968, irrespective of the dose used, was more effective than the maximum tolerated dose of irinotecan (60 mg/kg), in terms of tumor growth inhibition, against H460, MiaPaCa2, and HT29 models and, in terms of CR or cure rates, in A2780, HCT116, and H69 tumor xenografts. Comparable efficacies with those achieved by

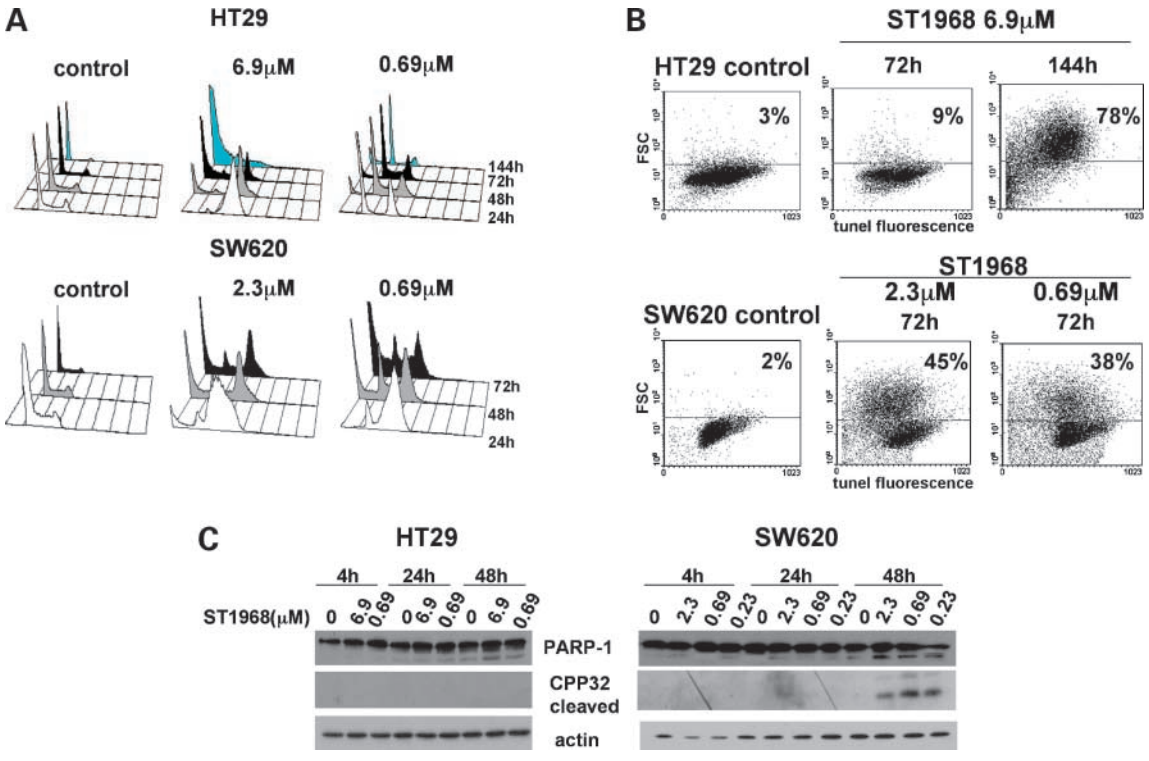

Figure 1. **A,** cell cycle analysis of drug-treated cells. Cells were treated for 1 h with ST1968 at equimolar (0.69 μmol/L) or equitoxic concentrations (IC_{80}, 2.3 and 6.9 μmol/L in SW620 and HT29 cells, respectively) and analysis was done at 24 h following treatment. Representative of three independent experiments. **B,** drug-induced apoptosis in SW620 and HT29 cells. Apoptosis was determined at 72 or 144 h following 1 h exposure to the drug by TUNEL assay and analyzed by flow cytometry. The results are expressed as percentage of apoptotic cells versus total cell number. Representative of three independent experiments. **C,** Western blot analysis of poly(ADP-ribose) polymerase-1 and caspase-3 cleavage. Representative of three independent experiments.

