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Potent Antitumor Activity and Improved Pharmacological Profile of ST1481, a Novel 7-substituted Camptothecin[1]

Michelandrea De Cesare, Graziella Pratesi, Paola Perego, Nives Carenini, Stella Tinelli, Lucio Merlini, Sergio Penco, Claudio Pisano, Federica Bucci, Loredana Vesci, Silvia Pace, Francesca Capocasa, Paolo Carminati, and Franco Zunino[2]

Istituto Nazionale per lo Studio e la Cura dei Tumori, 20133 Milano [M. D. C., G. P., P. P., N. C., S. T., F. Z.]; Dipartimento di Scienze Molecolari Agroalimentari, Universita' di Milano, 20133 Milano [L. M., S. P.]; and Sigma-Tau, 00040 Pomezia (Rome) [C. P., F. B., L. V., S. P., F. C., P. C.], Italy

ABSTRACT

Relevant drawbacks of the molecular structure and mechanism of the action of camptothecins are the instability of the E ring lactone and the reversibility of drug-target interaction. Such features are expected to limit the clinical efficacy of conventional camptothecins. In an attempt to overcome these limitations and to improve the pharmacological profile of camptothecins, a novel series of seven modified lipophilic analogues was synthesized based on the hypothesis that lipophilicity could promote a rapid cellular accumulation and stabilization of drug-target interaction. A novel analogue (ST1481) of the series, characterized by a potent antitopoisomerase and cytotoxic activity, was selected for preclinical development. A detailed preclinical study of ST1481 was performed in the H460 non-small cell lung tumor model using oral administration and various treatment schedules. Under all of the conditions, ST1481 exhibited an impressive efficacy in terms of tumor growth inhibition (tumor volume inhibition percentage > 99%), $\log_{10}$ cell kill, rate of complete responses (including "cures"), and an improvement of the therapeutic index compared with topotecan (used as the reference drug). The cytotoxic potency was also reflected by the *in vivo* potency, because the drug activity was observed at doses as low as 0.25 mg/kg with the daily schedule. In contrast to topotecan, no cross-resistance to ST1481 was found in ovarian carcinoma cells overexpressing P-glycoprotein (A2780/DX). A similar trend in the improvement of activity was also observed in the same tumor model growing *in vivo* with a 100% rate of complete tumor regressions. A rapid intestinal absorption and good oral bioavailability were supported by *in vivo* distribution studies, because the peak values of drug accumulation were found from 1 to 2 h after administration. The relevant liver accumulation may account for a marked effect of ST1481 against liver metastases induced by the ovarian carcinoma IGROV-1. In conclusion, the results support the hypothesis that a potent lipophilic camptothecin with a proper substituent at the position 7 may have therapeutic advantages likely related to a rapid intracellular uptake and tissue distribution, stabilization of the drug-target complex, and good oral bioavailability. Overall, the results support the preclinical interest of ST1481 in terms of efficacy, potency, toxicity profile, and ability to overcome multidrug resistance.

INTRODUCTION

The antitumor activity of camptothecins has been ascribed to their ability to interfere with the catalytic cycle of DNA topoisomerase I and stabilize the covalent DNA-enzyme complex by inhibiting DNA religation (1). During DNA synthesis, the reversible drug-enzyme-DNA complex (ternary complex) causes arrest of the replication fork and formation of double-strand DNA breaks (2). Although replication-independent effects have been described (3), camptothecins are recognized as S phase-specific agents (4).

The primary limitations of camptothecins are the formation of a labile drug target complex and instability of the lactone ring. On the basis of the reversibility of the ternary complex and formation of lethal lesions during DNA replication (5), optimal cytotoxic effects are expected with prolonged exposure to the drug. The validity of such expectation has been supported by preclinical and clinical studies using schedules with prolonged treatment (6, 7) or with formulations such as liposomal or conjugated camptothecins that allow a slow drug release (8, 9). In an attempt to optimize drug efficacy, possible strategies may involve chemical modifications of molecular structure. Analogues endowed with increased ability to induce topoisomerase I-mediated DNA cleavage and/or more persistent stabilization of the ternary complex compared with conventional camptothecins may result in more cytotoxic and possibly more effective antitumor drugs. A common feature of natural camptothecins is the intrinsic instability of the α-hydroxylactone, which in physiological conditions gives the ring-opened carboxylate form (10). The preferential binding of the carboxylate form to HSA shifts the hydrolysis equilibrium toward the inactive carboxylate in plasma. Such behavior represents a major drawback of drug activity, because a small amount of lactone is expected in plasma at equilibrium.

Thus, possible modifications can be aimed at: (*a*) stabilizing the lactone form of camptothecins and/or reducing the affinity of the drug for albumin; and (*b*) favoring the rapid drug uptake and/or enhancing drug interaction with the intracellular target. The latter approach was preferred in our design of novel molecules, because the lactone ring instability is a common feature of all of the camptothecins. Indeed, rapid uptake, enhanced intracellular accumulation, and stabilization of the drug-target complex are expected to stabilize the active lactone form (11) and to result in a potent cytotoxic activity. On the basis of such rationale, a novel series of camptothecins substituted in position 7 with lipophilic chains has been developed (12, 13).

