BIOCHEMICAL PHARMACOLOGY 73 (2007) 656–664

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Preclinical efficacy of ST1976, a novel camptothecin analog of the 7-oxyiminomethyl series

Michelandrea De Cesare[a], *Giovanni Luca Beretta*[a], *Stella Tinelli*[a], *Valentina Benedetti*[a], *Graziella Pratesi*[a], *Sergio Penco*[b], *Sabrina Dallavalle*[c], *Lucio Merlini*[c], *Claudio Pisano*[b], *Paolo Carminati*[b], *Franco Zunino*[a,*]

[a] *Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy*
[b] *Sigma-Tau, Pomezia, Italy*
[c] *Dipartimento di Scienze Molecolari Agroalimentari, Università di Milano, Milan, Italy*

ARTICLE INFO

Article history:
Received 12 July 2006
Accepted 6 November 2006

Keywords:
Camptothecins
DNA topoisomerase I
Antitumor activity
Cytotoxicity
BCRP
DNA cleavage

ABSTRACT

In previous studies, we have documented the potential therapeutic advantages of camptothecin analogs modified at the 7-position, i.e., 7-oxyiminomethyl derivatives. The present study was performed to explore the therapeutic potential of novel hydrophilic derivatives of this series. With one exception (ST1976), the tested camptothecins exhibited a reduced antiproliferative activity and all compounds retained ability to stabilize the topoisomerase I-mediated cleavable complex. The two analogs (ST1976 and ST1968) characterized by the presence of a free amino group in the side chain also exhibited the formation of persistent cleavable complexes. The most potent compound, ST1976 (7-(4-aminobenzyl)oxyiminomethylcamptothecin), was selected for evaluation of its preclinical profile of antitumor activity in a large panel of human tumor xenografts. As expected on the basis of the introduction of a hydrophilic substituent, the novel camptothecin was a substrate for BCRP. However, in spite of an apparent recognition by BCRP, ST1976 was effective following oral administration. The antitumor activity was evaluated using various schedules and routes of administration (i.v. and p.o.). ST1976 exhibited a remarkable activity in all tested tumors and was effective in a number of tumors which are resistant to irinotecan. The biological and pharmacological profile of ST1976 supports the therapeutic potential of camptothecins containing hydrophilic substituents at the 7-position. On the basis of its excellent activity in preclinical models, ST1976 is a promising candidate for clinical development.

1. Introduction

Camptothecins continue to be the subject of intense investigation [1]. The preclinical and clinical efficacy of topoisomerase I inhibitors has generated high expectations in the development of the novel generation of camptothecins. A major limitation of camptothecins is related to their peculiar chemical structure [2,3]. The chemical instability of the lactone form at physiological pH generates the carboxylate form which exhibits high affinity for human serum albumin [4]. This interaction shifts the equilibrium toward the open form, thus favoring the hydrolysis of the lactone and limiting the therapeutic level of the active form [2,4]. The drug-stabilized topoisomerase I-DNA cleavable complex, containing single-strand break, is reversible [5]. This lesion is converted in the more lethal double-stranded breaks during DNA synthesis [6]. Therefore, as a result of this mechanism of

* *Corresponding author*. Tel.: +39 02 23902267; fax: +39 02 23902692.
E-mail address: franco.zunino@istitutotumori.mi.it (F. Zunino).
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.11.004

action, stability of the cleavable complex (i.e., drug–enzyme–DNA ternary complex) or prolonged exposure to the active drug form are critical requisites for therapeutic efficacy of camptothecins [2,3].

We have reported that 7-substituted lipophilic camptothecins exhibit favorable molecular and pharmacological features resulting in potential therapeutic advantages [7–13]. The promising pharmacological profile of analogs of this series likely reflects a rapid intracellular accumulation (a favorable event to minimize drug–plasma protein interaction) and a persistent stabilization of the cleavable complex. However, recent studies on the molecular structure of the cleavable complex stabilized by topotecan have indicated the participation of water in the enzyme-mediated cleavable complex [14], which likely provides stabilization of the ternary complex. Based on this model, hydrophilic analogs modified at the 7-position able to form extensive hydrogen bond networks have been prepared [15]. The reported hydrophilic camptothecin analogs form stable cleavable complex with DNA and topoisomerase I and the *in vivo* activity of the tested 7-modified analogs was found promising.

The present study was performed to explore the influence of hydrophilic substituents at the 7-position of novel camptothecins of the 7-oxyiminomethyl series [9]. Among the tested camptothecins we selected a potent analog, ST1976, for detailed antitumor activity studies.

2. Materials and methods

2.1. Drugs

The procedure for the synthesis of 7 modified camptothecins has been previously described [7,9] and the chemical structures are presented in Fig. 1. The clinical preparations of topotecan and irinotecan were used as standard reference. ST1600, ST1587, and ST1968 were dissolved in distilled water; ST1976 was dissolved in DMSO and diluted in sterile distilled water before use with a final DMSO concentration of 10% for oral and 5% for i.v. delivery. Irinotecan was dissolved in sterile, distilled water keeping it under magnetic stirring for about two hours, and administered i.v. very slowly. All drugs were delivered in a volume of 10 ml/kg of body weight.

