Combination of trabectedin and irinotecan is highly effective in a human rhabdomyosarcoma xenograft

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Our objective was to evaluate *in vitro* and *in vivo* the effect of the combination of trabectedin (Yondelis, ET-743) and irinotecan (CPT-11) or its major metabolite SN-38 in a human rhabdomyosarcoma cell line. The schedule trabectedin (1 h) followed by irinotecan or SN-38 (24 h) and the opposite sequence (irinotecan or SN-38 24 h followed by trabectedin 1 h) were analyzed in a rhabdomyosarcoma cell line. *In vivo* studies were conducted with trabectedin and irinotecan at the doses of 0.2 and 20 mg/kg, respectively, simultaneously administered with a q4d × 3 schedule. *In vitro* studies indicated an overall additive effect [combination index (CI) relatively close to 1.0], with the former schedule slightly superior to the latter (at the IC_{50} effect levels: CI = 0.89 versus 1.07). Neither transcription nor expression of DNA topoisomerase I was affected by trabectedin treatment. *In vivo* the therapeutic results of the combination were certainly more impressive: trabectedin and irinotecan combination caused a strong and long-lasting effect on tumor growth (tumor volume inhibition = 89%, $\log_{10}$ cell kill = 1.6), whereas each drug given as a single agent was only marginally active. The discrepancy between the *in vitro* and *in vivo* results suggests possible mechanisms involving host cells, other than tumor cells. The striking effects of the combination observed *in vivo* could be related to a combination of a direct cytotoxic and an anti-inflammatory indirect effect. The very marked and long-lasting effect of the trabectedin and irinotecan combination *in vivo* suggests a basis for a clinical evaluation in pediatric patients with rhabdomyosarcoma. *Anti-Cancer Drugs* 16:811–815

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Introduction

Trabectedin (ET-743, Yondelis), a novel marine-derived compound, exerts a therapeutic effect through several pathways: through inhibiting promoter-specific induction of several important genes [1,2], by interfering with cellular transcription-coupled nucleotide excision repair [3], and by causing a slowing in S phase [4] and an arrest in the G_2/M phase with subsequent p53-independent apoptosis [2]. These characteristics are unique and distinguish trabectedin from all known DNA-interacting drugs. Trabectedin is currently undergoing phase II–III clinical trials for several solid malignancies including pediatric sarcomas [5,6]. Phase II studies conducted in the USA indicate long-lasting responses and promising survival rates (median survival 19 months) in soft tissue sarcoma treated with trabectedin [7]. In several preclinical systems, trabectedin combined with other drugs (i.e. cisplatin, paclitaxel and doxorubicin) [8–10] showed more than additive effects. Phase I trials of trabectedin in combination with standard chemotherapeutics are in progress and initial clinical results of the combination with cisplatin appear to confirm the preclinical findings [11].

Irinotecan is a water-soluble derivative of camptothecin that exerts its cytotoxic action targeting topoisomerase I (Topo I) and is converted by carboxyl esterases to SN-38, a metabolite with 1000 times higher Topo I-inhibiting ability than irinotecan [12]. A reported phase I trial of irinotecan in children utilized a protracted schedule of drug administration over 2 weeks, producing optimal anti-tumor activity [13]. Moreover, a study conducted with the same protracted schedule by the Memorial Sloan-Kettering (New York, USA) on heavily pre-treated pediatric patients reported six out of seven objective responses in patients with rhabdomyosarcoma [14].

The rationale for the ET-743 and irinotecan combination is based on the clinical activity demonstrated in

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preliminary data by ET-743 on rhabdomyosarcoma [5] and on the clinical finding that irinotecan is active in pediatric solid tumors, especially against rhabdomyosarcoma.

The present study was designed to investigate the effect of the combination of trabectedin and irinotecan (or SN-38) in a childhood rhabdomyosarcoma-derived cell line growing *in vitro* and in nude mice *in vivo*.

Materials and methods

Chemicals

Trabectedin, supplied by PharmaMar (Tres Cantos, Madrid, Spain), was prepared as a 1 mg/ml stock solution in ethanol. Irinotecan was obtained from Pharmacia (Kalamazoo, Michigan, USA) as a 20 mg/ml solution. SN-38, kindly provided by Aventis Pharma (Vitry sur Seine, France), was dissolved in DMSO to a final concentration of 10 mM.