Using as the screening system the NCI-H460 lung carcinoma cell line characterized by overexpression of topoisomerase I, a new analogue of the series (ST1481) was selected as a promising candidate for additional investigation. The analogue is endowed with markedly increased cytotoxic activity compared with camptothecin and is more effective in stimulation of topoisomerase I-mediated DNA cleavage. The topoisomerase I-DNA-cleavable complexes formed in the presence of ST1481 are less reversible than those induced by conventional camptothecins (13). The aim of the study was to investigate relevant aspects of the pharmacological profile and therapeutic features of ST1481 in preclinical systems. Oral administration was used to assess in athymic nude mice bearing the NCI-H460 lung tumor: (*a*) the potency and efficacy of ST1481; (*b*) the TI of ST1481; and (*c*) the schedule dependency of antitumor activity. The results of antitumor efficacy studies, together with the ability to completely overcome MDR[3] mediated by P-glycoprotein in

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[2] To whom requests for reprints should be addressed, at Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy. Phone: 39-02-23902267; Fax: 39-02-23902692; E-mail: zunino@istitutotumori.mi.it.

[3] The abbreviations used are: q4dx4, every 4th day for 4 times; qdx5/w, daily for 5 days/week; q8–10d, every 8th-10th day; TVI %, tumor volume inhibition percentage; MDR, multidrug-resistance; HSA, human serum albumin; HPLC, high-performance liquid chromatography; TPT, topotecan; TV, tumor volume; LCK, $\log_{10}$ cell kill; CR, complete regression; BWL, body weight loss; TI, therapeutic index; MTD, maximum tolerated dose; BCRP, breast cancer resistance protein.

in vivo models, indicate ST1481 as a promising compound for clinical development.

MATERIALS AND METHODS

Drugs

The synthesis of ST1481 (7-t-butoxyiminomethylcamptothecin) has been already described (13). The drug was dissolved in DMSO and stored at −20°C until use. Immediately before treatment, the drug was additionally diluted in sterile, distilled water (DMSO 10% final concentration). TPT, kindly supplied by Smith-Kline Beecham Pharmaceuticals, was dissolved in sterile, distilled water. Drugs were administered by gavage in a volume of 10 ml/kg according to three schedules: q4dx4, qdx5/w for 5–10 weeks (see "Results"), and q8−10d for 10 times.

Cell Lines and Drug Sensitivity Studies

The human tumor cell lines used in the study included: the non-small cell lung carcinoma NCI-H460 cell line; the ovarian carcinoma A2780 and IGROV-1 cell lines and the doxorubicin-resistant A2780/DX and IGROV-1/DX1 sublines, characterized by P-glycoprotein-mediated MDR phenotype (14); the prostate carcinoma PC-3 cell line; and the breast carcinoma MCF-7 cell line. All of the cell lines were maintained as monolayer cultures in RPMI 1640 supplemented with 10% FCS.

Cellular sensitivity to drugs was assessed by the growth-inhibition assay. Briefly, cells in the logarithmic phase of growth were seeded in duplicate in six-well plates and, 24 h after seeding, were exposed to drug for 1 h. When the effects of HSA were studied, it was added to the medium just before exposure to the drug. HSA itself at the used concentrations (≤30 mg/ml) did not affect cell proliferation. Cells were then washed and incubated in drug-free medium for 72 h. Adherent cells were trypsinized and counted by using a cell counter (Coulter Electronics, Luton, United Kingdom). IC_{50} was defined as the concentration required for 50% cell growth inhibition compared with control cells.

Animals

All of the antitumor activity experiments were carried out using female athymic Swiss nude mice, 8–10 weeks old (Charles River, Calco, Italy). 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 drug distribution studies, 5–6 week-old female BALB/c mice (Charles River) were used.

Cellular Pharmacokinetic Studies

Cells were plated in six-well plates at a subconfluent density corresponding to 6×10^5 and 8×10^5 cells/well for PC-3 and MCF-7, respectively. Cells (3 wells/sample) were then washed with serum-free medium and exposed for 1 h to 10 μM of ST1481 or TPT dissolved in 1% DMSO. Treated cells were washed three times with PBS to remove the drug, and they were maintained in drug-free medium for 24 h. At different times, washed cells were incubated for 30 min with TE buffer [10 mM Tris-HCl (pH 7.5) and 1 mM EDTA] and 1% NP-40 (30 μl/well). Cell lysates were collected and frozen at −20°C.

The extraction of ST1481 was performed according to a modified method described by Koshkina *et al.* (15). Briefly, 700 μl of 0.1% acetic acid: acetonitrile (1:4), pH 4.5, were added to each sample and extracted with 4 ml of methylene chloride. The mixture was centrifuged at 4500 × *g* for 5 min, and the organic phase was dried under vacuum. Dried extracts were reconstituted in 150 μl of acetonitrile, and 50 μl was injected into the HPLC column (Licrosphere 100; RP-18; 5 μm; Merck). The mobile phase consisted of water:acetonitrile (1:1), and the flow rate was 1.5 ml/min.

