Cancer Chemother Pharmacol (2003) 52: 131–138
DOI 10.1007/s00280-003-0636-6

ORIGINAL ARTICLE

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Effective combination of ET-743 and doxorubicin in sarcoma: preclinical studies

Received: 14 January 2003 / Accepted: 3 April 2003 / Published online: 29 May 2003

Abstract *Purpose*: To investigate the cytotoxic and antitumor effects of the combination of the novel anticancer drug ET-743 and doxorubicin (Dx) and to determine whether any pharmacokinetic interaction occurs in sarcoma-bearing mice. *Methods*: The cytotoxicity of each drug and of their combinations was assessed in the rhabdomyosarcoma cell line TE-671 by a clonogenic assay, and isobologram analysis was performed to detect any synergistic, additive or antagonistic effects. The antitumor activities of each drug and of the combinations were also evaluated in nude mice transplanted subcutaneously with human TE-671 rhabdomyosarcoma and in C3H female mice injected intravenously with UV2237 M fibrosarcoma or with the Dx-resistant subline UV2237 M-ADM which overexpresses Pgp. Antitumor activity was evaluated by monitoring the TE-671 tumor volume over time and, in the case of the murine fibrosarcomas, by evaluation of lung deposits at autopsy quantified by determining lung weight. Pharmacokinetic studies were performed in TE-671-bearing mice. ET-743 was determined in plasma by an HPLC-MS method and Dx in plasma and tissue by an HPLC method with fluorescence detection. *Results*: The combination of ET-743 and Dx was found to be additive with the average combination index slightly lower than 1 at all survival levels, suggesting weak synergism. In TE-671 tumors in vivo the activity of ET-743 or Dx given alone was marginal, whereas the combination produced a significant antitumor effect. The log cell kill (LCK) values were 0.13 and 0.33 for ET-743 and Dx alone, whereas they ranged from 0.85 to 1.12 for the combination. Giving ET-743 1 h before Dx slightly enhanced the effect (LCK 1.12) compared with giving the drugs simultaneously (LCK 0.85) or in the opposite sequence (LCK 0.92). In UV2237 M fibrosarcoma, both Dx and ET-743 showed an effect in reducing the weight of lung metastases, although the combination of the two drugs was not superior to each drug alone. In UV2237 M-ADM tumors neither of the two drugs was active, whereas the combination, particularly when the two drugs were given simultaneously, produced a significant effect. Plasma levels of ET-743 and Dx were not significantly different when the drugs were given alone or in combination. The concentrations of Dx in tissues including tumor, liver, heart and kidney were found to be the same whether the drug was given alone or in combination with ET-743. *Conclusions*: These results indicate that ET-743 and Dx in combination produce an additive effect against human sarcoma cells, reinforcing the idea that they act by a different mechanism of action. In mice no pharmacokinetic interaction between the two drugs was found. The observed activity in UV2237 M-ADM and in human TE-671 sarcoma suggests that the combination of the two drugs could be effective for tumors displaying low sensitivity to each drug given alone. Based on these findings a phase I study on the combination of the two drugs was recently initiated.

Keywords ET-743 · Doxorubicin · Combination · Sarcoma

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Introduction

Ecteinascidin-743 (ET-743) is a tristetrahydroisoquinoline isolated from the Caribbean marine tunicate *Ecteinascidia turbinata*. It has shown potent cytotoxic activity at nanomolar concentrations against a variety of tumor cell lines [12, 13] and antitumor activity against several human xenografts [23]. ET-743 is currently undergoing phase II/III clinical trials, the available data indicating therapeutic potential in patients with advanced pretreated sarcoma, and breast and ovarian cancer [3, 6, 26]. The mechanism of action of ET-743 has not yet been fully elucidated. It has been shown that ET-743 binds to the minor groove of DNA forming adducts at the N2 position of guanine with some degree of sequence-specificity [17, 18, 22], and interferes with gene transcription regulation in a promoter-dependent manner [14, 16]. It has recently been reported that cells deficient in transcription-coupled nucleotide excision repair (NER) are less sensitive to ET-743 than cells with a normal NER function [5, 28], although the precise mechanism underlying this effect has still to be clarified. ET-743 inhibits cell cycle progression with delayed traverse from G_1 to G_2 and a marked blockade in G_2/M that is not p53-independent [16].