2.2. Antiproliferative activity

Cell lines used were the non-small cell lung cancer cell line NCI-H460 (ATCC, HTB-177), the colorectal adenocarcinoma cell line HT29 (ATCC, HTB-38) and the corresponding mitoxantrone resistant variant HT29/mit [16].

Cells were cultured in RPMI-1640 containing 10% foetal calf serum. Cytotoxicity was assessed by growth inhibition assay after 1 h drug exposure. Cells in the logarithmic phase of growth were harvested and seeded in duplicates into 6-well plates. Twenty-four hours after seeding, cells were exposed to the drug and harvested 72 h after exposure and counted with a Coulter counter. IC_{50} is defined as the inhibitory drug concentration causing a 50% decrease of cell growth over that of untreated control.

2.3. Topoisomerase I-dependent DNA cleavage assay

A 3′-end labeled gel purified 751-bp *Bam*HI-*Eco*RI fragment of SV40 DNA was used for the cleavage assay. SV40 plasmid was first linearized with *Bam*HI enzyme and then 3′-labeled by using DNA polymerase I large (klenow) fragment (Invitrogen, Paisley, UK) in presence of dGTP and α^{32}P ddATP. The labeled DNA was then restricted with *Eco*RI enzyme and the corresponding 751-bp was purified on agarose gel. Topoisomerase I DNA cleavage reactions (20,000 cpm/sample) were performed in 20 μl of 10 mM Tris–HCl (pH 7.6), 150 mM KCl, 5 mM $MgCl_2$, 15 μg/ml BSA, 0.1 mM dithiotritol, and 640 ng of human recombinant enzyme (full length topoisomerase I) [17] for 30 min at 37 °C. Reactions were stopped by 0.5% SDS and 0.3 mg/ml of proteinase K for 45 min at 42 °C. Persistence of DNA cleavage at different time points was examined by adding 0.6 M NaCl after 30 min of incubation. After precipitation DNA was resuspended in denaturing buffer (80% formamide, 10 mM NaOH, 0.01 M EDTA and 1 mg/ml dyes) before loading on a denaturing 8% polyacrylamide gel in TBE buffer. Overall DNA cleavage levels were measured with a PhosphoImager 425 model (Molecular Dynamics).

2.4. Antitumor activity studies

All experiments were carried out using female athymic Swiss nude mice, 7–10 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 according to institutional guidelines [18].

Human tumor lines were maintained by serial s.c. passages of fragments (about 2 mm × 2 mm × 6 mm) of

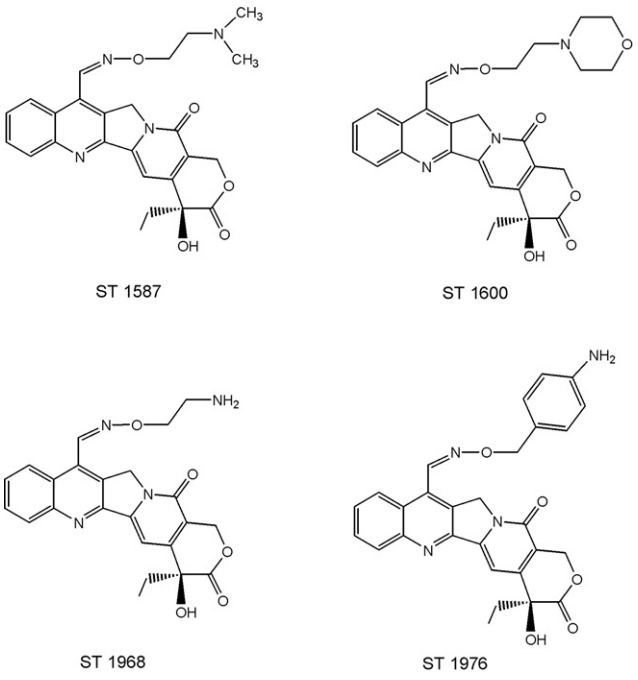

Fig. 1 – Chemical structures of novel hydrophilic camptothecins.