The extraction of TPT was performed according to the method described previously (16). Briefly, 450 μl of methanol, cooled at −20°C, was added to each sample. After centrifugation at 10,000 rpm for 10 min at 4°C, 50 μl of the supernatant was injected into the HPLC column (Nova-Pack C18; 3.9 × 150 mm; Waters). The mobile phase consisted of 0.1 M acetic acid (pH 3.5) with triethylammine:acetonitrile (20:80), and the flow rate was 1 ml/min. Analyses were performed at room temperature using a BioSys HPLC (Model PP-510; Beckman Coulter) and a Fluorescence Detector (Model RF10AxL; Shimadzu) using an excitation wavelength of 370 nm and emission at 510 nm for detection. Known amounts of drug were added to untreated samples processed as described above to estimate the extraction efficiency of the methods. Efficiency was calculated as: (amount of the drug added/amount of the drug obtained by HPLC analysis) × 100.

Pharmacokinetic Analysis and Tissue Distribution of ST1481

Healthy athymic Swiss mice were treated p.o. with 5 mg/kg of ST1481. Plasma levels of the drug were determined as the total lactone forms by using an HPLC fluorimetric method. The HPLC system consisted of two Model PU-980 pumps (Jasco International Co. Ltd., Tokyo, Japan), a Model FP-920 fluorescence detector (Jasco International Co. Ltd.), and a Model AS-950 autosampler (Jasco International Co. Ltd.). For the determination of plasma levels of ST1481, 50 μl of plasma were diluted with 50 μl of 0.2 N HCl, then 10 μl of internal standard solution were added. After vortexing, samples were transferred into autosampler vials and injected. The method consisted of an on-line purification of plasma sample followed by isocratic elution chromatography. The sample purification was obtained with a BioTrap 500 C18 20 × 4.0 mm column (ChromTech Ltd., Congleton, United Kingdom). The HPLC mobile phase for purification was prepared by mixing 900 ml of water with 100 ml of acetonitrile. The chromatographic separation was obtained with a Luna 5 μm Phenyl-Hexyl 150 × 4.60 mm column (Phenomenex, Torrance, CA). The HPLC mobile phase for chromatographic separation was prepared by mixing 500 ml of water and 500 ml of acetonitrile. The column eluate was monitored by fluorescence with the excitation wavelength set at 370 nm and the emission wavelength set at 510 nm, a gain of 1000. Chromatographic data processing was performed with Borwin Ver. 1.2.6 software (Jasco International Co. Ltd.) working on a Pentium PC. Using 50 μl of plasma, the lower limit of quantification for ST1481 was 2.5 ng/ml. The pharmacokinetic parameters were obtained using a model-independent approach. The pharmacokinetic analysis was carried out using WinNonlin Professional Edition, version 1.5 software (Scientific Consulting Inc.).

For *in vivo* distribution experiments, exponentially growing M109 murine lung carcinoma cells (3×10^5 cells/mouse) were injected i.m. into the right hind leg muscle of female BALB/c mice. A single dose of ST1481 (1.2 mg/kg in 10% DMSO) was administered by the oral route on day 13 from tumor injection. At various times after treatment, 5 mice/group were sacrificed. Selected tissues were homogenized in 1 ml of 0.1% acetic acid:acetonitrile, (1:4, v/v), pH 4.5, and centrifuged at 16,000 rpm for 10 min. Supernatant fractions were extracted by adding 10 ml of methylene chloride and processed as described above for cellular pharmacokinetics studies.

Antitumor Activity Studies on s.c. Growing Tumors

The NCI-H460 human lung carcinoma and the A2780/DX human ovarian carcinoma were used. Exponentially growing tumor cells were injected s.c. ($8–10 \times 10^6$ cells/flank) into nude athymic mice. The tumor line was maintained by serial s.c. passages of tumor fragments (~2 × 2 × 6 mm) in healthy mice, as described previously (14).

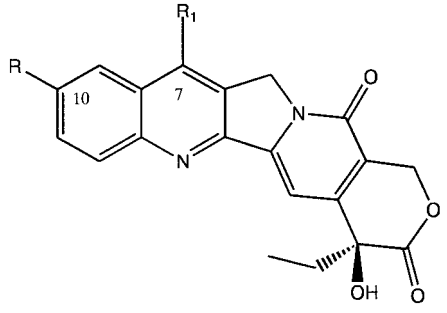
Antitumor Efficacy Studies. Experimental groups included four or five mice bearing bilateral s.c. tumors, if not otherwise indicated. Tumor fragments were implanted on day 0, and tumor growth was followed by biweekly measurements of tumor diameters with a Vernier caliper. TV was calculated according to the formula: TV (mm^3) = $d^2 \times D/2$, where d and D are the shortest and the longest diameters, respectively. Treatments started at different times after tumor injection, depending on the experimental design. For ethical reasons experimental groups were eliminated when the TV in the group was about 2000–3000 mm^3.

The efficacy of the drug treatment was assessed as: (*a*) TVI % in treated *versus* control mice, calculated as TVI % = 100 − (mean TV treated/mean TV control × 100); (*b*) LCK calculated by the formula LCK = (T − C)/3.32 × DT, where T and C are the mean times (days) required for treated (T) and control (C) tumors, respectively, to reach 1000 mm^3, and DT is the doubling time of control tumors (an LCK value >1 is indicative of an active compound); and (*c*) CR meaning no evidence of tumor lasting for ≥10 days [CRs at the end of the experiment (≥100 days) are considered "cures"].