Activity of ET-743 has been seen in phase I/II studies in soft-tissue sarcoma refractory to doxorubicin (Dx) and conventional alkylating agents [6, 15, 21]. Considering that Dx is the drug of choice for the therapy of sarcomas, it may appear attractive to combine ET-743 with Dx to enhance antitumor activity. This consideration prompted us to perform some preclinical studies aimed at excluding antagonism between ET-743 and Dx, to evaluate whether the combination proves more effective than each of the two drugs used alone and to investigate whether any pharmacokinetic interaction occurs between the two drugs.

Materials and methods

Chemicals

ET-743, supplied by Pharma Mar (Tres Cantos, Madrid, Spain), was prepared as a 1-mg/ml stock solution in ethanol and, prior to use, diluted with 0.05 *M* phosphate buffer (KH_2P0_4). Dx was obtained from Sigma Chemicals Co. (St. Louis, Mo.).

Mice

CD1 nu/nu male nude mice and C_3H female mice (Charles River, Calco, Italy) at 5 weeks of age were used throughout the study. All experimental animal investigations complied with the guidelines of the Istituto Superiore di Sanità (Rome, Italy) on experimental neoplasia in animals. The animals were maintained under specified pathogen-free conditions.

TE-671 xenografts and tumor growth

Mice were injected subcutaneously with 1.5×10^6 cells of the TE-671 cell lines in both flanks. Drug administration was started 10 days after transplantation when tumors weighed approximately 50–100 mg. For evaluation of antitumor activity, each control or drug-treated group consisted of eight to ten tumors. Tumor growth was monitored for up to 28 days after drug treatment and tumor weight (TW) determined by measuring the tumor diameters with a vernier caliper and using the formula $TW = d^2\times D/2$, where d and D represent the shortest and the longest diameter, respectively. When the tumors weighed around 50–100 mg, mice were treated intravenously (i.v.) with the drugs. The antitumor activity was evaluated according to two criteria:

1. TW inhibition (TWI) in treated (T) versus control (C) mice according to the formula: $100-(T/C\times100)$
2. Log_{10} cell kill (LCK): $(T-C)/3.32\times DT$, where T is the mean time (days) required for treated tumors and the control tumors (C) to reach an established weight (500 mg), and DT is the mean doubling time of control tumors.

Xenograft therapy

Compounds were prepared immediately prior to use and given by i.v. injection. ET-743 (0.1 mg/kg) and Dx (10 mg/kg) were combined using three different schedules: the two drugs were given simultaneously or one after the other at 1-h intervals in two opposite sequences. In each experiment the results of the combination of the two drugs were compared with those obtained with each drug alone and with the results in control mice treated with drug vehicle.

Experimental metastasis formation

Syngeneic C_3H female mice (18 ± 3 g body weight) were inoculated i.v. via the lateral tail vein with 2×10^5 viable tumor cells of the UV2237 M cell line and the ADM-resistant line, UV2237 M-ADM, selected and isolated in vitro by exposure of the parent line to the continuous presence of Dx [8]. Dx was dissolved in distilled water, and ET-743 was dissolved in ethanol and diluted with 0.05 *M* phosphate buffer (KH_2PO_4) prior to use. Dx was injected i.v. at 10 mg/kg 3 days after injection of tumor cells. ET-743 (0.2 mg/kg) was injected i.v. either 1 h after or 1 h before Dx. Mice were killed 4 weeks after transplant, and the lungs were removed, weighed and fixed in Bouin's solution, and the number of tumor nodules determined.

Pharmacokinetic study

For the pharmacokinetic study, ET-743 was administered at 0.1 mg/kg i.v. via a lateral tail vein, immediately followed by Dx at 10 mg/kg i.v. in a group of four mice, while two other groups of four mice received each drug alone. Serial blood samples were collected by cardiac puncture from four animals per time point at 1, 4, 8 and 24 h after the treatments. Tissues were removed from the same animals (tumor, heart, liver and kidney). Plasma and tissues were frozen at −20°C until assayed.

Analytical methods

Doxorubicin assay

Dx was determined in plasma and tissue of mice by an HPLC method after solvent extraction [4]. Tissues were washed with saline solution and homogenized in four parts of water using an Ultra Turrax apparatus, model TP18-10 (Jane and Krugel, Staufen, Germany). Plasma or homogenized tissue (0.5 ml) was mixed with 50 ng daunorubicin as internal standard, with 20 µl $AgNO_3$ (33% in water) in the case of tissue, and with 8 ml chloroform/isopropanol (1:1). After shaking for 20 min, the samples were centrifuged at 3000 rpm for 10 min, and the separated organic phase was

evaporated under vacuum. The residue was dissolved in 0.1 *M* H_3PO_4 and 50 µl injected into an HPLC apparatus consisting of a Waters 2690 separation module equipped with a fluorescence detector model 474 (Waters). Separation was obtained using a µBondapack C18 column (10 µm, 4.6×300 mm) and an isocratic solvent system of acetonitrile/water/0.1 *M* H_3PO_4 (30:44:26) at a flow rate of 1.4 ml/min. The limit of detection of the assay was 2 ng per milliliter or per gram.

