Antiangiogenic Effects of the Novel Camptothecin ST1481 (Gimatecan) in Human Tumor Xenografts

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Abstract

ST1481 (gimatecan) is a novel lipophilic camptothecin with a promising preclinical pharmacological profile. On the basis of its high antitumor efficacy when delivered by the oral route, the compound is suitable for prolonged administration. This schedule of treatment has been reported as the most appropriate to exploit the antiangiogenic effects of cytotoxic drugs. The aim of the study was to investigate the antiangiogenic and antitumor effects of oral ST1481 in human tumor xenografts. In spite of a marginal drug effect against the s.c. growing A549 lung carcinoma following administration with an intermittent schedule (q4dx4 times, maximum tolerated dose: 2 mg/kg), tumor growth was strongly inhibited by a daily low-dose (0.5 mg/kg) prolonged administration. Immunohistochemical analysis showed a reduced number of microvessels in tumors of both treated groups *versus* controls and a significantly higher reduction in the daily *versus* the q4dx4-treated tumors ($P < 0.0001$, by Student's t test). In our experimental model, the relation between microvessel density and tumor size ($r = 0.738$, by the Spearman rank test) suggests a role of inhibition of tumor vasculature in tumor response. Significant inhibition of tumor angiogenesis ($P < 0.0001$ *versus* control tumors) was observed even with a very low drug dose (0.06 mg/kg) in the orthotopically implanted (i.d.) MeWo melanoma, under conditions causing minimal tumor growth inhibition. Additional evidences of the antiangiogenic activity of ST1481 were provided by antimotility effects on endothelial cells, *in vivo* inhibition of vascularization in the Matrigel assay, and downregulation of the expression of the proangiogenic basic fibroblast growth factor in A549 tumor cells associated with inhibition of the pathway involving Akt. In conclusion, the available results support the possibility that the antiangiogenic properties of ST1481 contribute to its antitumor potential and that this effect might be enhanced by the continuous low-dose treatment.

Introduction

Camptothecins are clinically effective antitumor agents with a wide spectrum of antitumor activity against solid tumors (1). Their efficacy is related to the ability to inhibit the DNA topoisomerase I function by stabilizing the DNA-enzyme complex (2). From a novel series of 7-modified camptothecins, a lipophilic analog, ST1481 or gimatecan, has been selected for the marked cytotoxic potency and topoisomerase I inhibitory activity (3, 4). Preclinical studies with ST1481 in human tumor xenografts have shown pharmacological improvements over topotecan, a clinically established camptothecin (5, 6). As reported for other camptothecins (7), daily administration of the drug was the optimal schedule for antitumor activity of ST1481, although the analogue was very effective with various treatment schedules (5, 6). Reversibility of the drug-target complex, as well as low stability of the active molecule (lactone form), have been reported as possibly responsible for the therapeutic advantages generally observed by frequent administrations with drugs of this class (8). ST1481 is highly bioavailable by the oral route and such a property makes it very suitable for prolonged daily administration.

Inhibition of angiogenesis has been reported for camptothecins in *in vitro* and *in vivo* assays (9–13), but its role in the drug antitumor effect remains to be elucidated. Indeed, preclinical studies have indicated the ability of a number of established cytotoxic drugs, such as cyclophosphamide and vinblastine, to strongly inhibit the growth of sensitive and resistant tumors when administered according to an "antiangiogenic schedule," that is, continuous low-dose scheduling (14–16), and many antitumor chemotherapeutic agents have been tested for their antiangiogenic potential (17). The continuous low-dose scheduling has been described as the most appropriate to exploit the antiangiogenic potential of cytotoxic drugs (18, 19).

The aim of the study was to investigate the antiangiogenic effect of ST1481 and its role in the antitumor activity of the compound in human tumor xenografts. The drug was administered by the oral route. The A549 non-small cell lung cancer was used; it is a slowly growing tumor only moderately responsive to camptothecins administered by an intermittent treatment, but sensitive to the continuous drug treatment. In addition, an orthotopic model of melanoma (MeWo cells i.d. implanted) was also investigated. Both tumor models have been reported as suitable for angiogenesis studies *in vivo* (20, 21). The results indicated that angiogenesis inhibition may contribute to the antitumor efficacy of ST1481 in human tumor xenografts.

Results

Growth of the A549 tumor xenograft was affected differently by ST1481 when delivered by a daily or an intermittent treatment schedule. We have already reported that the low dose used in the daily schedule, 0.5 mg/kg, daily ×5d/week, was well tolerated even following a protracted treatment (6 weeks) and much more effective than the maximum tolerated dose (MTD) delivered q4dx4 (6). In the present study, we show that the superior efficacy was maintained even when the treatment with the daily low dose was started in the presence of large tumors [300 mm^3, mean tumor volume (TV)] and lasted shorter (2 weeks instead of 6 weeks; Fig. 1). Indeed, at day 36 (7 days after treatment ended), the mean TV in the daily treated group was significantly smaller than in the q4dx4-treated mice ($P < 0.01$, by Student's t test), in spite of the lower ST1481 total dose received in the former group (5 *versus* 8 mg/kg; Table 1).