The toxicity of the drug treatment was assessed as: (*a*) BWL calculated as

Table 1 *Cellular sensitivity of non-small-cell lung carcinoma H460 cell line to camptothecins and influence of HSA*

Cellular sensitivity was determined by growth inhibition assay after 1 h drug exposure. After 72-h incubation in drug-free medium adherent cells were trypsinized and counted. When HSA was used, it was added just before addition of the drug. See "Materials and Methods" for details.



Drug	R	R_1	IC_{50} (μM)
Camptothecin	H	H	0.33 ± 0.05
Topotecan[a]	OH	H	1.38 ± 0.19
SN38	OH	CH_2–CH_3	0.21 ± 01
ST 1481	H	CH = NOC$(CH_3)_3$	0.01 ± 0.006
ST 1602	OH	CH = NOC$(CH_3)_3$	0.13 ± 0.03
ST1481 + 1 mg/ml HSA			0.024 ± 0.01
ST1481 + 30 mg/ml HSA			0.063 ± 0.004

[a] TPT contains an additional substituent $CH_2NH(CH_3)_2$ at the position 9.

BWL % = 100 − (mean BW_{dayx}/mean BW_{day1}x100), where day 1 is the first day of treatment and day x is any day after (maximum BWL % values are reported in the "Tables;" mice were weighed twice/week throughout the experimental frame); and (*b*) lethal toxicity, *i.e.*, any death in treated groups occurring before any control death (mice were inspected daily for mortality).

For statistical analysis, Fisher's exact test was used for comparison of CR rates.

Assessment of TI. To evaluate the TI of the two drugs, a wide range of doses was tested according to the q4dx4 schedule on NCI-H460 tumor-bearing mice. TI was calculated by the formula TI = LD10/ED90, where LD10 is the dose causing 10% lethal toxicity and ED90 is the drug dose causing 90% TVI.

Activity Against Liver Metastases

The IGROV-1 human ovarian carcinoma was adapted to grow in mice as an i.p. model, where the tumor grows as diffuse carcinomatosis and ascites (17). Ascitic tumor cells were inoculated (10^6 cells in 0.5 ml of saline) in the spleen. Briefly, mice were anesthetized and the left flank opened. The spleen was carefully exposed, and tumor cells were injected under the spleen capsule via a 27-gauge needle. After cell injection (1 min), the spleen was removed, and the wound was closed (18). All of the mice developed liver tumor foci and ascites. Six days after surgery, drugs were given p.o. qdx5/wx4w. Control mice were treated with 10% DMSO. A few days after ascites onset, mice were sacrificed and livers removed and weighed. At day 80, all of the mice were sacrificed, including those without evidence of ascites.

RESULTS

Cellular Sensitivity Studies

Table 1 shows a comparison of sensitivity of the NCI-H460 cell line to ST1481 and to clinically relevant camptothecins after short-term exposure (1 h). The novel analogue was markedly more potent than camptothecin and TPT and substantially more potent than other molecules modified at the position 7 and containing a hydroxyl group at the position 10 (*i.e.*, SN38 and ST1602). Thus, because an appreciable reduction of the antiproliferative effect by the presence of the 10-hydroxy group was evident, the ST1481 compound, lacking modifications in the A ring, was selected for additional development despite expected affinity for serum albumin (19). Indeed, the antiproliferative effect was reduced in the presence of 30 mg/ml of HSA. However, under such conditions, drug potency was still higher than that of TPT or SN-38 in the absence of albumin.

Activity Against NCI-H460 Human Lung Carcinoma Xenograft

The effects of oral ST1481 against the NCI-H460 human lung tumor xenografts were investigated with various schedules in comparison with oral TPT used as the reference drug.

Effect of Protracted Daily Treatment (qdx5/w). The results of the antitumor activity studies with the qdx5/w schedule for several weeks are reported in Table 2 and Fig. 1*A*. Treatment with the lowest tested dose of TPT (1.2 mg/kg) was effective in slowing tumor growth (LCK = 2.4) but did not achieve CR. In animals treated with 2 mg/kg of TPT, 4 of 10 tumors regressed ~30 days from the start of treatment. Moreover, despite continuing drug delivery (≤10 weeks), the tumors started to regrow and all of the tumors were present at the end of the experiment (day 100). In mice treated with the low dose of ST1481 (0.25 mg/kg), 4 of 8 tumors completely regressed ~15 days, but all of the tumors started to regrow by the end of the experiment. When a higher dose (0.5 mg/kg) was delivered, all of the tumors (8/8) disappeared by day 30, and 5 of 8 were cured at day 100 despite the shorter treatment duration compared with that of TPT (5 *versus* 10 weeks). The cumulative doses delivered were 100 and 12.5 mg/kg for TPT and ST1481, respectively. No significant toxicity was induced by the tested doses of the two drugs according to this schedule.