ET-743 assay

ET-743 was determined in plasma using a liquid chromatography tandem mass spectrometry technique (HPLC-MS/MS) after solid-phase extraction (SPE) on a cyano cartridge [19]. Plasma (0.3 ml) was spiked with 50 µl ET-729 (10 ng/ml in MeOH) as internal standard, diluted with 0.7 ml 0.2 *M* ammonium acetate (pH 5) and loaded into the SPE column preconditioned with 1 ml 0.1 *M* HCl in methanol and with 2 ml 0.01 *M* ammonium acetate (pH 5). After washing the cartridge with 2 ml 0.01 *M* ammonium acetate (pH 5) and 2 ml acetonitrile, elution of ET-743 was performed with 2.5 ml 0.1 *M* HCl in methanol. The eluted sample was evaporated under nitrogen and the residue dissolved in 200 µl mobile phase. A 20-µl aliquot was injected into the HPLC-MS/MS system consisting of a liquid chromatograph (Perkin Elmer series 200) coupled to a triple quadrupole instrument with an electrospray ion source (Applied Biosystems API 3000). Chromatographic separation was obtained using an Inertsil ODS2 column (125×2 mm, 5 µm) (Agilent Technologies), with an isocratic solvent mixture of methanol/water (70:30) containing 5 m*M* ammonium acetate and 0.4% formic acid at a flow rate of 200 µl/min. The limit of detection of the assay was 10 pg/ml.

Calculations

The area under the curve of the plasma concentration vs time (AUC) of Dx and ET-743 was calculated by the trapezoidal rule. The statistical significance of differences between pharmacokinetic parameters was assessed by Duncan's test.

Drug combination studies in vitro

The human TE-671 rhabdomyosarcoma cell line was obtained from Dr. J.L. Biedler (Memorial Sloan Kettering, New York). The cell line was grown as monolayer cultures in RPMI-1640 medium with 10% fetal calf serum.

A factorial design was adopted for each experiment, where single concentrations of ET-743 were coupled with several concentrations of Dx and vice versa, with three replicated independent flasks. The experiment was performed twice. The data from each treatment were initially expressed as a percentage (or fraction unaffected, fu) of untreated samples, then analyzed using several approaches for assessment of drug interaction. Best fit values of the concentration-effect relationships of ET-743 or Dx were obtained using a non-linear fitting routine with the Hill function ($r^2 > 0.99$ for ET-743 and $r^2 > 0.93$ for Dx relationships).

Berembaum's interaction index

Best fit values of the concentration-effect relationships of ET-743 or Dx alone were used to calculate the interaction index according to Berembaum and the difference between the expected (based on the Lowe additivity criterion) and observed effects in each combination [1].

The interaction index is defined as:

$$I = D_{ET-743}/IC_{X,ET-743} + D_{Dx}/IC_{X,Dx}$$

where $D_{ET\text{-}743}$ and D_{Dx} are the concentrations of ET-743 and Dx used in the combination, X is the observed effect in the combination, $IC_{X,ET\text{-}743}$ and $IC_{X,Dx}$ are the concentrations of each individual drug that would produce the effect X if given alone.

ET-743 and Dx concentrations were grouped on the basis of the effect of the single drug in the low (L, fu >0.8), intermediate (M, fu ≤ 0.8 and >0.5), and high (H, fu ≤ 0.5 and >0.2) groups. Concentrations associated with fu lower than 0.2 were associated with measures that were not precise enough in either the data or the fitting procedure and were excluded from the analysis.

Eventually all combination data, referring to combinations of concentrations individually producing fu >0.2 in the two experiments entered in the analysis and the respective results were pooled in nine groups: $L_{ET\text{-}743} + L_{Dx}$, $L_{ET\text{-}743} + M_{Dx}$, $L_{ET\text{-}743} + H_{Dx}$, $M_{ET\text{-}743} + L_{Dx}$, $M_{ET\text{-}743} + M_{Dx}$, $M_{ET\text{-}743} + H_{Dx}$, $H_{ET\text{-}743} + L_{Dx}$, $H_{ET\text{-}743} + M_{Dx}$, L_{Dx}, and $H_{ET\text{-}743} + H_{Dx}$. The means and variances of the interaction indices (I) were calculated in each group. The significance of the difference of the mean from $I = 1$ was evaluated using a two-tailed *t*-test, indicating additivity when the mean value of I was not different from 1 at the 0.05 level of significance, or else synergism or antagonism when the mean values of I were, respectively, lower or higher than 1. Groups containing fewer than three data were not considered.