In an attempt to investigate the basis of the superior antitumor efficacy achieved with ST1481 by the daily compared with the intermittent administration, we studied the extent of apoptosis and tumor angiogenesis. In treated A549 tumors, immunohistochemistry (IHC) analysis for microvessel density (MVD) assessment clearly showed that the drug significantly decreased MVD *versus* control tumors ($P < 0.0001$) and that the "low-dose daily schedule" was significantly more effective than the "MTD-intermittent schedule" ($P < 0.0005$, Table 1). In contrast, the level of apoptosis induction by the drug administered according to either schedule was comparable, and the values of apoptotic index (AI) were doubled in both
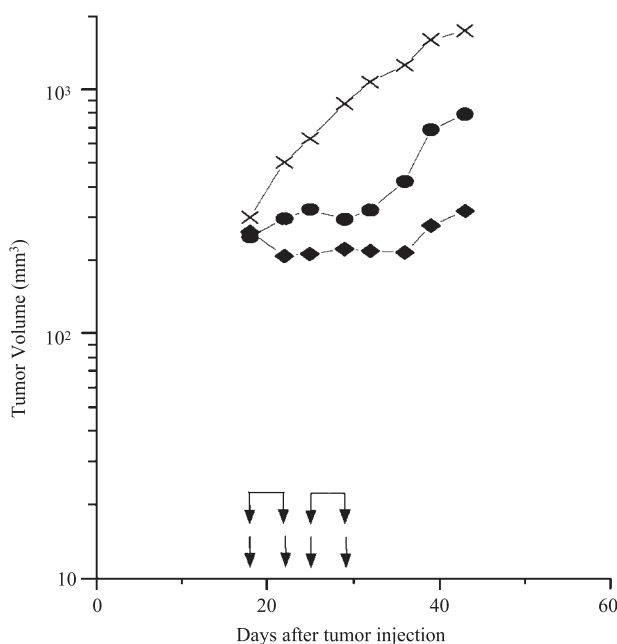
treated groups *versus* control tumors ($P < 0.0001$, Table 1). When A549 TVs were plotted *versus* MVD, a linear relation was found ($r = 0.738$, $P < 0.05$ by the Spearman rank correlation test, Fig. 2).

The inhibitory effect of the compound on tumor angiogenesis was confirmed in the experiment performed with i.d. MeWo melanoma-bearing mice treated daily with two low-dose levels of ST1481 for 2 weeks (Table 2). The drug doses used in the study (0.06 and 0.12 mg/kg) were 4- to 8-fold lower than those used in the A549 tumor. Nevertheless, the extent of vascularized area was significantly lower in both treated groups than in control tumors ($P < 0.0001$, by the Mann-Whitney test). In such experimental conditions, only the dose of 0.12 mg/kg was effective in inhibiting tumor growth, whereas the lower dose tested (0.06 mg/kg) was inactive (Fig. 3). Thus, this result documented that even very low doses of ST1481 were effective on tumor vascularization.

The effect of ST1481 on *in vivo* blood vessel development was investigated in the Matrigel plug assay, where the formation of new vessels was induced by a basic fibroblast growth factor (bFGF)-embedded pellet injected s.c. in mice. Daily treatment of ST1481 (0.12 mg/kg, p.o.) significantly reduced the angiogenic response evoked by bFGF. As shown in Fig. 4, a significant decrease of the hemoglobin level in bFGF-containing pellets was observed in ST1481-treated mice as compared to vehicle-treated mice ($P < 0.01$, by the Mann-Whitney test). The value observed in ST1481-treated mice was comparable to that present in Matrigel not embedded with bFGF, thus suggesting that ST1481 completely blocked the fibroblast growth factor (FGF)-induced vessel formation.

To investigate the cellular mechanisms responsible for the antiangiogenic effect of ST1481, the inhibitory effect of the drug on endothelial cell proliferation and motility was investigated in cell systems. In endothelial cells, 1 h exposure to the compound induced an antiproliferative effect about 20-fold greater than topotecan, the IC$_{50}$ values being 45 and 87 ng/ml for ST1481 on bovine aortic endothelial cells (BAEC) and b-End, respectively, and 1200 ng/ml for topotecan on both cell lines. Since long-term exposure has been reported as the most appropriate to achieve endothelial cell hypersensitivity to chemotherapeutic drugs (22), the inhibition of cell proliferation was determined also on endothelial and tumor cells after short- and long-term exposure to the drug. As shown in Table 3, endothelial cells did not exhibit an increased sensitivity over tumor cells at any time. This result may suggest that the *in vivo* antiangiogenic effect is not only related to inhibition of endothelial cell proliferation.