Effect of Every 8th-10th Day Treatment (q8–10d × 10 Times). The results of the antitumor activity studies delivering the drugs by a very spaced schedule are reported in Table 2. Two dose levels of TPT were investigated: they were effective in reducing tumor growth (LCK values of 2.5 and 3.1 with 18 and 22 mg/kg, respectively) but not in achieving CR. Both doses were slightly toxic. In contrast, the two investigated doses of ST1481 (5 and 6 mg/kg) induced a very high rate of CR, most of which resulted in no evidence of disease at the end of the experiment (day 100). As a consequence, LCK values

Table 2 *Effects of ST1481 and TPT, delivered orally (for day 3), using daily or intermittent schedule on the NCI-H460 human tumor xenograft*

Drug	Schedule	Dose (mg/kg)		CR at day[a]				LCK[b]	Maximum	Lethal toxicity[d]
		Single	Total	15	30	60	100	(1000 mm^3)	BWL[c] %	(day)
TPT	qdx5/wx10w	1.2	60	0/8	0/8	0/8	ND[e]	2.4	0	0 /4
		2	100	0/10	4/10	2/10	0/10	6.0	0	0 /5
	q8-10dx10	18	180	0/8	0/6	0/6	ND[e]	2.5	9	1/4 (24)
		22	220	0/10	0/8	0/8	ND[e]	3.1	14	1/5 (27)
ST1481	qdx5/wx5w	0.25	6.25	4/8	4/8	2/8	ND[e]	4.0	0	0 /4
		0.5	12.5	7/8	8/8	6/8	5/8	6.3	3	0 /4
	q8-10dx10	5	50	0/8	4/8	5/8	5/8	8.6	9	0 /4
		6	60	0/10	6/10	7/10	4/8	7.9	8	1/5 (61)

[a] CR, no. of completely regressed tumors/total no. of tumors. Days are calculated from the day of tumor implantation.
[b] See "Materials and Methods" for LCK formula.
[c] Maximum BWL % after drug treatment.
[d] No. of mice dead for toxicity/total no. of mice. Days are calculated from the day of tumor implantation.
[e] ND, not determined. Mice were eliminated by this time because of tumor burden.

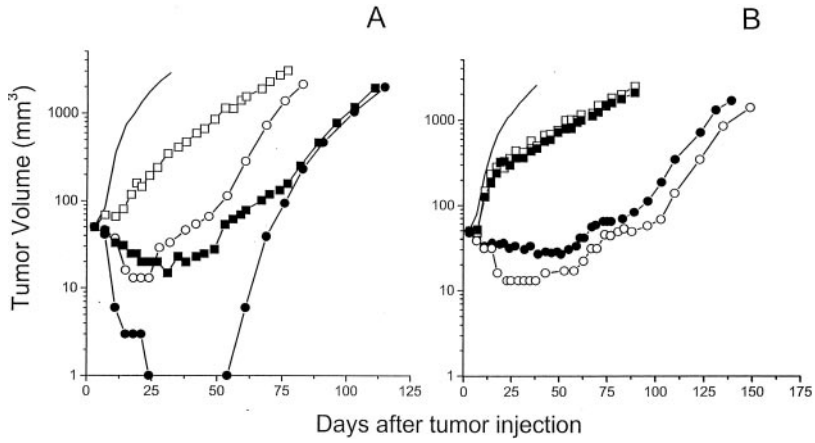


Fig. 1. Effects on tumor growth of TPT and ST1481 delivered p.o. (from day 3) against the NCI-H460 non-small cell lung carcinoma. *A*, treatment schedule qdx5/w administrations: TPT, 1.2 (□) and 2 (■) mg/kg × 10 weeks; ST1481, 0.25 (○) and 0.5 (●) mg/kg × 5 weeks. *B*, treatment schedule q8–10dx10 administrations: TPT, 18 (□) and 22 (■) mg/kg; ST1481, 5 (○) and 6 (●) mg/kg.

were much higher than in TPT-treated tumors. The marked differences in antitumor activity between the two drugs are clearly documented by the growth curves (Fig. 1*B*).

Effect of Every 4th Day Treatment (q4dx4). With the q4dx4 schedule, four dose levels of TPT were investigated, and a clear dose-dependent antitumor effect was observed in terms of TVI % and LCK values (Table 3). Tumor growth was stopped (≤20 days) by the highest tolerated dose, *i.e.*, 15 mg/kg (total dose, 60 mg/kg), but no CR was achieved. The dose of 18 mg/kg was highly toxic. The analogue ST1481 induced CR in 5 of 8 tumors at a well-tolerated dose (2 mg/kg) and in all of the surviving animals at a higher toxic level (2/5 toxic deaths among mice treated with 4 mg/kg).

Assessment of TI. To better define the potential advantages in the therapeutic profile of ST1481, the relation between drug tolerability and efficacy was evaluated in NCI-H460 tumor-bearing mice. The MTDs of TPT and ST1481 delivered p.o. according to the q4dx4 schedule were deduced by observations of lethal toxicity in several independent experiments. Pooling together these observations the MTDs (roughly corresponding to the LD10) were 3 mg/kg for ST1481 (4 dead in 52 treated mice) and 15 mg/kg for TPT (6 dead in 173 treated mice), thus indicating that the novel analogue is ~5-fold more potent than TPT. The MTD was confirmed also in nontumor-bearing mice (data not shown). When we calculated the TI for each drug as LD10:ED90 (dose inducing 90% TVI %, see Table 3), a favorable profile of tolerability at effective doses was found for the novel analogue, because the TI was 3 for ST1481 (*i.e.*, 3/1) and 1.7 for TPT (*i.e.*, 15/9).