Table 2. Pattern of antitumor activity of i.v. ST1968 in human tumor xenografts of diverse tumor type

Tumor type	Model	Drug*	Dose (mg/kg)	TVI (%)†	CR‡	NED§	Body weight loss‖ (%)
Ovarian carcinoma	A2780	ST1968	3.75	83	0/8		1
			7.5	96	2/8	0/8	3
			15	100	8/8	0/8	7
			20	100	8/8	4/8	8
			30	100	7/7	7/7	11
			35	100	7/7	7/7	15
		CPT11	60	100	7/7	1/7	9
		TPT	15	99	2/6	0/6	16 (2/8)
	A2780/DX	ST1968	15	99	6/8	1/8	4
			30	100	8/8	0/8	6
		TPT	10	85	1/6	0/6	4 (1/7)
	A2780/DDP	ST1968	15	100	8/8	5/8	6
			30	100	7/7	7/7	13 (1/8)
		TPT	10	93	1/8	1/8	4
	IGROV-1	ST1968	25	72¶	0/10		4
			30	93¶	3/10	0/10	0
		CPT11	50	40	0/10		5
Lung carcinoma	H69 (small cell lung cancer)	ST1968	17.5	100	8/8	8/8	6
		CPT11	60	100	6/8	3/8	7
	A549 (non-small cell lung cancer)	ST1968	25	83¶	0/8		11
		CPT11	50	42	0/8		4
	H460 (non-small cell lung cancer)	ST1968	12.5	71	0/8		5
			25	88	0/8		7
			35	97¶	0/7		20 (1/7)
		CPT11	60	85	0/8		8
		TPT	15	82	0/8		12 (1/8)
Colon carcinoma	CoBA	ST1968	25	92	0/8		5
		CPT11	50	86	0/8		2
	CoRA	ST1968	30	82	0/10		15
		CPT11	50	74	0/10		8
	LoVo	ST1968	30	98¶	0/12		9
		CPT11	50	93	0/12		3
	SW620	ST1968	15	100	8/8	2/8	6
			30	100	8/8	3/8	10
		CPT11	30	100	8/8	0/8	2
			60	100	8/8	2/8	10
Colon carcinoma	HCT116	ST1968	3.12	74	0/8		2
			6.25	84	0/8		3
			12.5	97	1/8	1/8	5
			25	99	2/8	2/8	9
			35	97	4/6	2/6	10 (2/8)
		CPT11	60	97	1/8	1/8	8
	HT29	ST1968	25	85	0/8		8
			35	97¶	3/7	0/7	13 (1/7)
		CPT11	60	79	0/8		7
Mesothelioma	MESO	ST1968	30	92¶	2/12	2/12	12
		CPT11	50	76	0/12		7
Bladder carcinoma	HT1736	ST1968	30	77¶	1/12	1/12	12
		CPT11	50	46	0/10		9
Pancreas carcinoma	MiaPaCa2	ST1968	30	89¶	0/8		7
		CPT11	60	69	0/8		0
Melanoma	501Mel	ST1968	30	68	0/10		16
		CPT11	50	70	0/10		18
Larynx carcinoma	Hep2	ST1968	7.5	86	2/8	1/8	6
			15	100	8/8	8/8	10
		TPT	12	82	2/7	1/7	9 (1/8)

(*Continued on the following page*)

Table 2. Pattern of antitumor activity of i.v. ST1968 in human tumor xenografts of diverse tumor type (Cont'd)

Tumor type	Model	Drug*	Dose (mg/kg)	TVI (%)†	CR‡	NED§	Body weight loss‖ (%)
Prostate carcinoma	DU145	ST1968	30	75	0/8		13
		CPT11	60	82	0/8		2
	DU145RC1	ST1968	30	65	0/8		15
		CPT11	60	45	0/8		10
	PC3	ST1968	25	97	1/8	1/8	10
		CPT11	60	99	1/8	1/8	8