The isobologram method

The isobologram method relies on the extrapolation or fitting of the combined concentrations of ET-743 and Dx that cause a given effect (in our choice 70%, 50% and 30% "survival" or fu 0.7, 0.5 or 0.3). For each experimental concentration of ET-743, the Dx concentration causing the desired effect in combination was found by non-linear fitting of the concentration-effect relationship of Dx to the given ET-743 concentration, and vice versa, for each experimental concentration of Dx, the ET-743 concentration causing the desired effect in combination was found by fitting the concentration-effect relationship of ET-743 to that particular Dx concentration. In each case, several analytical functions were tested (Hill, Fermi, exponential, linear-quadratic and multicomponent functions) for best fitting of the data.

In this way, multiple couples of isoeffect drug concentrations were found. For each pair of drug concentrations $\{D_{ET\text{-}743}, D_{Dx}\}$, producing the effect X in combination, the combination index (CI) (based on the Lowe additivity criterion) was calculated as follows:

$$CI = D_{ET-743}/IC_{X,ET-743} + D_{Dx}/IC_{X,Dx}$$

where $IC_{X,ET\text{-}743}$ and $IC_{X,Dx}$ are the concentrations of the each individual drug that would produce the effect X if given alone.

Thus each experiment generated a set of CI values for a particular effect level. The CI values obtained from all experiments with a given cell line were pooled and the means and variances were calculated at the 30%, 50% and 70% survival levels. The significances of the differences of the means from $CI = 1$ were evaluated using a two-tailed *t*-test.

Results

In vitro studies

IC_{50} values of ET-743 and Dx in TE-671 cells (rhabdomyosarcoma) were 1.0 ± 0.2 n*M* and 54.9 ± 0.5 µ*M*, respectively. Two drug interaction experiments were performed. Included in the analysis were 102 independent combination data, referring to combinations of concentrations individually producing survival $>20\%$. Table 1 summarizes the results of Barembaum's interaction index. The combination of high ET-743 with low Dx concentrations showed weak synergism, low or

Table 1 Interaction indices of the combination of ET-743 and DX in TE-671 cells. ET-743 and Dx concentrations were grouped on the basis of the effect of the single drug in the low (L, survival >80%), intermediate (M, survival 50–80%), high (H, survival 20–50%) groups. The claim of synergism, additivity or antagonism in each group was based on the statistical significance at the $P = 0.05$ level of the difference from I = 1

Group	L_{DX}			M_{DX}			H_{DX}		
	No. of data	Interaction index (mean ± SE)	Interaction	No. of data	Interaction index (mean ± SE)	Interaction	No. of data	Interaction index (mean ± SE)	Interaction
$L_{ET\text{-}743}$	12	0.93 ± 0.04	Additivity	6	0.91 ± 0.06	Additivity	12	1.10 ± 0.03	Antagonism
$M_{ET\text{-}743}$	11	1.03 ± 0.04	Additivity	6	1.14 ± 0.06	Additivity	12	1.06 ± 0.03	Antagonism
$H_{ET\text{-}743}$	20	0.90 ± 0.04	Synergism	15	1.04 ± 0.04	Additivity	8	0.96 ± 0.03	Additivity

intermediate ET-743 with high Dx concentrations showed weak antagonism, and the other six groups showed additivity. The global average interaction index was 1.00 ± 0.02. Figure 1 and Table 2 show the results obtained with the isobologram method. The data indicate that the average CI was slightly lower than 1 at all survival levels, but not significant at the 0.05 level of significance.

Taken together, the results of these experiments with combinations of ET-743 and Dx at clinically achievable concentrations exclude significant antagonism between the two drugs and indicate an additive or slightly synergistic effect of the drugs in combination.

In vivo studies

After a number of experiments aimed at evaluating the optimal doses of ET-743 and Dx given alone, it was evident that TE-671 was not sensitive to ET-743 even at doses of 0.2 mg/kg, which displayed toxicity. The optimal activity of Dx, which was moderate, was obtained at 10 mg/kg. Therefore in TE-671-bearing mice we investigated the combinations of ET-743 and Dx at the doses of 0.1 mg/kg and 10 mg/kg, respectively. The combinations were more effective than each drug given alone in inhibiting tumor growth (Fig 2a). No relevant differences were found between the three different schedules (drugs given simultaneously or one after the other). Both tumor weight inhibition and LCK were higher after treatment with the combinations than after treatment with each drug alone (Table 3). The combinations caused a higher weight loss of mice than each drug given alone (Fig. 2b). When ET-743 was given 1 h after Dx there was less toxicity than observed with the other schedules.