Table 1 – Antiproliferative activity of 7-modified camptothecins

Drug	IC_{50} (μM)[a]				log P[b]
	H460	HT29	HT29/Mit	RI[c]	
Topotecan	0.31 ± 0.11	1.35 ± 0.66	46.29 ± 21	34	1.394 ± 0.812
SN38	0.22 ± 0.013	0.67 ± 0.36	11.5 ± 2.7	17	2.625 ± 0.787
ST1976	0.025 ± 0.0014	0.038 ± 0.016	0.32 ± 0.1	8	3.006 ± 0.778
ST1600	0.077 ± 0.008	0.17 ± 0.08	1.01 ± 0.39	6	2.272 ± 0.80
ST1968	0.51 ± 0.15	0.46 ± 0.16	28.8 ± 4.84	62	1.938 ± 0.778
ST1587	0.3 ± 0.086	0.97 ± 0.32	4.32 ± 2.16	4	2.665 ± 0.80

[a] IC_{50}, drug concentration required for 50% reduction of cell growth as compared with untreated controls after 1-h exposure to the drug. Means ± S.D. are reported from at least three experiments.
[b] log *P* was calculated using Advanced Chemistry Development (ACCD/Labs) Software V8.14 for Solaris. P, partition coefficient between n-octanol and water
[c] RI, resistance index in HT29/Mit.

regrowing tumors in healthy mice [19]. Groups of four/six mice bearing bilateral s.c. tumors were employed for experiments. Tumor fragments were implanted on day 0 and 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 the longest diameter, respectively. ST1976 was delivered orally or i.v. and irinotecan i.v. Several schedules of administration were employed, starting treatments when tumors were just palpable (about 50 mm^3).

The efficacy of drug treatment was assessed as:

(i) Tumor volume inhibition percentage (TVI%) in treated versus control mice, calculated as: TVI% = 100 − (mean TV treated/mean TV control × 100).
(ii) $\log_{10}$ cell kill (LCK) calculated by the formula: $LCK = (T - C)/3.32 \times DT$, where *T* and *C* are the mean times (days) required for treated (*T*) and control (*C*) tumors, respectively, to reach an established TV and DT is the doubling time of control tumors.
(iii) Complete regression (CR), i.e., disappearance of the tumor lasting at least 10 days after the end of treatments.

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

Students' *t*-test (two tailed) and Fisher's exact test were used for statistical comparison of tumor volumes and CR, respectively.

3. Results

3.1. Antiproliferative activity

The antiproliferative effects of the tested camptothecins were determined at 72 h following 1 h-exposure to the drugs. The 7-modified analogs exhibited a different potency in inhibiting cell growth under these conditions (Table 1). The most water-soluble analog ST1968, which contains an aliphatic amine in the side chain, was characterized by a low potency comparable to that of topotecan or SN38, the active metabolite of irinotecan. In contrast, the less polar ST1976, which contains a primary aromatic amine as hydrophilic substituent in the side chain at 7-position, retained a marked potency. The tested hydrophilic analogs were apparently substrates of BCRP, because all compounds exhibited cross-resistance in the HT29/Mit subline which overexpresses BCRP, a transport system implicated in resistance to conventional camptothecins. This finding is consistent with the previous observation that other camptothecin analogs with high polarity may be substrates for BCRP [20,21]. Indeed, the less marked cross-resistance of ST1587, ST1976 and ST1600 is consistent with their lower polarity compared with ST1968.

Since human serum albumin is known to tightly bind the ring opened carboxylate form of camptothecins, thus favoring the lactone ring hydrolysis [22], a reduction of the cytotoxic effects of the camptothecin in the presence of human serum albumin provides indirect information on the lactone stability. Using this assay, no change in cytotoxic effect was observed for ST1600, ST1968 and ST1587. The IC_{50} value of ST1976 increased in the presence of 30 mg/ml HSA (Table 2), thus suggesting a reduced stability of the lactone. However, even in the presence of the high albumin concentration, ST1976 exhibited an antiproliferative potency comparable to other analogues and substantially higher than that of topotecan.

Table 2 – Effects of human serum albumin on antiproliferative activity of 7-modified camptothecins against H460 lung carcinoma cells

Drug	IC_{50} (μM)[a]		
	no HSA	HSA (1 mg/ml)	HSA (30 mg/ml)
Topotecan	0.52	0.74	1.13
ST1976	0.025 ± 0.0014	0.03 ± 0.0024	0.15 ± 0.004
ST1600	0.077	0.077	0.077
ST1968	0.24 ± 0.15	0.35 ± 0.15	0.21 ± 0.026
ST1587	0.3	0.3	0.3

[a] IC_{50}, drug concentration required for 50% reduction of cell growth as compared with untreated controls after 1-h exposure to the drug. Where reported, means ± S.D. are referred to at least three experiments.