In an attempt to identify other cellular effects that may be relevant for the antiangiogenic activity of the camptothecin, we investigated endothelial cell migration following long-term exposure to pharmacologically relevant drug concentrations. In the Boyden chamber assay (Fig. 5), BAEC cells showed a high capability of migration which was increased by the presence of serum in the medium. Exposure to ST1481 for 144 h caused a migration inhibition of about 40% compared to untreated cells.

Since endothelial cell migration has been related to the production of a number of growth factors secreted from tumor cells and neighboring stromal and normal tissues (23), we examined the effect of the camptothecin analogue on the

FIGURE 1. Antitumor effects of ST1481 in the treatment of A549 non-small cell lung carcinoma. Oral treatments started when tumor size was about 300 mm^3. ×, controls; ●, ST1481, 2 mg/kg, q4dx4; ◆, ST1481, 0.5 mg/kg, qdx5. *Arrows* indicate the days (↓) or weeks (↓↓↓) of treatment.

Table 1.   Antitumor Effects of Oral ST1481 Delivered by Two Different Treatment Schedules Against the A549 Human Lung Carcinoma Xenograft

Drug	Schedule	Dose (mg/kg)	TVI%[a,b]	LCK[c]	AI[a,d]	MVI%[a,e]
10% DMSO					2.6 ± 1.1	
ST1481	q4dx4	2	67	0.7	5.5 ± 1.2***	45***
ST1481	qdx5/wx2w	0.5	83*	>>1.2	5.6 ± 0.7***	61**·***

Note: Treatments from day 18 (mean TV ≅ 250 mm³).
[a]Thirty-six days after the tumor inoculum, *i.e.*, 7 days after treatment ended.
[b]TVI% = TV inhibition percentage in treated *versus* control mice.
[c]LCK = $\log_{10}$ cell kill (to 600 mm³ of tumor).
[d]AI = Apoptotic Index = number of apoptotic nuclei/total number of cells × 100. Mean values ±SD are reported.
[e]MVI% = microvessel (CD31⁺ cells) inhibition percentage = 100− (mean MV density in treated/mean MV density in control tumors × 100).
*$P < 0.01$ *versus* q4dx4 drug-treated mice.
**$P < 0.0005$ *versus* q4dx4 drug-treated mice.
***$P < 0.0001$ *versus* control mice, by Student's *t* test.

production of proangiogenic factors in tumor A549 cells. The expression levels of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and bFGF were determined by Western blotting at different times (6, 24, and 72 h) following 1 h exposure to drug concentrations corresponding to the IC_{50} and IC_{80}. The results indicated a specific modulation of bFGF expression by ST1481. In fact, although VEGF (Fig. 6) and platelet-derived growth factor (not shown) were not affected by treatment, bFGF appeared to be down-regulated in a time- and dose-dependent manner. In the same experimental conditions, ST1481 treatment strongly reduced Akt activation, as detected by its dephosphorylation at Ser 473, whereas the overall levels of Akt protein were not affected (Fig. 6). Similar to the drug effect on bFGF expression, the inhibition of Akt activation was dose- and time-dependent, and persisted up to 72 h in A549 cells treated with the higher drug concentration.
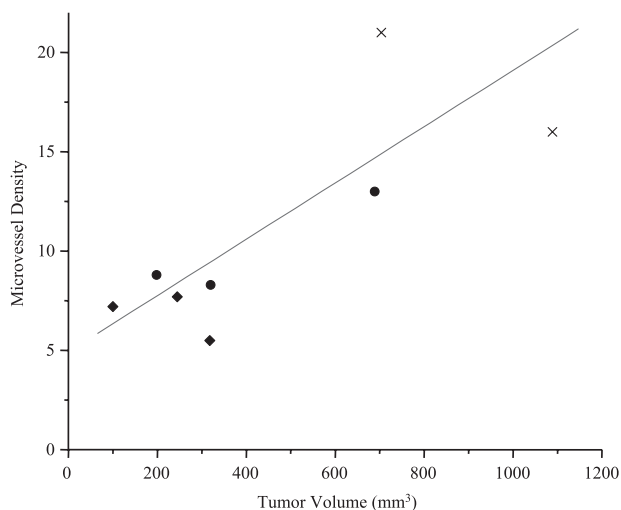
FIGURE 2.   Relationship between TV and MVD in A549 lung carcinoma xenograft. *Points,* tumors, including untreated controls (×) and ST1481-treated tumors, 2 mg/kg, q4dx4 (●), 0.5 mg/kg, qdx5 (◆). $r = 0.738$, $P < 0.05$, by Spearman rank correlation test.