Table 4 shows the effects of ET-743 or Dx given alone or in combination against i.v.-injected UV2237 M and UV2237 M-ADM fibrosarcomas. Both ET-743 and Dx showed the ability to reduce the tumor lung deposits of UV2237 M, assessed by weighing the lungs. The combinations of ET-743 and Dx produced an activity that was comparable with that of each drug given alone. Conversely, in UV2237 M-ADM, both drugs were inactive when given alone, whereas the combinations caused a significant reduction in lung weight. Simultaneous treatment with the two drugs appeared to be slightly more effective than both sequential treatments.

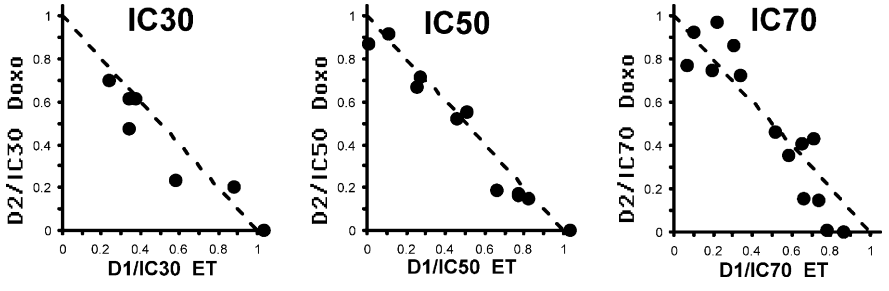
Pharmacokinetic studies

In order to evaluate whether there was a pharmacokinetic interaction between Dx and ET-743, the levels of the two drugs were determined in plasma of TE-671 tumor-bearing mice, receiving either each drug alone or

Table 2 Combination indices of the combination of ET-743 and DX in TE-671 cells at the 70%, 50%, 30% effect levels

Effect	No. of points	Combination index (mean ± SE)	P value (H_0 CI = 1)
Survival 70%	7	0.95 ± 0.04	0.227
Survival 50%	11	0.96 ± 0.02	0.079
Survival 30%	14	0.98 ± 0.04	0.569

Fig. 1 Isobolograms of the combination ET-743-DX at the IC_{30}, IC_{50} and IC_{70} levels. *Abscissa* ET-743 concentrations normalized to the IC_{30}, IC_{50} or IC_{70}. *Ordinate* Dx concentrations normalized to the IC_{30}, IC_{50} or IC_{70}. *Dashed lines* separate the region of synergism (below) and the region of antagonism (above)