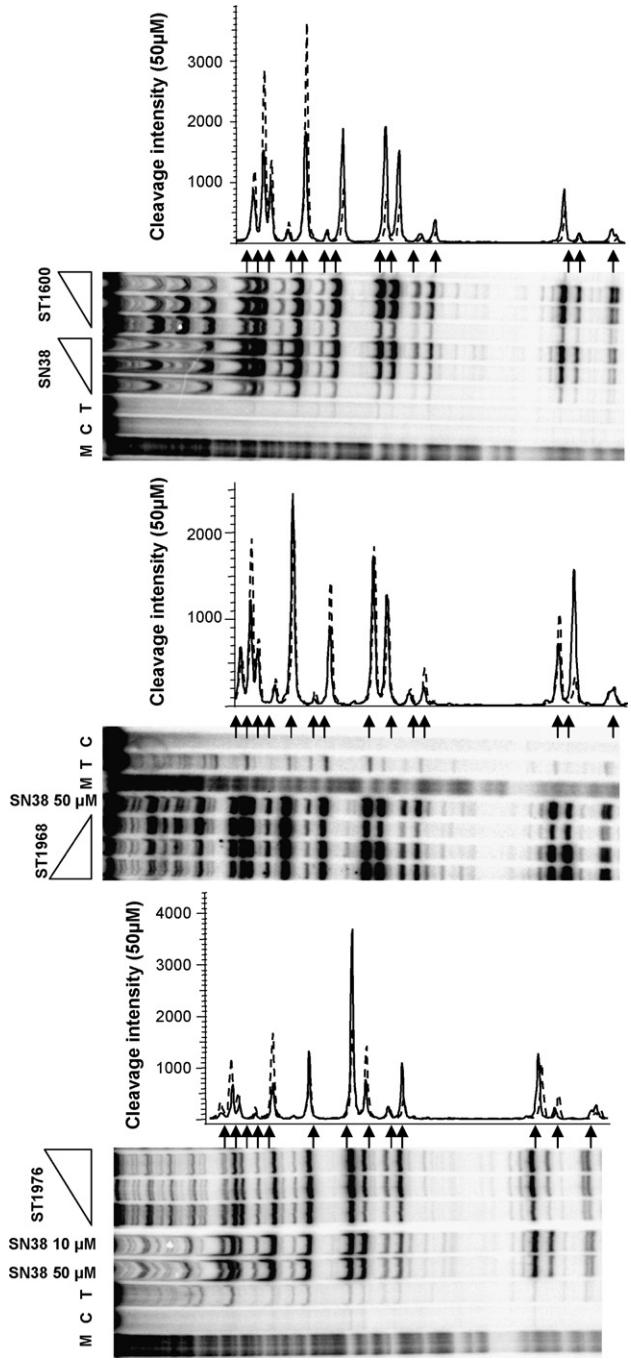

Fig. 2 – Topoisomerase I-mediated DNA cleavage by SN38, ST1968, ST1600 and ST1976. Samples were reacted with 1, 10 and 50 μM drug at 37 °C for 30 min. Reaction was than stopped by adding 1% SDS, 0.3 mg/ml of proteinase K and incubating for 45 min at 42 °C before loading on a denaturing 8% polyacrylamide gel. C, control DNA; T, reaction without drug; M, purine markers; solid line, novel camptothecin analogs (ST1976, ST1600 and ST1968); dashed line, SN38.

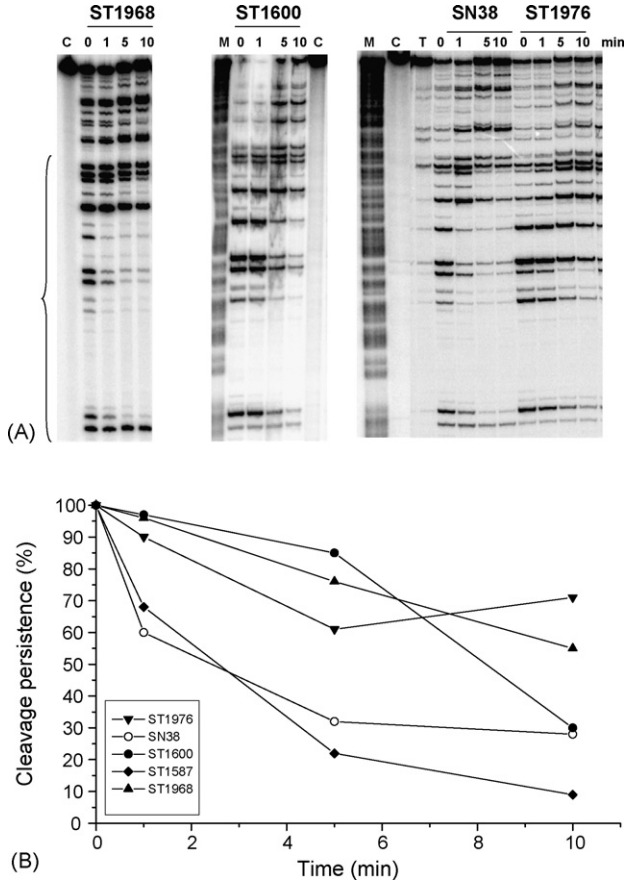

Fig. 3 – Persistence of topoisomerase I-mediated DNA cleavage in the presence of SN38, ST1968, ST1600, ST1587 and ST1976. (A) Time-course analysis of DNA cleavage induced SN38 and camptothecin analogs. The samples were reacted for 30 min with 10 μM drug. DNA cleavage was then reversed by adding 0.6 M NaCl. The 100% value is referred to the extent of DNA cleavage at 30 min of incubation. C, control DNA; T, reaction without drug; M, purine markers. (B) Comparison of cleavage persistence in presence of SN38 and camptothecin analogs. Each value was obtained by densitometric analysis of the site indicated in bracket. The experiment was repeated 3 times and the results of a representative value are reported.