Discussion

Our study documents that the novel camptothecin, ST1481, has a potent antiangiogenic activity, since it produced an inhibitory effect on vasculature development in human tumor xenograft at a very large range of low and suboptimal doses delivered by daily protracted treatment. Such property was clearly demonstrated in the early phase of development of the melanoma model, MeWo. Under conditions where the drug produced a marginal effect on tumor size (0.06 mg/kg), a strong inhibition of angiogenesis was observed. At a higher suboptimal dose (0.12 mg/kg), the drug-induced inhibition of angiogenesis was also associated with a marked reduction of tumor growth, possibly because of a substantial contribution of a direct cytotoxic effect on tumor cells. This drug action may be of particular relevance for therapeutic implications and suggests optimal schedules to exploit the therapeutic potential of camptothecins. Indeed, in the treatment of A549 lung tumor, ST1481 delivered at a therapeutic dose level (0.5 mg/kg) according to the daily low-dose schedule (6) was more effective in inhibiting blood vessel density and tumor growth than at the MTD according to an intermittent schedule. Although tumor size is not necessarily expected to be closely related to vascularization density (24), in the A549 model a linear relation between TVs and MVD was found, thus supporting that the antitumor effects of ST1481 may be related to inhibition of angiogenesis. This interpretation is also consistent with the observation that in spite of a comparable extent of apoptotic cells in tumors treated with ST1481 delivered by the two different schedules, blood vessel density was significantly lower with the continuous treatment.

In vitro ST1481 exhibited a potent antiproliferative activity against endothelial cell cultures, substantially higher (about 20-fold) than that of topotecan. However, the relative cytotoxic potency of ST1481 was similar to that achieved in A549 and MeWo tumor cells and in other tumor cell lines after short-term (5, 25) or prolonged exposure times (144 h). Thus, a differential cytotoxicity did not account for the antiangiogenic effect of ST1481 in *in vivo* systems. Although a direct cytotoxic activity of the camptothecin analogue on tumor endothelium may cause a vascular damage, the inhibitory

Table 2. Effect of Oral ST1481, Delivered qdx5/wx2w, on MeWo Melanoma Xenograft

Drug	Dose (mg/kg)	Vascular Area[a]	
		%	mm^2
10% DMSO		7.8 ± 0.29	8.55 ± 0.31
ST1481	0.06	4.8 ± 0.55*	5.24 ± 0.60*
ST1481	0.12	3.5 ± 0.33**	3.82 ± 0.36**

[a]Vascular area was calculated as described in "Materials and Methods," 14 days after tumor inoculum. Mean ± SE.
*$P < 0.01$ *versus* control mice, by the Mann-Whitney test.
**$P < 0.001$ *versus* control mice, by the Mann-Whitney test.

effect of the drug on tumor angiogenesis *in vivo* seems influenced by additional mechanisms, such as inhibition of endothelial cell motility, which was clearly documented in the study, or host-mediated effects.

Tumor neovascularization and endothelial cell migration are known to be promoted by a number of angiogenesis factors produced in part by the tumor cells. The specific ST1481-induced down-regulation of bFGF in A549 tumor cells suggested an additional mechanism contributing to the antiangiogenic activity of the novel camptothecin. Indeed, the ability of the drug to inhibit bFGF-mediated blood vessel development (Fig. 4) supports the relevance of inhibiting expression of bFGF, which may be released by tumor cells as well as by neighboring stromal cells and normal tissues (23).