Activity Against Doxorubicin-resistant Human Ovarian Carcinoma Models

To examine the ability of ST1481 to overcome drug resistance in tumor cells with the MDR phenotype, we used human ovarian carcinoma variants selected for resistance to doxorubicin. Both sublines displayed a pronounced resistance to the selecting agent (Table 4) and were characterized by a typical MDR phenotype. A marked cross-resistance to TPT was observed only in IGROV-1/DX. The higher degree of resistance of IGROV-1/DX cells was likely related to an increased level of P-glycoprotein expression as compared with A2780/DX. BCRP expression was not detected in these cell systems. In both cell systems, no cross-resistance to ST1481 was observed. The lack of influence of P-glycoprotein expression on tumor response to ST1481 was confirmed in the A2780/DX tumor xenografts. In this model all of the tumors (8/8) completely regressed under treatment with the MTD (3 mg/kg; q4dx4 schedule) of the novel camptothecin.

Table 3 *Effects of ST1481 and TPT, delivered orally q4dx4 times (days 3, 7, 11, and 15), on the NCI-H460 human tumor xenograft*

Drug	Dose/injection (mg/kg)		TVI %[a] (day 21)	CR[b] (day 25)	LCK[c] (1000 mm^3)	Maximum BWL[d] %	Lethal toxicity[e] (day)
	Single	Total					
TPT	5	20	80	0/8	1.3	0	0/4
	9	36	91	0/8	1.7	0	0/4
	15	60	98	0/8	2.1	8	0/4
	18	72	ND[f]	ND[f]	ND[f]	23	4/5 (18, 18, 19, 20)
ST14781	1	4	90	0/8	1.4	0	0/4
	2	8	99	5/8	2.4	1	0/4
	4	16	100	8/8	2.5	23	2/5 (17, 31)

[a] TVI % in treated *versus* control mice.
[b] CR, no. of completely regressed tumors/total no. of tumors in surviving animals at the indicated day. Days are calculated from the day of tumor implantation.
[c] See "Materials and Methods" for LCK formula.
[d] Maximum BWL % because of the drug treatment.
[e] Days of death in parentheses.
[f] ND, not determined.

Table 4 *Cellular sensitivity of ovarian carcinoma cell lines with MDR phenotype to camptothecins*[a]

	IC_{50} (μg/ml)			IC_{50} (μg/ml)		
	A2780	A2780/DX	RI[b]	IGROV-1	IGROV-1/DX	RI[b]
TPT	0.14 ± 0.04	0.21 ± 0.1	1.5	0.30 ± 0.05	8.15 ± 2.6	27
ST1481	0.015 ± 0.002	0.013 ± 0.01	0.9	0.017 ± 0.008	0.018 ± 0.002	1
Doxorubicin	0.24 ± 0.12	2.3 ± 1.8	10	0.39 ± 0.06	8.8 ± 1.6	22

[a] Assessed by growth inhibition assay after 1 h of drug exposure. Values are the mean (±SD) of at least three experiments.
[b] RI, resistant index: ratio between the IC_{50} values of the resistant and parental cell lines.

TPT, in the same experimental conditions, was significantly less effective against the tumor (2/10 CR).

Activity Against Liver Metastases

The high efficacy exhibited by ST1481 delivered by the oral route suggested the potential therapeutic interest of the novel analogue in the treatment of liver metastases. The study was performed with the ovarian carcinoma IGROV-1 model characterized by low level of expression of P-glycoprotein and only moderate responsiveness to agents recognized by this transport-system (*e.g.,* Taxol® and doxorubicin; Ref. 14). Mice developed lethal ascites because of liver invasion by tumor with formation of multiple foci. A daily oral treatment with TPT or ST1481 strongly influenced the onset of ascites as a consequence of a substantial reduction in liver tumor burden. The effect was more marked in ST1481-treated mice, because only 1/5 mice presented visible liver foci at the end of the experiment (day 80). The pattern was reflected by marked differences in liver weight of treated animals (Table 5).

Cellular Accumulation Studies

Cellular drug contents were determined in MCF-7 breast carcinoma cells and in PC-3 prostate carcinoma cells after 1-h exposure to equimolar concentrations of each drug (10 μM). The drug concentration was used because it allowed a reproducible determination of cellular drug content under our conditions. The results presented in Table 6 indicated a markedly (20-fold) higher intracellular accumulation of ST1481 than TPT, suggesting a rapid drug uptake. In addition, after a 24-h incubation in drug-free medium, the intracellular content of ST1481 was still higher than that found immediately after a 1-h exposure to TPT.