3.2. *Topoisomerase I-mediated DNA cleavage*

Topoisomerase I-mediated DNA cleavage assays with purified human topoisomerase I were used to investigate the ability of camptothecin analogs to stimulate the DNA damage. SN38 was used as a reference compound. The compounds revealed an intensity of DNA damage comparable to that of SN38, as determined by densitometric analysis (Fig. 2). Cleavage pattern was found identical to SN38 for all the tested camptothecins. Since the drug interaction with the DNA enzyme complex is expected to be reversible, the drug potency is likely related to the drug stabilization of the ternary complex. Therefore, the stabilization of the cleavable complex was evaluated after the addition of high salt concentration (0.6 M NaCl), which favors the dissociation of the ternary drug–enzyme–DNA complex. As compared with SN38, ST1976, ST1600 and ST1968 revealed a more stable ternary complex. Such a feature was particularly evident for ST1976 and ST1968, characterized by the presence of a free amino group, which

Table 3 – Effects of oral ST1976 (q4dx4 times) against human gastrointestinal tumor xenografts

Tumor model	Dose (mg/kg)	TVI%[a]	CR[b]	LCK[c]	B.W. loss%[d]	Tox[e]
MKN28 gastric carcinoma	6	78	0/8	0.6	0	0/4
	12	92	0/8	1.0	4	0/4
CoBA colon carcinoma	15	94	0/8	1.3	20	0/4
	18	–	–	–	25	3/4
Panc-1 pancreatic carcinoma	14	98	5/8	1.6	16	0/4

[a] Tumor volume inhibition % in treated over control mice.
[b] Complete responses: i.e., disappearance of tumors lasting at least 10 days.
[c] Gross $\log_{10}$ cell kill to reach 1000 mm^3 of tumor volume.
[d] Body weight loss % induced by drug treatment.
[e] Number of dead mice/total number of mice.

revealed a DNA damage persistence around 60–80% after 10 min (Fig. 3). The less polar analog ST1587, bearing a methylated amino group in the side chain, exhibited a reduced stability.

3.3. *Antitumor activity studies*

On the basis of its antiproliferative potency, ST1976 was selected for further preclinical development. The antitumor activity of oral ST1976 was studied in a large panel of human tumor xenografts using various treatment schedules. The maximum tolerated dose (MTD) was determined with the intermittent q4dx4 schedule. Against MKN-28 gastric carcinoma the antitumor activity was dose-dependent at well tolerated doses, up to 12 mg/kg (Table 3). Against CoBA colon carcinoma, a higher dose was tested, 15 mg/kg, which was effective, without lethal toxicity, but with a substantial body weight loss. The dose of 18 mg/kg was highly toxic in most treated mice (3/4). With the dose of 14 mg/kg against the pancreatic carcinoma model Panc-1, an excellent antitumor activity was achieved (in 5/8 treated animals) without evidence of substantial toxicity. Although no toxic death were observed up to 15 mg/kg, the doses of 14–15 mg/kg caused a marked body weight loss.

We investigated the antitumor activity of ST1976 in comparison with irinotecan as a standard camptothecin, in a panel of tumor xenografts of various tumor types, including tumor models resistant to conventional camptothecins (Table 4). Irinotecan, was delivered i.v., q4dx4, 50 mg/kg (which in our experimental conditions is the MTD with such schedule). The same schedule (q4dx4) and the q11dx3 schedule were used for oral ST1976 delivering 10 and 15 mg/kg, respectively. Under these conditions ST1976 was well tolerated and no toxic death occurred. When the compounds

Table 4 – Pattern of antitumor activity of oral ST1976 and i.v. irinotecan (CPT11)

Tumor model	Drug	Dose (mg/kg)		TVI%[a] (day)	CR[b]	LCK[c]	B.W. loss %[d]
		q4dx4	q11dx3				
IGROV-1 ovarian carcinoma	CPT11	50		49 (26)	0/10	0.5 (300)	4
	ST1976	10		96*	0/8	1.4	17
			15	88 (35)	0/8	1.2	21
HT1376 bladder carcinoma	CPT11	50		46 (28)	0/10	0.2 (300)	9
	ST1976	10		85*	0/8	0.9	12
			15	76 (35)	1/8	0.9	16
CoRa colon carcinoma	CPT11	50		60 (37)	0/12	0.8 (500)	8
	ST1976	10		89*	0/10	1.5	14
LoVo colon carcinoma	CPT11	50		93 (34)	0/12	1.5 (700)	3
	ST1976	10		97	1/10	2.0	13
MESO mesothelioma	CPT11	50		78 (30)	0/12	0.8 (200)	7
	ST1976	10		80	0/8	0.8	20
			15	73 (63)	0/8	0.9	16
A431 epidermoid carcinoma	CPT11	50		100 (29)	12/12	6.8 (1000)	7
	ST1976	10		97**	4/8	2.0	10
			15	76	0/8	0.8	3