The capacity of camptothecins to modulate the expression of important factors controlling cell proliferation and tumor invasion is an important aspect of their antitumor efficacy (9–13). Although DNA topoisomerase I inhibitors have been traditionally regarded as non-specific DNA-damaging agents, recent observations support that such agents are able to differentially modulate specific genes (26, 27). Such behavior could be attributed to a role of the target enzyme as a transcriptional regulator and to its involvement in cellular response to DNA damage (28). In particular, FGF is a well-known proangiogenic factor (29), but the FGF signaling pathway also appears to play an important role in tumor development and progression. Deregulation of the pathway may contribute to the promotion of cell growth as a consequence of the overexpression of FGF receptors with the possibility of autocrine stimulation (29). A specific inhibition of bFGF could account for the impressive efficacy on tumor
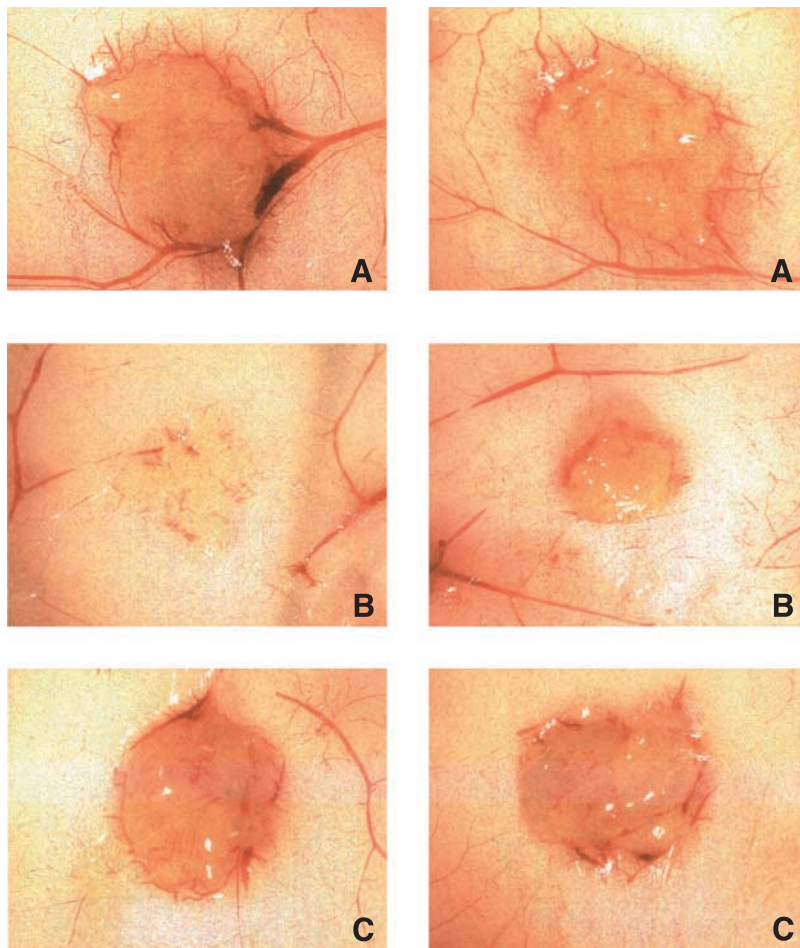
FIGURE 3. Inhibitory effect of ST1481 on angiogenesis of i.d. MeWo melanoma xenograft. Tumors and surrounding skin sections were photographed to visualize the vascularization area (14 days after tumor cell inoculum, *i.e.*, the day after the treatment ended). **A.** Untreated control tumors. **B.** Tumors treated with ST1481 (0.12 mg/kg). **C.** Tumors treated with ST1481 (0.06 mg/kg). ST1481 was delivered by oral route, qdx5/wx2w. Two tumors/group are shown.
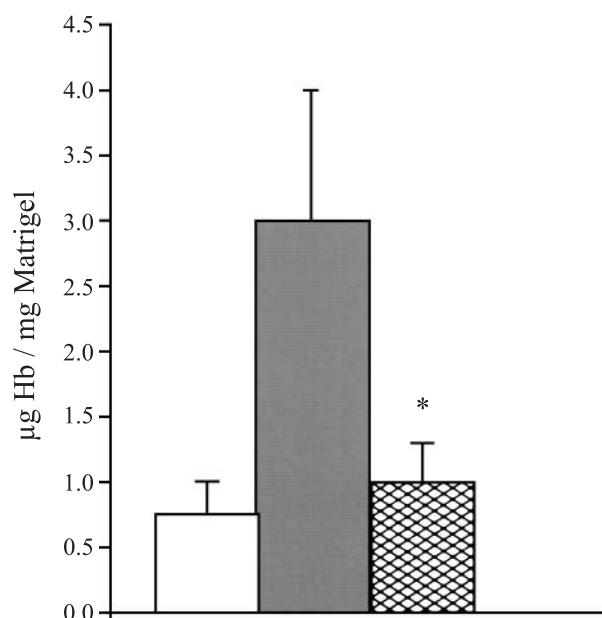
FIGURE 4. Inhibitory effect of ST1481 on bFGF-induced angiogenesis in the Matrigel plug assay. Matrigel without (□) or with bFGF (150 ng/pellet) was implanted s.c. in mice. Mice with bFGF-containing Matrigel were treated with vehicle (■) or ST1481 (▨) 0.12 mg/kg, p.o., daily × 6. The day after treatment ended, pellets were surgically removed, and the hemoglobin level was assessed. *Columns,* mean hemoglobin content (μg Hb/mg pellet) ± SE. *, $P < 0.01$ *versus* vehicle-treated mice (Mann-Whitney U test).