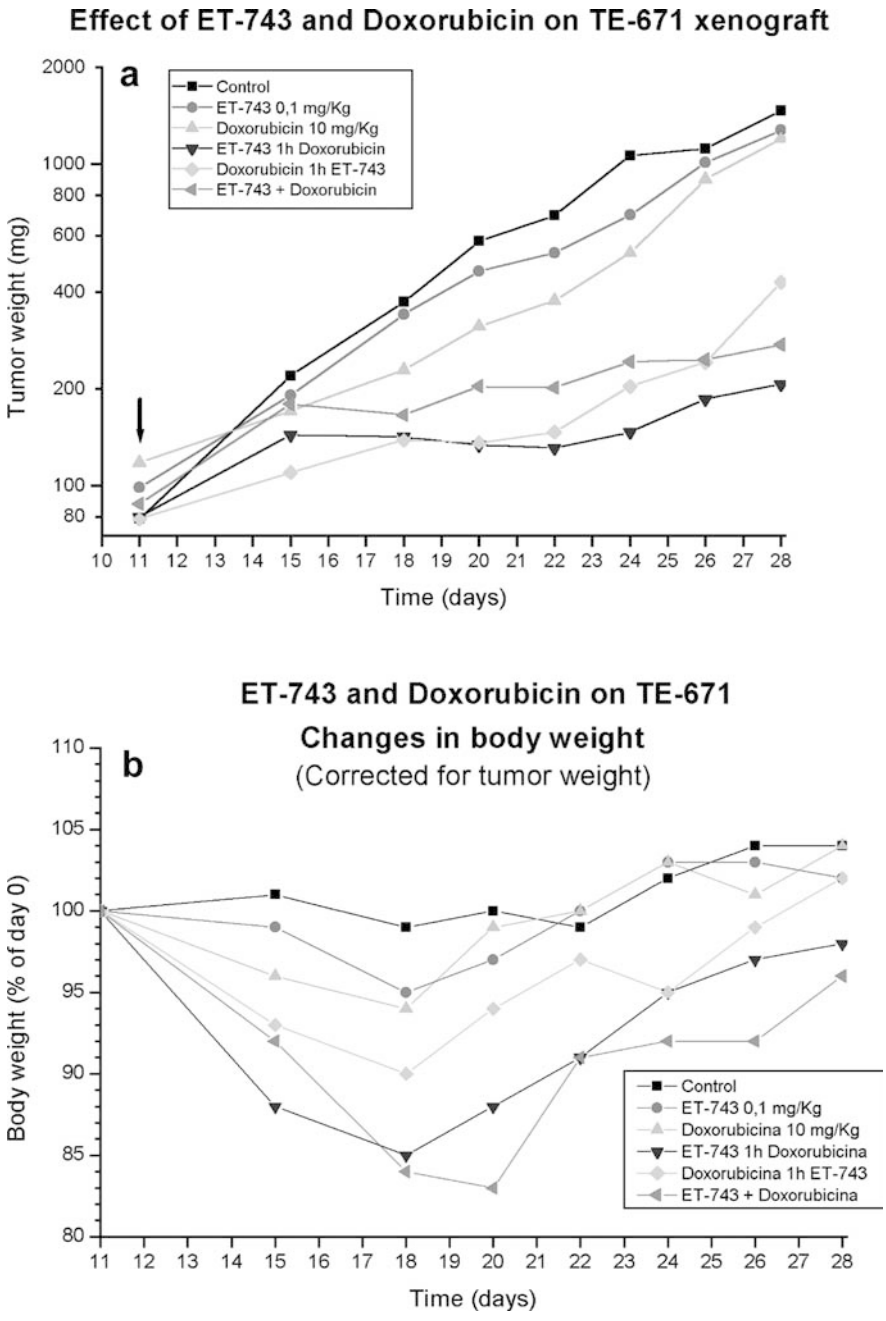


Fig. 2a Effect of simultaneous or alternate administration of ET-743 and Dx on the growth of TE-671 xenografts. Growth curves of treated and untreated tumors (eight to ten tumors per group). ET-743 was given at 0.1 mg/kg and Dx at 10 mg/kg (■ control group, ● ET-743 alone, ▲ Dx, ▼ ET-743 1 h before Dx; ♦ Dx 1 h before ET-743, ◀ ET-743 + Dx). **b** Toxicity of ET-743 and Dx, alone and in combinations. Group body weights ($n = 7$) for single-agent ET-743 0.1 mg/kg (●), Dx 10 mg/kg (▲), or the combinations ET-743 1 h before Dx (◆), Dx 1 h before ET-743 (♦) and ET-743 + Dx (◀) are expressed as percentages of those on the day of treatment

the two drugs combined. ET-743 plasma levels were not different at any time-point when the drug was given alone or in combination with Dx (Fig 3a). Therefore the AUC values of ET-743 were essentially the same in mice treated with ET-743 alone and in those treated with ET-743 and Dx (9.3 vs 10.1 ng/ml·h). Dx plasma levels were slightly higher when Dx was given alone than when it was given in combination with ET-743 (Fig 3b). Consequently the AUC of Dx, when the drug was given alone, appears to be higher than the AUC of Dx after the combined treatment (2.9 vs 1.9 μg/ml·h). The differences, however, were not statistically significant. Dx concentrations were also determined in tumor, heart, liver and kidney, and the levels were found to be similar ($P > 0.05$) when Dx was given alone and in combination with ET-743 (Fig. 4).

Discussion

In recently published phase I/II studies ET-743 has been found to be active in patients with soft-tissue sarcoma resistant to Dx and to alkylating agents [6, 7, 27]. As Dx is the most effective available drug for treating soft-tissue sarcoma, clinical trials with Dx combined with ET-743 are of potential interest in these diseases, although the preclinical data available on this combination are very limited. To our knowledge the only

Table 3 Effect of ET-743 and Dx alone and in combinations on the growth of subcutaneous human TE761 xenograft in nude mice (*TWI* tumor weight inhibition in treated versus control mice, *LCK* $\log_{10}$ cell kill)

Group	Dose (mg/kg)[a]	TWI (%)	LCK
ET-743 alone	0.1	46	0.13
Dx alone	10	50	0.33
ET-743, 1 h, then Dx	0.1/10	82	1.12
Dx, 1 h, then ET-743	10/0.1	75	0.85
Dx + ET-743	10/0.1	77	0.92