*$P < 0.05$; **$P < 0.01$ vs. CPT11-treated mice, by Student's *t*-test.
[a] Tumor volume inhibition % in treated over control mice; in parenthesis the day on which it was assessed.
[b] Complete responses: i.e., disappearance of tumors lasting at least 10 days.
[c] Gross $\log_{10}$ cell kill to reach an established tumor volume (in parenthesis, mm^3).
[d] Body weight loss % induced by drug treatment; the highest change is reported.

Table 5 – Effects of oral ST1976 against slowly growing human tumor xenografts

Tumor model[a]	Single dose (mg/kg)	Days of treatment	Total dose (mg/kg)	TVI%[b]	CR[c]	LCK[d]	BWL%[e]	Tox[f]
A549 NSCLC	1.25	4 → 8, 11 → 15, 18 → 22, 25 → 29, 32 → 36, 39 → 43	37.5	50	0/8	0.6	2	0/4
	1.5	4 → 8, 11 → 15, 18 → 22, 25 → 29, 32 → 36, 39 → 43	45	70	0/8	0.9	4	0/4
	1.8	4 → 8, 11 → 15, 18 → 22, 25 → 29, 32 → 36, 39 → 43	54	83	0/8	1.5	12	0/4
	15	4, 11, 18, 25, 32, 39	90	71	0/6	1.2	7	1/4
CoBA colon carcinoma	2	5 → 9, 19 → 23, 33 → 37, 47 → 51, 61 → 65	50	92	1/8	2.7	9	1/4
	2.5	5 → 9, 19 → 23, 33 → 37, 47 → 51, 61 → 65	62.5	96	1/8	2.7	10	0/4

[a] Doubling time, around 6.5 days for both tumors.
[b] Tumor volume inhibition % in treated over control mice.
[c] Complete responses, disappearance of tumor lasting at least 10 days.
[d] Gross $\log_{10}$ cell kill to reach an established tumor volume, i.e., 900 mm^3 for A549 and 1000 mm^3 for CoBA-bearing mice.
[e] Body weight loss % induced by tumor and drug treatment, the highest change is reported.
[f] Number of dead mice/total number of mice.

were delivered with the same schedule (q4dx4), oral ST1976 showed superior efficacy over i.v. irinotecan against IGROV-1 ovarian carcinoma, HT1376 bladder carcinoma and CoRa colon carcinoma ($P < 0.05$ in TVI% values), all quite resistant to irinotecan. ST1976 was partially superior to irinotecan even against LoVo colon carcinoma. The two drugs had a comparable activity against MESO mesothelioma, whereas irinotecan exhibited a superior efficacy only against A431 epidermoid carcinoma, a tumor model highly responsive to camptothecins. When delivered at the dose of 15 mg/kg with the less frequent treatment schedule (q11dx3), ST1976 showed lower efficacy than when delivered at the dose of 10 mg/kg, q4dx4, in spite of comparable total doses (45 and 40 mg/kg).

We have previously shown that slow-growing tumor xenografts are more sensitive to daily protracted administration of camptothecins [12]. Thus, a daily prolonged treatment schedule with low-dose levels of ST1976 was tested against the A549 lung carcinoma (Table 5). With this schedule the highest dose tested (total dose, 54 mg/kg) was significantly effective. In contrast, the weekly treatment (q7dx6) with 15 mg/kg was less effective, in spite of the higher total dose delivered (90 mg/kg). In mice bearing the CoBA colon carcinoma, the doses of 2 and 2.5 mg/kg with the daily schedule were safely employed and ST1976 showed relevant efficacy.

In an attempt to identify optimal treatment conditions, the antitumor activity of ST1976 was investigated against the ovarian carcinoma IGROV-1 and the bladder carcinoma HT1376 using different schedules and comparing i.v. and oral administration. In IGROV-1 tumor, when ST1976 was delivered orally, the best antitumor effect was achieved with the daily