Drug combination studies *in vitro*

The human rhabdomyosarcoma cell line TE-671 (Dr J. L. Biedler, Memorial Sloan-Kettering, New York, USA) was grown as monolayer culture in RPMI 1640 medium with 10% fetal calf serum (FCS). The effect of the combination trabectedin and irinotecan or its active metabolite SN-38 was evaluated by the cloning assay, utilizing the following schedule: cells were exposed to trabectedin for 1 h, washed in fresh medium once and immediately exposed to irinotecan (or SN-38) for 24 h. The reverse sequence (exposure to irinotecan or SN-38 for 24 h followed by a 1 h exposure to trabectedin) was also performed. A factorial experimental design was adopted in each experiment, where single concentrations of trabectedin were coupled with several concentrations of irinotecan and vice versa, with three independent flasks. The data from each treatment were initially expressed as a percentage [or fraction 'unaffected' (f_u)] of untreated samples, then analyzed using both the isobologram method and the combination index (CI) for assessment of drug interaction. Best fit values of the concentration–effect relationships of trabectedin or irinotecan were obtained using a non-linear fitting routine with the Hill function ($r^2 > 0.95$).

The isobologram method and the CI

The isobologram method relies on the measure of the combined concentrations of trabectedin and irinotecan that cause a given effect (in our choice: 50 and 30% 'survival' or $f_u = 0.5$ or 0.3). For each experimental concentration of trabectedin, the irinotecan concentration causing the desired effect in combination was found by non-linear fitting of the concentration–effect relationship of irinotecan to the given trabectedin concentration. Vice versa, for each experimental concentration of irinotecan, the trabectedin concentration causing the desired effect in combination was found by fitting the concentration–effect relationship of trabectedin to that particular irinotecan concentration. In this way, multiple couples of drug concentrations that achieved the same isoeffect were found and plotted in an isobologram together with the theoretical straight line marking additivity. In order to quantify the interaction, we calculated the CI for each pair of drug concentrations ($D_{trabectedin}$, $D_{irinotecan}$), producing the effect X (i.e. 70, 50 or 30% survival) in combination, using the formula: $CI = D_{trabectedin}/IC_{X,trabectedin} + D_{irinotecan}/IC_{X,irinotecan}$, where $IC_{X,trabectedin}$ and $IC_{X,irinotecan}$ are the concentrations of each individual drug that would produce the effect X if given alone. Thus, each experiment generated a set of CI values for each (50 or 30%) effect level. The significance of the difference of the mean from CI = 1 was evaluated using a two-tailed *t*-test.

In vivo study

Combination studies were carried out using CD1 *nu/nu* male nude mice (Charles River, Calco, Italy). Mice were kept in laminar flow rooms, and had free access to food and water. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Istituto Superiore di Sanità (Rome, Italy). Each control or drug-treated group included five/seven mice bearing bilateral tumors, obtained by s.c. injection of 1.5×10^6 cells of the TE-671 cell line. Tumor growth was monitored up to 30 days after treatment and tumor volume (TV) determined by measuring the tumor diameters and using the formula: TV (mm^3) = $d^2 \times D/2$ (where d and D represent the shortest and the longest diameter, respectively). When tumors measured around 50–100 mm^3, mice were treated i.v. with the drugs. The anti-tumor activity was evaluated according to two criteria. (i) Tumor volume inhibition (TVI) in drug-treated versus control mice as: TVI (%) = 100 – (mean TV treated/ mean TV control × 100). (ii) Log_{10} cell kill (LCK): $(T - C)/3.32 \times DT$, where T and C are the mean time (in days) required for treated (T) and control (C) tumors to reach an established volume, and DT is the doubling time of control tumors. Trabectedin (0.2 mg/kg) and irinotecan (20 mg/kg) were administered simultaneously by i.v. injection every fourth day for a total of three doses (q4d × 3). The results of the combination of the two drugs were compared with those obtained with each drug alone and with control mice. For statistical analysis, tumor volumes were compared by Student's *t*-test.

Results

In vitro study

IC_{50} values of trabectedin, irinotecan and SN-38 in TE-671 cells were 6.0 ± 1.3 nM, 0.73 ± 0.1 μM and 1.12 ± 0.3 nM, respectively. At least two drug interaction experiments were performed for each schedule and drug combination [trabectedin 1 h → irinotecan (or SN-38)

Table 1 Number of data points and mean ± SE of the CI of trabectedin, irinotecan and SN-38 in TE-671 cells at the IC_{50} and IC_{70} effect levels