*Drugs were administrated i.v. with the intermittent q4d × 4 schedule.
†%TVI in treated over control mice.
‡CR: disappearance of tumors lasting at least 10 d.
§Number of free tumor mice on total mice at the end of the experiment.
‖Body weight loss % induced by drug treatment (lethal toxicity in parentheses: number of dead mice / total number of mice).
¶$P < 0.05$ versus CPT11, by Student's t test (two tailed).

the reference drugs (irinotecan or topotecan) were observed in the colon carcinoma SW620, CoBA, and CoRA, in melanoma 501Mel, and in the prostate carcinoma DU145 and PC3. Table 3 summarizes the antitumor efficacy profile of ST1968 and irinotecan in the tumor panel.

Some of the tumors with natural or acquired resistance included in the panel (A2780/DX, A2780/DDP, IGROV-1, and the prostate carcinomas) in *in vivo* studies were much more responsive (Table 2) than could be expected based on *in vitro* chemosensitivity studies (Table 1), thus suggesting that mechanisms other than a direct tumor cytotoxic effect could contribute to the antitumor activity of ST1968. To clarify the effects of ST1968 at the cellular level, a histologic analysis of treated and control tumors was done on two tumor xenografts with different sensitivity, the highly responsive ovarian carcinoma A2780 and the moderately responsive prostate carcinoma DU145. Tumors were removed and fixed 24 h after the second treatment (30 mg/kg q4d). Tumors slices were analyzed to assess the mitotic index, the apoptosis induction (by TUNEL assay), and the microvessel density (by $CD31^+$ vessel cells; Table 4). The mitotic index was significantly reduced only in the sensitive tumor while remained unchanged in the prostate tumor. Apoptosis was stimulated by ST1968 in both tumor xenografts, with a higher effect in the ovarian tumor A2780 ($P < 0.01$ versus control tumor). A strong reduction of microvessel density was observed in the highly sensitive ovarian carcinoma A2780 ($P < 0.001$ versus untreated tumors) as well as in the less responsive prostate carcinoma DU145 ($P < 0.05$ versus untreated tumors). A comparable effect on angiogenesis was observed in KB model, a human squamous cell carcinoma characterized by a low susceptibility to apoptosis (Table 4). Such results suggest that the antiangiogenic effect is a relevant component of the antitumor efficacy of ST1968.

ST1968 Inhibits the Viability of Yeast Cells Expressing K720E Mutant Human DNA Topoisomerase I

To evaluate the activity of ST1968 on mutant forms of human topoisomerase I, *S. cerevisiae* yeast cells lacking endogenous topoisomerase I and transformed with YCp-GAL1, YCpGAL1-hTOP1, YCpGAL1-hTOP1G363C, YCp-GAL1-hTOP1K720E, or YCpGAL1-hTOP1A653P were used (26–28). Yeast spot test indicated that all the tested CPT reduced the growth of yeast cells expressing wild-type human topoisomerase I (Fig. 2A and B). Cells transformed with YCpGAL1-hTOP1G363C or YCpGAL1-hTOP1A653P were resistant to all the CPT, whereas only ST1968 was active in reducing the viability of yeast cells expressing the mutant K720E enzyme.

In the attempt to understand the molecular basis of the activity of ST1968 against the K720E mutant enzyme, the binding of the drug with topoisomerase I-DNA complex was examined by molecular docking to predict the conformation of the analogue when bound in the ternary complex (Fig. 2C). In this model, Lys^{720} is far from the drug-binding site (minimum distance 12 Å). When the

Table 3. Comparison of antitumor efficacy of ST1968 and irinotecan (CPT11)