Table 6 – Effects of ST1976 against human tumor xenografts

Tumor model	Route	Days of treatment	Dose (mg/kg)		TVI%[a]	CR[b]	LCK[c]	BWL%[d]	Tox[e]
			Single	Total					
IGROV-1 ovarian carcinoma	i.v.	4, 8, 11, 15	4	16	84 (22)	0/8	0.6	9	0/4
		4, 8, 11	5	15	–	–	–	34	4/4
		4, 15, 27, 39	6.25	25	98 (55)	3/8	2.7	24	0/4
		4	7.5	7.5	78 (15)	0/6	0.5	20	1/4
	Oral	4 → 8, 11 → 15, 18 → 22, 25 → 29	1.25	25	89 (40)	0/8	1.5	6	0/4
		4 → 8, 11 → 15, 18 → 22, 25 → 29	2	40	96 (40)	0/8	1.9	16	0/4
		4, 8, 12, 16	10	40	96 (25)	0/8	1.4	17	0/4
		3, 14, 24	15	45	88 (35)	0/8	1.2	21	0/4
HT1376 bladder carcinoma	i.v.	6, 10, 14, 18	4.2	16.8	68 (27)	0/8	0.5	10	0/4
		6, 10, 14, 18	4.5	18	91 (31)	1/8	1.3	14	0/4
		6, 13, 20, 27, 34	5	25	83 (46)	0/8	1.3	6	0/4
	Oral	7, 11, 15, 19	10	40	85 (28)	0/8	0.9	12	0/4
		7, 18, 28	15	45	76 (40)	1/8	0.9	16	0/4

[a] Tumor volume inhibition % in treated over control mice, in parenthesis the day on which it was assessed.
[b] Complete responses: i.e., disappearance of tumors lasting at least 10 days.
[c] Gross $\log_{10}$ cell kill to reach 300 mm^3 of tumor volume.
[d] Body weight loss % induced by drug treatment; the highest change is reported.
[e] Number of dead mice/total number of mice.

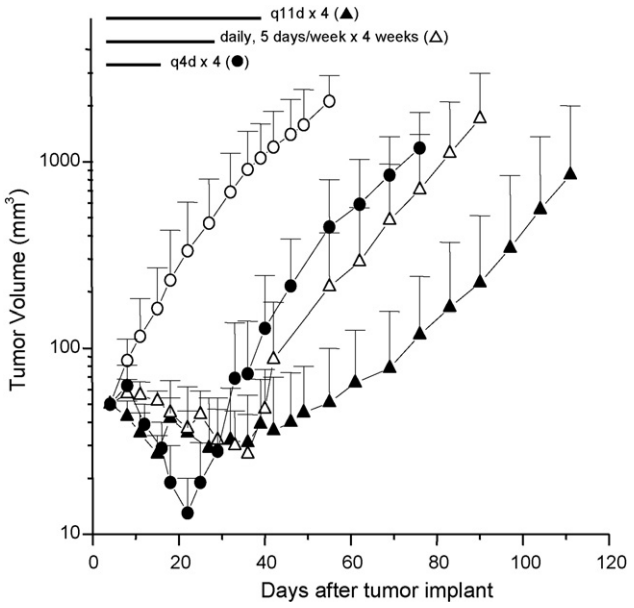

Fig. 4 – Effects of ST1976 on s.c. growing IGROV-1 human ovarian carcinoma. (○) Control untreatred tumors; (▲) i.v. ST1976, 6.25 mg/kg, q11dx4; (●) oral ST1976, 10 mg/kg, q4dx4; (△) oral ST1976, 2 mg/kg, daily for 5 days/week for 4 weeks. Bars, standard deviation.

low-dose schedule (Table 6 and Fig. 4). Following i.v. administration, a relevant therapeutic outcome (3/8 CR) was achieved even with an intermittent treatment schedule (q10-12d) at the maximum tolerated dose (6.25 mg/kg). Against the less responsive HT1376 bladder tumor, optimal effects were obtained by i.v. administration according to the q4d schedule (Table 6 and Fig. 5). In general, for both administration routes, less frequent administrations were less effective, and the drug was more toxic following i.v. than oral administration.

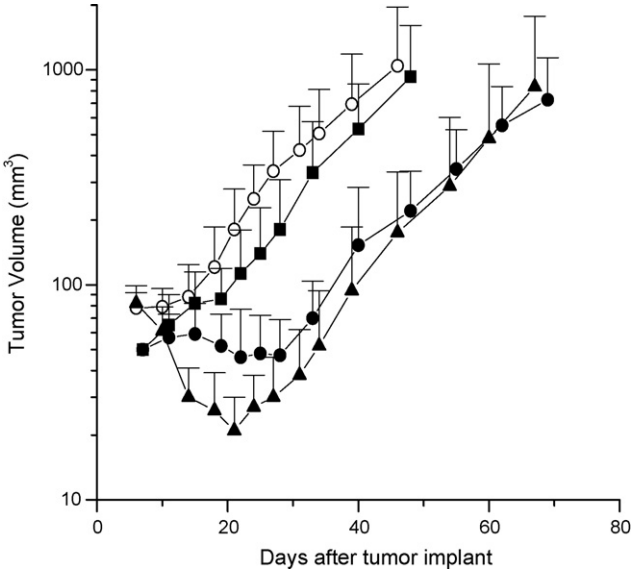

Fig. 5 – Effects of ST1976 and irinotecan on s.c. growing HT1376 human bladder carcinoma. Treatments were delivered q4dx4 times. (○) Control untreatred tumors; (■) i.v. irinotecan, 50 mg/kg; (●) oral ST1976, 10 mg/kg; (▲) i.v. ST1976, 4.5 mg/kg. Each point represents the mean value of 10 tumors. Bars, standard deviation.