Schedule	Effect	No. points	Mean ± SE	*p* value (H_0CI = 1)
Trabectedin (4 h) → irinotecan (24 h)	IC_{50}	11	0.89 ± 0.02	< 0.01
	IC_{70}	19	0.85 ± 0.02	< 0.01
Irinotecan (24 h) → trabectedin (4 h)	IC_{50}	22	1.07 ± 0.02	< 0.01
	IC_{70}	30	0.98 ± 0.03	NS
Trabectedin (4 h) → SN-38 (24 h)	IC_{50}	6	0.78 ± 0.04	< 0.01
	IC_{70}	9	0.83 ± 0.03	< 0.01
SN-38 (24 h) → trabectedin (4 h)	IC_{50}	27	1.29 ± 0.05	< 0.01
	IC_{70}	32	1.09 ± 0.05	< 0.01

24 h, irinotecan (or SN-38) 24 h → trabectedin 1 h]. As can be seen in Table 1, the CI appears to be lower than 1.0 in the majority of cases. Although the statistical analysis indicates a significant synergism of the two-drug combination, the fact that the CI was relatively close to 1.0 suggests that the two drugs have an effect that is essentially additive. A slight superiority was observed when trabectedin treatment occurred before irinotecan or SN-38, compared to the opposite sequences. This slight difference could be the result of trabectedin modulating the transcription and expression of DNA Topo I, with consequent increased cytotoxicity of the inhibitor. The experiments performed indicated that the intracellular concentrations of Topo I assessed by Western blotting were not significantly different before and after trabectedin treatment (data not shown); this excludes the cause of the interaction being due to a modulation of the enzyme.