Tissue Distribution and Pharmacokinetic Analysis

The cellular behavior was consistent with the *in vivo* distribution of the drug (Fig. 2). Again, a rapid tissue distribution was found in all of the examined organs. The extent of drug content in these tissues was clearly related to the route of administration. Indeed, the highest concentration found in the liver was expected after oral administration. The drug levels achieved in 1 h were persistent for ≥4 h. A relevant observation of this study was the appreciable amount of drug found in the central nervous system.

Table 5 *Effects of ST1481, 0.5 mg/kg, and TPT, 2 mg/kg, (oral administration, from day 6, qdx5/wx4w) on experimental liver metastases of the human ovarian tumor IGROV-1*

Drug	Median day of ascites onset[a]	Day of evaluation[b]	No. of mice with metastases/total	Liver weight (g)		
				Mean ± SD	Median	Range
Solvent	20	24	8/8	3.7 ± 1.6	3.4	1.6–7.1
TPT	76	80	3/5	2.1 ± 1	2.5	1.2–3.9
ST1481	>80	80	1/5	1.8 ± 0.6	1.5	1.4–2.9

[a] Mice develop ascites as a consequence of tumor growth in the liver. Mice with no evidence of ascites at the end of the experiment are indicated as >80.
[b] Time at which mice are sacrificed for liver inspection.

Table 6 *Intracellular content of ST1481 and TPT in PC-3 and MCF-7 human tumor cells*[a]

Cell line	Time (h)	ng/mg protein[b]	
		ST1481	TPT
Prostate cancer PC-3	1	52 ± 4	2.6 ± 0.1
	6	3.7 ± 0.3	0.3 ± 0.02
	24	4.1 ± 0.2	0.2 ± 0.03
Breast cancer MCF-7	1	28 ± 1	1.4 ± 0.06
	6	2.6 ± 0.3	0.3 ± 0.07
	24	1.5 ± 0.1	0.3 ± 0.04

[a] Intracellular drug content was determined after 1 h exposure to 10 μM drug. Drug content at 6 and 24 h refers to the amount of drug retained by the cell after removal of extracellular drug.
[b] The values are expressed as mean ± SE of three independent experiments.

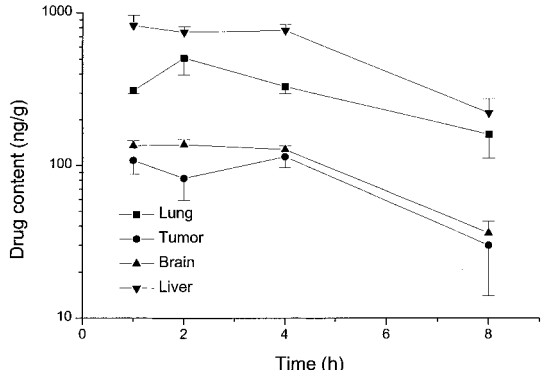


Fig. 2. Tissue level of ST1481 after a single oral administration (1.2 mg/kg) in M109 tumor-bearing BALB/c mice. Values refers to the mean of drug content in tissues from five animals; *bars*, ± SD.

Table 7 *Pharmacokinetic parameters of ST1481 and TPT obtained after oral administration of therapeutic doses*

Drug	Dose (mg/kg)	C max[a] (ng/ml)	$AUC_{0\text{-}inf}$[b]	$t_{1/2}$[c] (h)
TPT	15	118.91 ± 30.64	552.29	2.77
ST1481	5	307.46 ± 69.79	2458.66	11.8

[a] Maximal plasma concentration. Mean ± SD from three mice.
[b] Area under the concentration-time curve from 0 to infinity.
[c] Apparent terminal half-life.

The pharmacokinetic analysis of ST1481 in plasma was assessed after oral administration of 5 mg/kg (Table 7). For purpose of comparison, the results achieved by TPT administered p.o. at the dose of 15 mg/kg are also reported (16). Both agents reached the maximum plasma concentrations within 1 h after administration. Despite a lower dose, ST1481 reached a substantially higher plasma concentration, thus suggesting a good bioavailability. An additional advantage of ST1481 was the long persistence as documented by area under the curve and $t_{1/2}$ values.

DISCUSSION

Whereas previous chemical efforts to exploit substitutions at the C-7 position focused on development of water-soluble derivatives of camptothecin, recent studies have suggested pharmacological interest of lipophilic camptothecins (20, 21). Among a series of 7 substituted camptothecins, the novel lipophilic analogue ST1481 was selected for preclinical development on the basis of promising features at cellular and molecular levels, including potent inhibition of topoisomerase I, persistence of DNA cleavage (13), and rapid cellular uptake of the drug. It is likely that the cytotoxic potency exhibited by ST1481 is the result of the combination of these features rather than of the stability of the active lactone form, although lipophilicity is expected to improve the stability profile (19). The present study provides evidence that such features are also reflected by a promising pharmacological profile and therapeutic behavior.

Indeed, ST1481 retained a strong potency and a superior antitumor efficacy in terms of tumor growth inhibition and rate of complete response against the NCI-H460 tumor xenograft. ST1481 was ~5-fold more potent than TPT when delivered by intermittent treatment schedules (every 4–8 days), whereas with the daily schedule a marked activity was observed at doses as low as 0.25 mg/kg/day and

cumulative doses in the range of 6–12 mg/kg, *i.e.*, ~10 times lower than those of TPT.