	HS* (%)	S* (%)	PR* (%)	R* (%)	CR† (%)	NED‡ (%)
ST1968	6/21 (29)	11/21 (52)	4/21 (19)	0/21 (0)	6/21 (29)	4/21 (19)
CPT11	3/18 (17)	6/18 (33)	5/18 (28)	4/18 (22)	2/18 (11)	0/18 (0)

Abbreviations: HS, highly sensitive, 100% TVI; S, sensitive, TVI, 80%-99%; PR, partially resistant, TVI, 50-79%; R, resistant, <50% TVI.
*Number of tumor models / total number of tested tumors.
†CR: number of models exhibiting 100% of CR / total tumor models.
‡NED, number of models with occurrence of 100% of NED at autopsy / total tumor models.

Table 4. Histologic analysis of tumor xenografts from different tumor types treated with ST1968 (30 mg/kg, q4d×2, iv)

Tumor type	Model	Treatment	Mitotic index	Apoptosis (TUNEL/mm²)	Vascularization CD31⁺ vessels/mm²
Ovarian carcinoma	A2780	Untreated	51.4 ± 1.2	19.4 ± 1.2	17.7 ± 1.0
		ST1968	5.1 ± 0.9*	28.2 ± 0.7†	7.2 ± 0.8*
Prostate carcinoma	DU145	Untreated	35.7 ± 4.3	35.5 ± 3.5	30.4 ± 3.7
		ST1968	30.3 ± 2.8	53.5 ± 3.2	10.4 ± 6.2‡
Squamous cell carcinoma	KB	Untreated	N.D.	14.2 ± 1.1	19.7 ± 1.4
		ST1968		15.3 ± 1.0	7.3 ± 0.6*

NOTE: Tumor lesions were collected at a volume of ~250 to 400 mm³ 24 h after the last administration. Mean ± SE of 14 samples (untreated) and 14 samples (treated groups).

*$P < 0.001$ versus untreated (Mann-Whitney).

†$P < 0.01$ versus untreated (Mann-Whitney).

‡$P < 0.05$ versus untreated (Mann-Whitney).

Lys[720] was substituted with Glu, the obtained refined structure was very similar to the one of the wild-type enzyme; also in this case, there was no direct interaction between ST1968 and Glu[720].

Discussion

The 7-substituted CPT analogue ST1968 was selected for preclinical development based on its hydrophilic properties and persistent topoisomerase I-DNA cleavage complex (15). The stabilization of the cleavable complex is consistent with the hypothesis that the free amino group at the 7-position is capable to form hydrogen bonds with water as observed for other analogues (29). Although the incorporation of the hydrophilic groups in the side chain at the 7-position is expected to reduce drug uptake and intracellular accumulation, ST1968 retained an appreciable growth-inhibitory potency comparable with that of SN38 in some cell lines (A2780 and HT29) and generally superior to that of topotecan.

The results presented in this work indicate that ST1968 is very effective against several human tumor models, including tumors relatively resistant to irinotecan. In a panel of s.c. implanted human tumor xenografts, the activity of ST1968 was generally superior to that of irinotecan and topotecan. Moreover, in the most responsive tumors (A2780, H69, Hep2, and SW620), an outstanding efficacy was achieved even with suboptimal doses, which produced a high rate of complete tumor regression and a persistent effect (no evidence of tumor regrowth). Such results support a favorable therapeutic index of ST1968, because, in contrast to irinotecan, curative efficacy could be achieved at doses even lower than 50% of the maximum tolerated dose. The finding may represent an important feature for the clinical development of ST1968, taking into account the well-known dose-limiting toxicities of CPT (30–32). The improved efficacy and the good therapeutic index of ST1968 could support an increased tumor selectivity, which likely reflected a favorable drug accumulation in the tumor rather than a differential sensitivity of tumor cells (20). Because there is no evidence of drug metabolism *in vivo* (data not shown), the mechanism of cytotoxic activity is expected to be similar to that of conventional CPT. The lack of metabolism and the favorable pharmacokinetic behavior of ST1968 could account for the improved toxicity profile. In contrast to irinotecan, the primary side effect of ST1968 was myelosuppression (as already observed with other CPT analogues of the same series; refs. 6, 33), whereas damage to gastrointestinal mucosa was only a delayed event associated with toxic doses.[6]