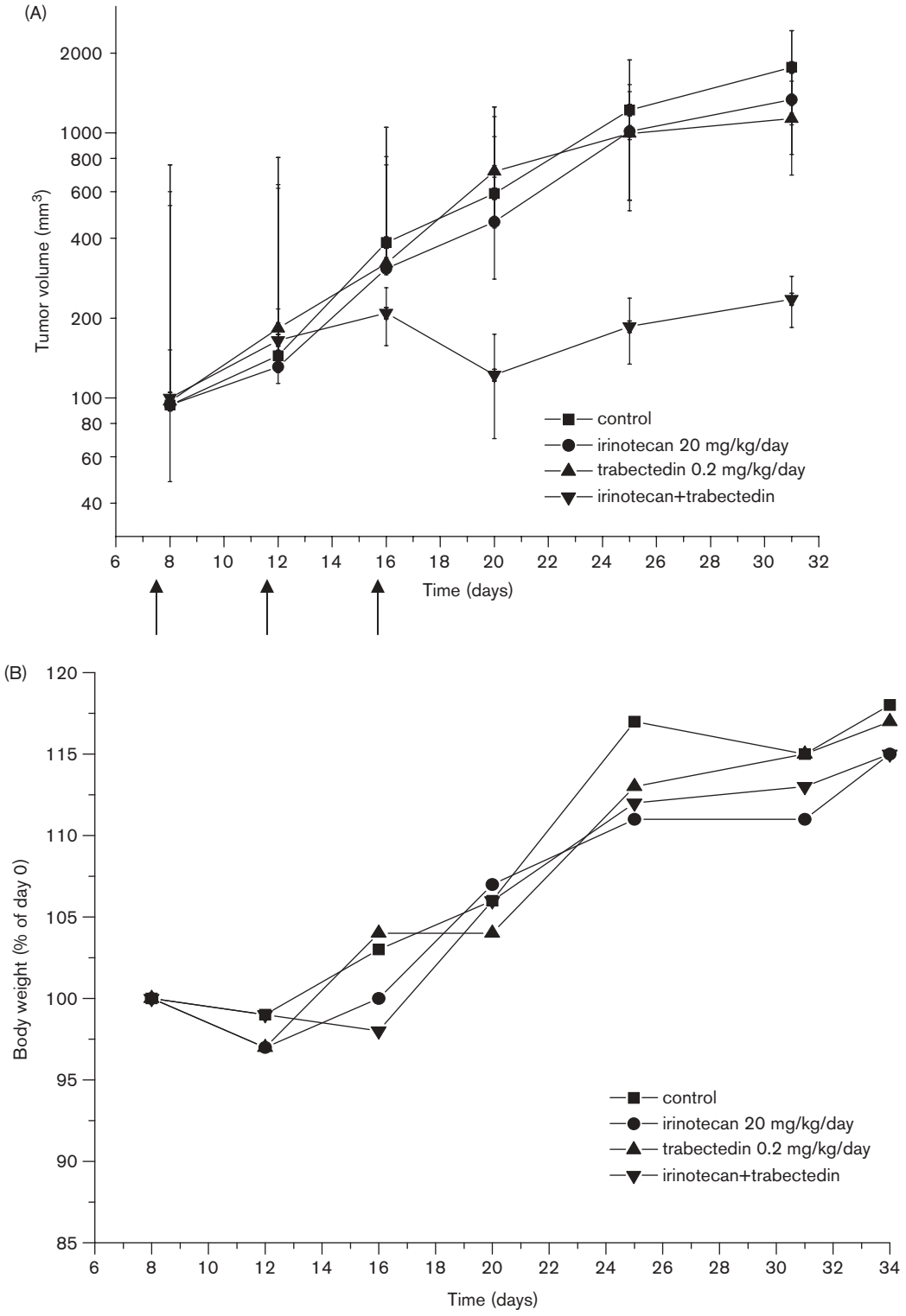
In vivo study

Preliminary experiments performed to evaluate the optimal doses of trabectedin and irinotecan when given as a single agent revealed that TE-671 was not sensitive to trabectedin even at the maximum tolerated dose (0.2 mg/kg/day). Irinotecan was administered at a dose and schedule (20 mg/kg/day, q4d × 3) such that the single-agent treatment was ineffective in inhibiting tumor growth. In TE-671 tumor-bearing mice we investigated the combination of trabectedin and irinotecan at the doses of 0.2 and 20 mg/kg, respectively, both administered with the q4d × 3 schedule and concurrently. The results are shown in Fig. 1(A), and reveal that the combination of trabectedin and irinotecan was very effective in inhibiting tumor growth and causing prolonged stabilization of the disease. The combination effect was already evident some days after the second administration (day 16) and became more striking after the third dose (TVI = 79% and LCK = 0.91 at day 20). In addition, following the third treatment there was a prolonged stationary status of the tumor (TVI = 89% and LCK = 1.6 at day 31). The doses utilized for the drug combination were well tolerated and no significant body weight loss was observed as compared to both controls and to single-agent administration (Fig. 1B).

Discussion

In vitro studies have shown that the combination of either irinotecan or SN-38 with trabectedin results in an additive or weakly synergistic effect against the rhabdomyosarcoma cell line TE-761. There was a slight superiority of the sequence of trabectedin followed by irinotecan or SN-38 with CI values around 0.8/0.9, whereas the opposite gave a CI around 1.0/1.3. In contrast, the results obtained against the same tumor growing *in vivo* as xenografts showed that the two drugs in combination produced a much greater effect than each drug alone. In these experiments both trabectedin and irinotecan produced a moderate anti-tumor effect when given alone, whereas the combination caused a very marked inhibition of tumor growth. Notably, the effect of the combination was evident already at the second dose and became striking after the third dose. In addition, following the last treatment there was a prolonged stationary status of the tumor. The mechanism underlying the synergism between trabectedin and irinotecan has not yet been elucidated, but we tend to exclude this mechanism as being related to an increase in Topo I expression. We found that trabectedin did not modify the expression of DNA Topo I in cancer cells exposed to the drug. In addition, if the observed potentiation were due to a modulation of DNA Topo I enzyme levels it would have been expected to have a similar effect both *in vitro* and *in vivo*, whereas only *in vitro* was an additive effect found. It is interesting to note that a similar finding was previously reported by us when we evaluated the combination of trabectedin with cisplatin, i.e. some experiments suggested much better results of the combination *in vivo* than *in vitro*, where only an additive effect was found [10]. The discrepancy between *in vitro* and *in vivo* results suggests the involvement of host-mediated mechanisms. We have recently found that trabectedin causes apoptosis in macrophages present in the tumor, and at very low concentrations selectively inhibits the production of some inflammatory cytokines that might enhance tumor cell proliferation and survival [15]. On the basis of these findings one can speculate that the strong and long-lasting therapeutic effect observed in a rhabdomyosarcoma xenograft treated with the combination of trabectedin and irinotecan is related

Fig. 1



Effect of trabectedin and irinotecan administration on the growth of TE-671 xenografts. (A) Growth curves of treated and untreated tumors. Each control or drug-treated group included five/seven mice bearing bilateral tumors. Trabectedin was given at 0.2 mg/kg/day and irinotecan at 20 mg/kg/day with the schedule q4d × 3. (B) Toxicity of trabectedin and irinotecan, as a single agent and in combination. Group body weights (n=5/7) are expressed as percentages of those on the day of treatment.

to a combination of a direct cytotoxic and an indirect anti-inflammatory effect. Further experiments will be planned to verify this hypothesis. The marked and long-lasting effect of the trabectedin and irinotecan combination *in vivo* may represent the basis for a clinical evaluation in relapsed pediatric patients with rhabdomyosarcoma.

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