[a]Maximum tolerated dose with the indicated schedule

Table 4 Activity of ET-743 and Dx given alone or in combination against lung metastasis of the murine UV2237 M and UV2237 - M-ADM fibrosarcoma. Values are means ± SE

	Lung weight (g)	
	UV2237 M	UV2237 M-ADM
Controls	0.564 ± 0.125	0.597 ± 0.090
ET-743	0.207 ± 0.042**	0.561 ± 0.055
Dx	0.257 ± 0.055**	0.426 ± 0.059
Dx + ET-743 (simultaneous)	0.200 ± 0.029**	0.308 ± 0.069**
Dx + ET-743 (after 1 h)	0.173 ± 0.029**	0.386 ± 0.069*
ET-743 + Dx (after 1 h)	0.333 ± 0.094*	0.381 ± 0.054*

*$P < 0.05$, **$P < 0.01$, Fisher's test

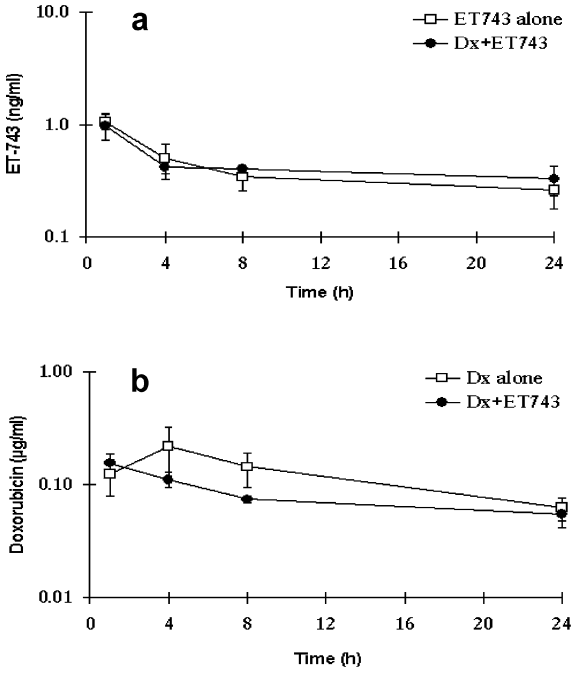


Fig. 3a,b Plasma levels of ET-743 (**a**) and Dx (**b**) after administration of 0.1 mg/kg ET-743 alone or in combination with 10 mg/kg Dx (**a**) and of 10 mg/kg Dx alone or in combination with 0.1 mg/kg ET-743 (**b**). The data are presented as means ± SE

available reports are by Takahashi et al. who found that in two sarcoma cell lines exposed in vitro to the combination of ET-743 and Dx for 72 h there was an additive effect in one cell line and synergism in the other cell line [21]. In addition, Takahashi et al. found that activity was enhanced if ET-743 was given for 24 h and followed by Dx for the next 48 h.

In the present study we investigated the combination of ET-743 and Dx both in vitro and in vivo against the human TE-671 rhabdomyosarcoma, which has a low sensitivity to the available anticancer drugs. In vitro, the two drugs showed an additive effect, as indicated by CI values close to one. In nude mice transplanted with TE-671 cells the combination was certainly more effective than each drug given alone at the optimal dosage schedule. However, it should be noted that the combination of the two drugs also caused increased toxicity, assessed as weight loss, that was reversed within a few days.

Further experiments were conducted in UV2237 M murine fibrosarcoma and its variant UV2237 M-ADM resistant to Dx, obtained and characterized previously for its mechanism of resistance related to the overexpression of Pgp [10, 11]. The experimental model was of relevance to our studies in view of the reported finding