The novel analogue was highly effective against the models of the tumor types known to be responsive to CPT in the clinical setting (ovarian and colorectal carcinomas). It is worth noting that the growth of the two tumor sublines of A2780 with acquired resistance to doxorubicin or cisplatin were completely inhibited by ST1968 (100% TVI); in A2780 and A2780/DDP, tumors never regrew, resulting in 100% cures at experiment end. In contrast, topotecan and irinotecan only inhibited tumor growth, with very few cured tumors. Even the poorly responsive IGROV-1 tumor was significantly more sensitive to ST1968 than to the reference CPT investigated.

The present results show an impressive antitumor effect in tumors derived from exquisitely sensitive cells (e.g., A2780) in which the drug stimulated a fast apoptotic response. However, the drug produced a significant effect (65% TVI) also in tumors derived from intrinsically resistant cells (e.g., prostate carcinoma). A plausible explanation for *in vivo* efficacy is the drug-induced angiogenesis inhibition observed in tumors with diverse responsiveness, which could be a relevant component of the tumor response.

Interestingly, ST1968 exhibited a unique ability to inhibit the growth of yeast cells expressing K720E mutant human topoisomerase I. Although the crystal structure of the ternary complex in the presence of topotecan indicated that K720 is not directly involved in enzyme-CPT interactions (34), this lysine is located adjacent to Y723 and N722, two

[6] In preparation.

amino acids that play a key role in topoisomerase function (35). K720E mutant enzyme has been reported to show 50-fold reduction in relaxation activity and thus CPT resistance (27, 35). It could be speculated that the charged free amino group of ST1968 is critical in overcoming CPT resistance of K720E mutant enzyme. Although the molecular modeling analysis did not provide evidence of a direct interaction of the amino acid involved in the mutation, the change of the electrostatic charge produced by the amino acid mutation (substitution of Lys for Glu) could affect protein conformation or influence enzyme interaction with DNA. Although many mutations of human topoisomerase I have been reported in cell lines resistant to CPT, the relevance of these mutations in patient response to CPT treatment is, at the moment, unknown.

Although the possible implications of the peculiar interactions of ST1968 at target level remain to be defined, the preclinical evaluation of efficacy supports therapeutic interest of the novel CPT analogue. The results indicate that ST1968 is potentially more effective than either irinotecan or topotecan. In addition, the potential therapeutic advantages of ST1968 over irinotecan are (*a*) the improvement of the therapeutic index as reflected by an increased efficacy at equitoxic doses and outstanding efficacy in a wide range of well-tolerated doses and (*b*) different toxicity profile characterized by a less severe gastrointestinal toxicity. Indeed, ST1968 is a water-soluble CPT, which, in contrast to irinotecan, does not require hepatic metabolism. Relevant to this point is the observation that, as a consequence of the higher conversion rate of the drug into the active metabolite, SN38, in mice compared with humans, the activity of irinotecan may be overestimated in preclinical studies (36).

In conclusion, taking into account the difficulty to identify novel topoisomerase I inhibitors (4, 37), the peculiar features of ST1968 may have obvious therapeutic implications in view of a possible clinical development.