FIGURE 5. Effect of ST1481 on endothelial cell migration. BAEC cells were incubated for 144 h with (▨) or without (□) the drug (1 ng/ml) and then allowed to migrate for 3 h in Boyden chamber in the presence (+) or absence (−) of FCS. *Columns,* means of duplicate determinations of three independent experiments; *bars,* SD.
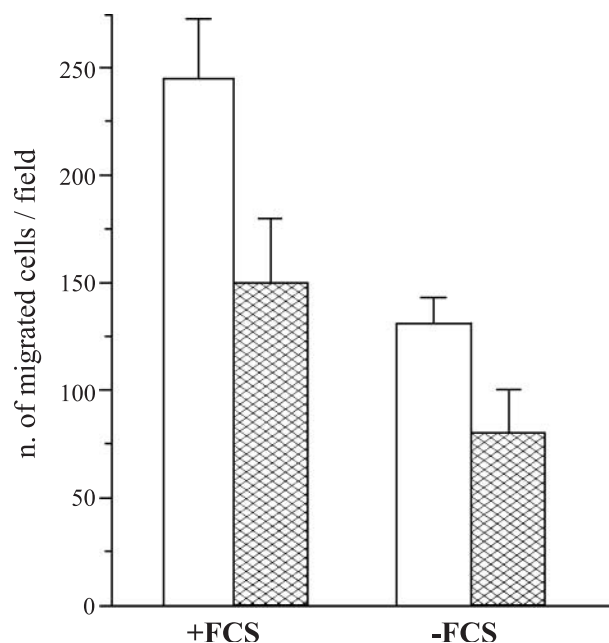
growth of ST1481 against most tumor types, including melanoma, glioma, and prostate carcinoma (6, 27), in which a paracrine or autocrine stimulation of tumor growth has been reported (29). In addition, our data indicate that the cellular response to the novel camptothecin resulted in an inhibition of the pathway involving Akt activation. Such effect, previously described for topotecan (30), could therefore represent a feature, shared by compounds of this class, contributing to their cytotoxicity.

In conclusion, the study shows that a novel camptothecin analogue, ST1481, when delivered p.o. by a "daily low-dose schedule," was able to strongly inhibit the growth and the blood vessel density of slow-growing human tumor xenografts, and that such vascular effects were not solely related to a

cytotoxic effect but were possibly mediated by inhibition of bFGF expression and Akt-dependent pathways. This mechanism, together with the well known cytotoxic activity of camptothecins in tumor cells (2), possibly contributed to the antitumor effect of ST1481. These results may have therapeutic

Table 3.   Antiproliferative Activity of ST1481 on Endothelial (BAEC and b-End) and Tumor (A549 and MeWo) Cell Lines

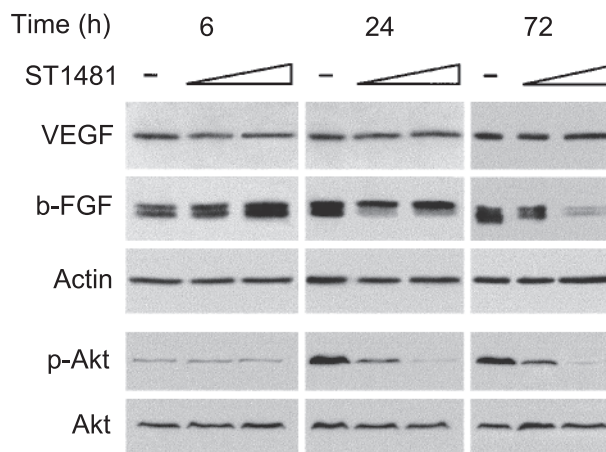| Cell Lines	IC$_{50}$ (ng/ml)	
	1 h*	144 h*
BAEC	45 ± 10	0.55 ± 0.07
b-End	87 ± 3	0.60 ± 0.09
A549	72 ± 11	0.50 ± 0.12
MeWo	63 ± 21	0.45 ± 0.15

*Time of drug exposure. Cells were counted at 144 h after the beginning of treatment.

FIGURE 6. Effect of ST1481 on expression of VEGF and bFGF and activation of Akt in A549 tumor cells. Cell lysates were prepared 6, 24, and 72 h after 1 h of treatment with solvent only (−) or two concentrations of ST1481, IC$_{50}$ (11 ng/ml), and IC$_{80}$ (250 ng/ml). Samples were resolved by SDS-PAGE and subjected to Western blotting with the indicated antibodies. Protein loading by actin expression is shown. Anti-phospho-Akt blots were stripped and reprobed with anti-Akt antibody.

implications. Indeed, the good oral availability and the favorable pharmacokinetic properties observed in Phase I clinical trials (31, 32) make ST1481 a promising candidate for the metronomic scheduling of chemotherapy.