The marked potency and outstanding efficacy of ST1481 were observed with all of the tested schedules; however, as expected for camptothecins, the best schedule was the daily prolonged treatment, which produced CR in all of the treated animals with a high rate of long-term disease-free survivors. A comparable therapeutic effect was achieved with an intermittent treatment schedule (every 8–10 days) using higher dose levels. The latter schedule was substantially less effective for TPT, because no CR was achieved by the drug under such conditions.

Favorable pharmacokinetic and pharmacodynamic properties have been described for camptothecins in mice compared with humans, and they are possibly responsible for the discrepancies in antitumor efficacy between the two species (22). Considering that camptothecins are more myelosuppressive in humans than in mice (23), an improvement of the TI in mice may represent a promising feature for the therapeutic use in humans. ST1481 exhibited an improvement of the TI as compared with TPT, because it exhibited a marked efficacy (TVI % $\geq$ 90) in a wide range of tolerated doses and with different schedules. Moreover, in contrast to the strong increase in cytotoxic potency of ST1481 *versus* TPT in tumor cells (Table 1), the two compounds exhibited comparable IC_{50} values in human bone marrow progenitor cells *in vitro* (data not shown).[4]

The enhanced lipophilicity of ST1481 compared with TPT, resulted in a more efficient drug uptake by tumor cells and in a favorable tissue distribution, with particular reference to the ability to cross the blood-brain barrier, as supported by *in vivo* distribution studies. The ability may represent a favorable feature for the treatment of central nervous system tumors, as already documented in preclinical models of glioma with the use of silatecans, a novel series of lipophilic camptothecins (24). Despite the expected lactone hydrolysis and drug interaction with serum albumin, ST1481 in the presence of HSA retained a marked cytotoxic activity higher than that of TPT and SN-38 in the absence of albumin. The reduction of cytotoxic potency was apparently less marked than that reported for other lipophilic camptothecins (19).

It is likely that the cytotoxic potency of ST1481 is the result of a combination of multiple factors, including a potent topoisomerase I inhibitory activity, stability of DNA-topoisomerase I cleavable complexes, and lipophilicity, resulting in enhanced drug uptake and lactone stability. The cytotoxic potency may be a critical requirement for *in vivo* drug efficacy, because a substantial reduction in the amount of the active lactone form in human plasma is conceivable even for camptothecins with an improved stability profile (19, 25).

An additional therapeutic advantage of ST1481 is a complete lack of recognition by transport systems involved in the typical MDR phenotype. Overexpression of P-glycoprotein plays a variable role in sensitivity to camptothecins. Indeed, a number of well-known camptothecins (TPT, 9-amino-camptothecin, and CPT11/SN38) shows a partial cross-resistance in P-glycoprotein-mediated drug resistance (26, 27). In contrast, the cytotoxic activity of ST1481 was not influenced by P-glycoprotein in ovarian carcinoma cells, which overexpress the MDR-1 gene, and the feature was also reflected by the impressive responsiveness of A2780/DX tumor growing s.c. and of liver metastases produced by the P-glycoprotein-positive IGROV-1 tumor. These resistant cell sublines did not express detectable levels of BCRP that is important in the transport of TPT and SN38 (28). We have recently found that the novel camptothecin ST1481 is able to overcome cellular resistance in the mitoxantrone-selected cell line characterized by high level of BCRP expression (29). The lack of recognition by BCRP is consistent with the oral efficacy, because this transport system is likely implicated in the regulation of intestinal absorption of camptothecins.

The *in vivo* study was performed using the oral route of administration, which has been reported as favorable for TPT in human tumor xenografts (16) as well as in clinical studies (30). A formulation suitable for i.v. injection is being developed and should allow a comparative pharmacokinetic analysis after i.v. or oral administration. Additionally, the antitumor efficacy observed for ST1481 at low dose levels ($\ll$1 mg/kg) and pharmacokinetic analysis suggest a good oral bioavailability. The oral administration should be regarded as an advantage rather than as a limitation for the clinical use of camptothecins, because the optimal activity of this class of drugs can be achieved by multiple daily doses, which ensure a prolonged exposure time (31, 32). An outstanding efficacy of ST1481 against liver metastases produced by the IGROV-1 ovarian carcinoma suggests an additional advantage of the oral treatment, because intestinal absorption could ensure a high drug concentration in the liver, which is a common site of metastatic involvement for several tumor types.

In conclusion, the preclinical profile of ST1481 compares favorably with known camptothecins; indeed, the 7-modified analogue exhibited an impressive antitumor efficacy in preclinical models of human tumors, as documented by its striking curative potential. The comparison of antitumor effects of ST1481 and TPT clearly evidenced a superior antitumor activity of the novel analogue, because a high rate of complete tumor regressions was observed. In particular, the pharmacological interest in ST1481 is related to its ability to overcome drug resistance mediated by P-glycoprotein, to an improved TI, and to obvious advantages related to drug potency, thereby allowing the oral delivery of substantially lower doses than those required for TPT. On the basis of this preclinical efficacy profile, ST1481 is a promising candidate for clinical evaluation.

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