4. Discussion

The results presented in this work indicate that the novel camptothecin ST1976 was very effective against several human tumor models, including tumors relatively resistant to irinotecan. The activity of ST1976 evaluated in conventional s.c.-transplanted tumor models was generally superior to that of irinotecan in various tumor types. More detailed information on the therapeutic advantages of the novel camptothecin could be obtained by the use of orthotopic models [12]. ST1976 and other tested analogs (Table 1) were apparently substrates for BCRP, as indicated by cross-resistance in HT29/Mit cells, which overexpresses BCRP [16]. However, the degree of resistance to ST1976 was substantially lower than that to other more hydrophilic analogs (e.g., topotecan and ST1968) (Table 1). Indeed, in spite of the presence of an amino group in the side chain at the position 7, ST1976 was characterized by limited water solubility which is consistent with a lower polarity (Table 1). The behavior of the tested camptothecins is consistent with the hypothesis that the substrate specificity of BCRP is dependent on the polarity of the camptothecins and not only on the presence of hydroxyl group at positions 10 and 11 [20,21]. In fact the most polar compound of this series ST1968 was apparently the best substrate for BCRP, thus indicating that even substitutions at position 7 confer recognition by BCRP. The partial recognition of ST1976 by this transport system, which is known to be expressed in the intestinal mucosa [23], provides a plausible explanation of the superior drug efficacy and potency following i.v. administration as compared with oral administration. Nevertheless, ST1976 retained an outstanding activity also with oral treatment, as for other lipophilic analogs of the 7-oxyiminomethyl-substituted camptothecins [8–13]. Indeed, a preliminary comparative study of ST1976 and gimatecan, the lead compound of this series, indicated a comparable antitumor activity against the gastric carcinoma MKN28, the pancreatic carcinoma Panc-1 and the colon carcinoma CoBA. ST1976 was characterized by an improved therapeutic index. However, the relative therapeutic advantages of ST1976 remain to be documented. Comparing the antitumor effects of ST1976 delivered according to different schedules, the best results were achieved by the daily prolonged low-dose schedule. Such behavior is a general observation which applies to most camptothecins [11] and is likely related to their antiangiogenic effects as a consequence of inhibition of HIF-1α [24,25]. With the i.v. treatment, the tolerability was markedly dependent on the treatment schedule rather than on the cumulative dose. Indeed an increase of interval between treatments allowed the administration of doses which were toxic with a more intensive treatment schedule (Table 6).

The camptothecin analogs, characterized by the presence of a free amino group in the side chain at the 7 position (e.g., ST1968 and ST1976), exhibited a remarkable ability to stabilize

the cleavable complex. This observation is consistent with the hypothesis that the drug ability to form hydrogen bonds at the 7-position would result in stable cleavable complexes as documented for other analogs modified at the 7-position [15]. Thus, although the incorporation of hydrophilic groups in the side chain at the 7-position is expected to reduce drug uptake and intracellular accumulation, the tested analogs retained the ability to poison topoisomerase I. Both ST1600 and ST1968 analogs exhibited a divergent behavior between cell growth inhibition, as detected by reduced antiproliferative potency, and molecular interaction at target level, as documented by topoisomerase I inhibitory efficacy in assays with purified enzyme. In contrast, the less water-soluble analog ST1976 retained a remarkable efficacy at both cellular and molecular level. The different behavior of ST1976, in comparison with the two more hydrophilic analogs, supports the interpretation that, in addition to the inhibitory activity at the target level, cellular pharmacokinetics is a determinant of the antiproliferative potency of active camptothecins.

Our original working hypothesis in the development of novel camptothecins was based on putative advantages of the drug lipophilicity related to rapid intracellular accumulation of the active lactone form [26]. Lipophilicity is also expected to influence the stability of the lactone form and the pharmacological behavior. The potent cytotoxic activity and the excellent antitumor efficacy of ST1976, characterized by the presence of a hydrogen-bonding group but by a limited hydrophilicity, likely reflect a combination of favorable features and support that both stable drug–target interactions and pharmacological behavior are critical events to enhance therapeutic efficacy of camptothecins.

In conclusion, on the basis of the peculiar preclinical profile, showing versatility with both oral and i.v. administration and efficacy against tumors resistant to irinotecan and a good therapeutic index, ST1976 is a promising candidate for clinical development.

Acknowledgements

This work was partially supported by the Associazione Italiana per la Ricerca sul Cancro, Milan, and by the Ministero della Salute, Rome, Italy.

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