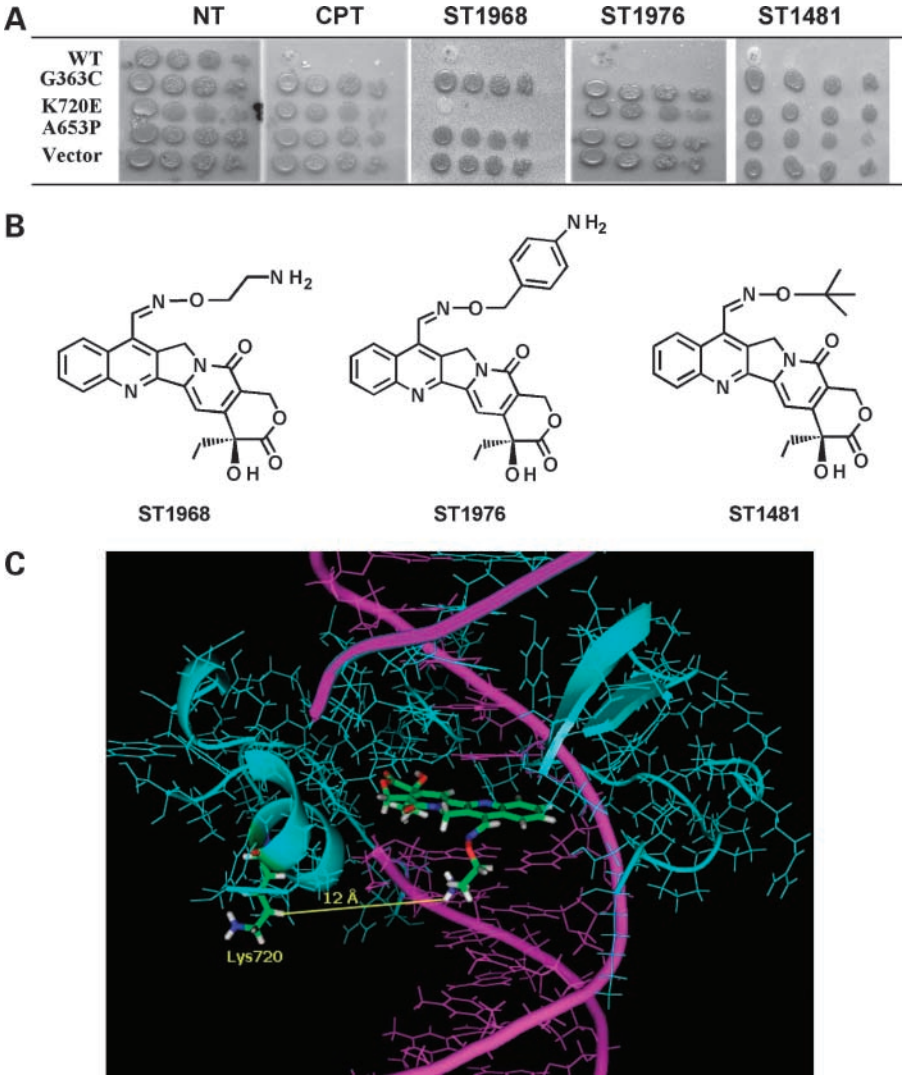

Figure 2. **A,** sensitivity of *S. cerevisiae* EKY3 yeast strain in spot test. EKY3 yeast strain was transformed with YCpGAL1 (*Vector*), YCpGAL1-hTOP1 (*WT*), YCpGAL1-hTOP1G363C (*G363C*), YCpGAL1-hTOP1A653P (*A653P*), or YCpGAL1-hTOP1K720E (*K720E*). Yeast strain was transformed by lithium acetate treatment and selected on complete medium (uracil-) plate plus 2% glucose. Transformants were grown to an A_{595} of 0.3. Five milliliters aliquots of serial 10-fold dilutions were spotted onto complete medium (uracil-) plates plus 2% galactose containing 45 μmol/L of the drugs. Two references CPT of the same series (ST1976 and ST1481) were included in the test. **B,** chemical structure of the tested CPT analogues. **C,** model of the proposed binding of ST1968 in the topoisomerase I-DNA cleavable complex.

Disclosure of Potential Conflicts of Interest

C. Pisano, S. Penco, L. Vesci, R. Foderà, F.F. Ferrara, M.B. Guglielmi, P. Carminati: employees of Sigma-Tau. The other authors disclosed no potential conflicts of interest.

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