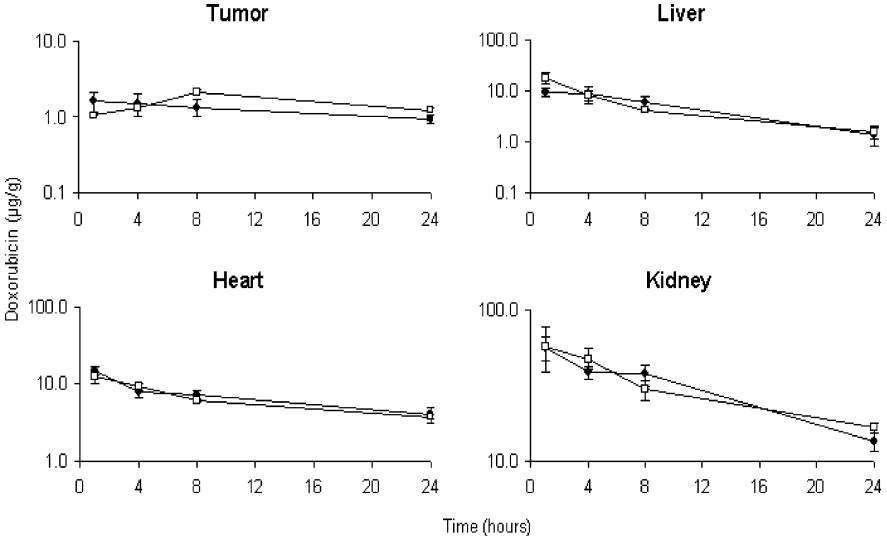


Fig. 4 Dx concentrations (means ± SE) in tissues of mice treated with 10 mg/kg Dx alone (◆) or Dx in combination with 0.1 mg/kg ET-743 (□)

that ET-743 inhibits the induction of mdr-1 gene transcription, thus possibly indicating its ability to counteract the resistance mechanisms due to Pgp expression. The inhibition of transcription induction of the mdr-1 gene has been reported to be very rapid, occurring within minutes [14], and therefore the experiments were performed by giving ET-743 and Dx either simultaneously or one after the other with an interval of 1 h.

In UV2237 M fibrosarcoma both Dx and ET-743 were effective and the combination produced an effect similar to that of each drug alone. In UV2237 M-ADM neither Dx nor ET-743 produced any effect, whereas the combination showed a significant activity. If ET-743 were effective in reducing Pgp expression one would expect to achieve maximum potentiation by giving ET-743 first and then Dx. Instead, the highest activity was observed when the two drugs were given in succession in the opposite sequence. In addition we failed to observe any change in Pgp expression in UV2237 M-ADM after exposure to ET-743 (data not shown). It should be noted that Jin et al. [14] have reported that ET-743 is able to inhibit the induction of the mdr-1 gene by histone deacetylase inhibitors, but no evidence was reported that the constitutive expression of mdr-1 gene is inhibited by ET-743. This is further supported by the recent report that ET-743 can preferentially affect the transcription of induced genes [9]. That Pgp overexpression can cause resistance to ET-743 has been shown for an ovarian cancer cell line rendered resistant to ET-743 after prolonged drug exposure [8]. The observed activity of the combination in UV2237 M-ADM might be due to the fact that both drugs are substrates of Pgp and therefore their combined treatment can partially inhibit Pgp, thus favoring the retention of the two drugs, as has been shown with some Pgp inhibitors that bind the protein (e.g. cyclosporin).

Another finding of possible clinical interest is that ET-743 and Dx did not show any significant pharmacokinetic interaction in mice. It has previously been found that the combination of cyclosporin and Dx increases the level of Dx in several tissues, including heart, with possible toxicological implications. In the case of combination with ET-743, the Dx levels in all the tissues examined were found to be very close to those found after Dx treatment alone, excluding a pharmacokinetic related increase in toxicity.

The AUC of Dx in mice treated with 10 mg/kg found in the present study (around 2 μg/ml·h) is similar to the values reported in patients receiving a Dx dose of 60 mg/m^2, a dose currently used in clinical practice [20]. The AUC of ET-743 found in mice (around 10 μg/ml·h) is instead approximately four to five times lower than that found in patients receiving doses between 1100 and 1500 μg/m^2 that are currently under investigation in phase II trials [24, 25]. Therefore it appears that, as previously reported [2], at similar AUC values of Dx there is a similar toxicity in mice and humans. In contrast, for ET-743 it seems that at the maximum tolerated dose the drug exposure is significantly higher in humans than in mice, and in principle this should be advantageous from a therapeutic point of view.

Initial pharmacokinetic data obtained in patients with soft-tissue sarcoma treated with Dx and ET-743 in combination in a phase I clinical trial indicate that in humans, as shown in the present study in mice, there is also no pharmacokinetic interaction between Dx and ET-743 (Silvia Marsoni, SENDO, personal communication).

In conclusion, the present study showed that ET-743 and Dx are not antagonistic in vitro or in vivo and can also be combined in the therapy of tumors having a low sensitivity to each drug given alone. The lack of pharmacokinetic interaction between ET-743 and Dx observed in this study is a further positive element in support of clinical trials using the two drugs in combination.

Acknowledgements This work was supported by the Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.), the Consiglio Nazionale per le Ricerche (C.N.R.), and the Ministero della Salute, Fondazione per l'Oncologia Pediatrica (F.O.P.).

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