Materials and Methods

Drug

ST1481 (7-*t*-butoxyiminomethylcamptothecin), supplied by Sigma-Tau (Pomezia, Rome), was dissolved in dimethylsulfoxide (DMSO) and stored at −20°C until use (see Ref. 4 for the synthesis). For *in vivo* studies, the drug was thawed and suspended in sterile distilled water (DMSO 10% final concentration), and then delivered p.o. by gavage, in a volume of 10 ml/kg of body weight. For *in vitro* studies, the stock solution was diluted in culture medium (DMSO 0.2% final concentration).

Mice

Adult (5–6 weeks old) C57Bl/6J mice were used for Matrigel implant. Adult (8–10 weeks old) female Swiss nu/nu mice and adult (6–8 weeks old) male BALB/c nu/nu mice were used for the studies with A549 or MeWo human tumors, respectively. All mice were supplied by Charles River Laboratories (Calco, Italy) and were maintained in a laminar flow room at constant temperature and humidity, with free access to sterilized food and water. Experimental protocols were approved by the Ethic Committee for Animal Experimentation of the Istituto Nazionale Tumori of Milan, according to the Italian and European policies. Experiments were conducted according to the UKCCCR guidelines (33).

In Vivo Studies

The Matrigel plug assay method described by Passaniti *et al.* (34) was used with some modifications. Briefly, Matrigel (BD Biosciences, Bedford, MA; 10.5 mg/ml, 0.5 ml) without or with bFGF (R&D Systems Inc., Minneapolis, MN; 150 ng/pellet) was injected s.c. in the ventral side of C57BL/6J mice. Mice (eight animals/group) received vehicle (10% DMSO) or ST1481 (0.12 mg/kg) daily, by oral route, for 6 days after Matrigel implantation. At day 7, pellets were removed and hemoglobin content was measured by Drabkin's method (Drabkin reagent kit, Sigma, St. Louis, MO) normalized by the weight of Matrigel. Results are presented as mean of hemoglobin values (μg Hb/mg of pellet) ± SE.

The human non-small cell lung carcinoma cell line A549 (American Type Culture Collection) was maintained in RPMI 1640 supplemented with 10% fetal bovine serum. Exponentially growing tumor cells were injected s.c. ($8–10 \times 10^6$ cells/flank) into nude athymic mice, and a tumor line *in vivo* was established by serial s.c. passages of tumor fragments (about 2 × 2 × 2 mm) in healthy mice, as previously described (35). For *in vivo* experiments, tumor fragments were s.c. implanted in both flanks of Swiss mice. Each group consisted of 8–10 tumors. 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 diameter,

respectively. Drug treatment started when mean TV was about 250–300 mm^3. ST1481 was delivered following two different treatment schedules: every fourth day for four times (q4dx4) or everyday for five times a week (qdx5/w) for 2 weeks. Control mice were treated with 10% DMSO. Drug efficacy was assessed as: (*a*) percentage TV inhibition (TVI%) in drug-treated *versus* control mice expressed as: TVI% = 100 − (mean TV treated/mean TV control × 100); (*b*) log$_{10}$ cell kill (LCK) calculated by the formula: LCK = $(T − C)/3.32 \times$ DT, where T and C are the mean time (days) required for treated (T) and control (C) tumors, respectively, to reach a determined volume (600 mm^3), and DT is the doubling time of control tumors (=7.2 days). Tumor growth curves were obtained by plotting TVs *versus* time in treated and control groups. Reduction in body weight following drug treatment never exceeded 10%.

The human melanoma cell line MeWo (ECACC) was maintained in monolayer culture in EMEM supplemented with 2 mM glutamine, 1% nonessential amino acids, and 10% FCS. Exponentially growing cells (5×10^5 cells in 30 μl of Ca^{2+}/Mg^{2+}-free HBSS) were inoculated i.d. in the right flank of BALB/c mice by using a 100-μl Hamilton syringe. Each group consisted of nine mice. ST1481 was administered from day 1, according to the qdx5/w schedule for 2 weeks. Mice were sacrificed 14 days after cell inoculation and the skin around the inoculation site was removed. Tumors consisted of very small volumes (*i.e.*, less than 50 mm^3 in control mice) and were located with a stereomicroscope SZX12 (Olympus, America Inc., Melville, NY) and imaged with color CoolSnap-Pro camera. The extent of blood vessel development around tumors was measured by Image Pro Plus 4.5 Software (Media Cybernetics, Silver Springs, MD). Tumor angiogenesis was quantified as vascularized area expressed in square millimeters or percentage of vascularized area/field.

For histological studies in the A549 tumor, mice bearing tumors of 250–300 mm^3 were treated with ST1481 according to the two schedules. Seven days after treatment ended, three mice/group were sacrificed by cervical dislocation and one tumor/mouse was excised, weighed, and fixed. Half of each tumor was fixed in formalin for H&E staining and terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP nick end labeling (TUNEL) reaction (see below), half was fixed in zinc fixative for IHC with CD31/PECAM-1 antibody, as previously described (36). Briefly, for IHC, blood vessels were stained using a rat anti-mouse CD31 monoclonal antibody (kindly supplied by Dr. A. Vecchi, Mario Negri Institute, Milan, Italy). MVD was determined using previously described criteria. Briefly, for each tumor, regions with elevated vessel density (hot spot areas) were identified and scanned. Using an Optimas Image Analysis software, microvessels were quantified within 12 random fields (0.159 mm^2 fields, 400× magnification). Delineated CD31-positive cells or cell clusters were identified as having the highest density of brown staining. Any brown-stained endothelial cell or cell cluster, clearly separated from adjacent microvessels, tumor cells, and other connective tissue elements, was regarded as a single, countable microvessel. Three tumors/group were analyzed. The results were expressed as mean number ± SD. Percentage microvessels

inhibition (MVI%) in drug-treated *versus* control mice was calculated as: MVI% = 100 − (mean MVD in treated/mean MVD in control tumors × 100). Neither vessel lumen nor RBC were used to define a microvessel.

For the TUNEL reaction, the *in situ* cell death detection POD kit (Roche Diagnostic GmbH, Mannheim, Germany) was used in sections of A549 tumors, as previously described (37). AI was determined by selecting, in a light microscope at 400× magnification, 10 fields of non-necrotic areas in each section. In each field, the number of apoptotic nuclei was recorded and the AI was expressed as a percentage of apoptotic nuclei relative to the mean number of cells per field. Scoring of histological tumor sections was conducted by two independent observers, without knowledge of the experimental group, with an interobserver reproducibility >95%.

For statistical analysis, TVs, microvessel, and apoptotic nuclei numbers (in A549 tumors) were compared by the unpaired Student t test (two-tailed). Vascular areas (in MeWo tumors and Matrigel pellets) were compared by the nonparametric Mann-Whitney test. The Spearman rank correlation analysis was used to determine the relationship between TVs and microvessel numbers (38).

In Vitro Studies

Endothelial cells BAEC (kindly supplied by Dr. M. Mariani, Pharmacia Italia, Milan, Italy) and b-End5 (mice brain endothelial cells-retrovirus-immortalized, from the ECACC collection) were maintained in DMEM + 10% FCS (added with 5 μM 2-mercaptoethanol + 1 μM sodium pyruvate + 1% nonessential amino acids for the b-End5). For cell proliferation assay, b-End5 (1.5×10^4 cells/ml), BAEC (8×10^4 cells/ml), A549 (2×10^4 cells/ml), and MeWo (2×10^4 cells/ml) were seeded in complete culture medium and treated 24 h later with different drug concentrations for 1 or 144 h. The drug antiproliferative effect was determined by cell counting at 144 h after the beginning of treatment.

For cell migration assay, BAEC cells were treated for 144 h with 1 ng/ml ST1481 and surviving cells were seeded (10^5/well) in Boyden chamber on polycarbonate filters previously coated with 5% gelatin (39). The drug was added in the upper and lower compartment of the chambers at the same concentration as for treatment. After 3 h of incubation at 37°C in the presence or absence of FCS, filters were cleaned on the upper side with a cotton swab, fixed in 95% ethanol, and stained with 2% crystal violet in 70% ethanol. After mounting on smear slides, cells were counted by an inverted microscope in six randomly chosen fields. All samples were performed in duplicate.

For Western blot analysis, A549 tumor cells were seeded and 24 h later exposed for 1 h to the vehicle or to different concentrations of ST1481 corresponding to the IC_{50} (11 ng/ml) and IC_{80} (250 ng/ml) evaluated at 72 h. Cells were then washed and incubated in drug-free medium. Expression of angiogenic factors (VEGF and bFGF) and activation of Akt, as detected by its phosphorylation at Ser 473, were determined after 6, 24, and 72 h. Whole cell extracts were prepared by lysing cells in Laemmli buffer (40). Equal amounts of proteins were fractionated by SDS-PAGE and blotted on nitrocellulose sheets.

Filters were incubated overnight with the following primary antibodies: rabbit polyclonal anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-bFGF (Upstate Biotechnology, Lake Placid, NY); rabbit anti-actin (Sigma); and rabbit polyclonal anti-phospho-Akt (Ser 473; Cell Signalling Technology, Beverly, MA). Akt expression was checked on stripped anti-phospho-Akt blots using mouse monoclonal anti-Akt antibody (Transduction Laboratories, Lexington, KY). Immunoreactive bands were revealed by an appropriate horseradish peroxidase-conjugated secondary antibody using enhanced chemiluminescence detection system from Pierce (Rockford, IL).

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