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Ecteinascidin-743 (ET-743), a natural marine compound, with a unique mechanism of action

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Abstract

The mode of action of Ecteinascidin-743 (ET-743), a marine tetrahydroisoquinoline alkaloid isolated from *Ecteinascidia turbinata*, which has shown very potent antitumour activity in preclinical systems and encouraging results in Phase I clinical trials was investigated at a cellular level. Both SW620 and LoVo human intestinal carcinoma cell lines exposed for 1 h to ET-743 progress through S phase more slowly than control cells and then accumulate in the G_2M phase. The sensitivity to ET-743 of G_1 synchronised cells was much higher than that of cells synchronised in S phase and even higher than that of cells synchronised in G_2M. ET-743 concentrations up to four times higher than the IC_{50} value caused no detectable DNA breaks or DNA–protein cross-links as assessed by alkaline elution techniques. ET-743 induced a significant increase in p53 levels in cell lines expressing wild-type (wt) (p53). However, the p53 status does not appear to be related to the ET-743 cytotoxic activity as demonstrated by comparing the drug sensitivity in p53 (−/−) or (+/+) mouse embryo fibroblasts and in A2780 ovarian cancer cells or the A2780/CX3 sub-line transfected with a dominant-negative mutant *TP53*. The cytotoxic potency of ET-743 was comparatively evaluated in CHO cell lines proficient or deficient in nucleotide excision repair (NER), and it was found that ET-743 was approximately 7–8 times less active in *ERCC3*/*XPB* and *ERCC1*-deficient cells than control cells. The findings that G_1 phase cells are hypersensitive and that NER-deficient cells are resistant to ET-743 indicate that the mode of action of ET-743 is unique and different from that of other DNA-interacting drugs. © 2001 Published by Elsevier Science Ltd.

Keywords: Ecteinascidin-743; Natural product; DNA-interacting drug; Cell cycle; DNA repair

1. Introduction

Ecteinascidin-743 (ET-743) is a marine tetrahydroisoquinoline alkaloid isolated from *Ectenascidia turbinata*, a tunicate that grows on mangrove roots throughout the Caribbean sea [1–3]. At nanomolar concentrations, ET-743 is active against a variety of solid tumour cell lines, including melanoma, non-small cell lung carcinoma (NSCLC), ovarian and colon cell lines [4] and against a variety of surgically derived human tumour specimens growing in primary cultures [5]. Furthermore, ET-743 is very active *in vivo* against several types of human solid tumour xenografts [6]. On the basis of these preclinical data, the drug was selected for clinical development. The results obtained so far in phase I clinical trials in Europe and the USA are quite encouraging in view of the many objective responses that have been observed at tolerable drug doses in a variety of human tumours including soft tissue sarcomas, osteosarcoma, melanoma and breast cancers [7,8].

The mechanism of action of ET-743 has yet to be fully elucidated. However, it has been shown that ET-743 binds to the minor groove of DNA forming covalent adducts at the N_2 position of guanine [9,10]. At relatively high concentrations, ET-743 causes disorganisation of microtubule assembly and is a topoisomerase I poison [11,12].

Most mechanistic studies performed so far have been done using ET-743 concentrations much higher than the optimal cytotoxic concentrations and, therefore, possibly irrelevant for explaining the antitumour activity of this compound.

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The present study was performed to investigate the cell cycle perturbations and the mechanism of cytotoxicity after a short exposure with relatively low drug concentrations.

Several factors such as cell cycle phase, p53 and nucleotide excision repair (NER) status have been examined and the overall picture that is emerging from these studies is that the primary mechanism of action for ET-743 is distinct from the other known DNA-interacting drugs.

2. Materials and methods

2.1. Cells and culture conditions

The human colon adenocarcinoma LoVo cell line was grown in a monolayer in Ham's F12 medium supplemented with 10% (v/v) fetal calf serum (FCS), 1% (v/v) L-glutamine (200 mM), 1% (v/v) vitamins (BME vitamin solution; 100×; Gibco Europe, Paisley, UK) at 37°C in a humidified 5% CO_2 atmosphere in T25 cm^2 tissue flasks (IWAKI, Bibby Sterilin, Staffordshire, UK). The human colon adenocarcinoma SW620 cell line was grown in monolayer in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) FCS in T25 cm^2 tissue flasks. The human ovarian carcinoma A2780 and its sub-line A2780/CX3 were grown in monolayer in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 1% (v/v) L-glutamine (200 mM) at 37°C in a humidified 5% CO_2 atmosphere in T25 cm^2 tissue flasks. A2780/CX3 cell line was obtained after transfection of A2780 cells with pc*TP53*-$CX3_3$ plasmid (kindly supplied by A. Levine, Princeton University, Princeton, NJ, USA) containing val–ala 143 mutant-p53. The MEF p53 (+/+) and the p53 (−/−) cell lines (kindly supplied by T. Jack, HHMI, MIT, Cambridge, USA) were grown in monolayer in Dulbeccos modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated FCS. The Chinese hamster ovary (CHO) parental cell line (CHO-AA8) and the ultraviolet (UV)-sensitive DNA-repair deficient mutant cell lines, CHO-UV23, CHO-UV61 and CHO-UV96 (hereafter referred to as UV23, UV61 and UV96) [13] were maintained in Ham-F-10 medium (Gibco Europe, Paisley, UK), with 10% (v/v) FCS and cultured at 37°C with 5% CO_2.

2.2. Drug, growth inhibition and clonogenicity test

ET-743 (Fig. 1) was kindly supplied by Pharma Mar, S.A. Tres Cantos, Spain.

The effect of the ET-743 treatment on A2780, A2780/CX3, MEF p53 (+/+) and MEF p53 (−/−) cell lines was evaluated by a standard growth inhibition test. Exponentially growing cells were treated for 1 h with different concentrations of ET-743. After treatment, cells were washed twice with phosphate-buffered solution (PBS) and a fresh drug-free medium was added to the cells. At 24 and 48 h after drug washout the number of viable cells was evaluated by counting the cells with a Coulter Counter instrument. The effect of the ET-743 treatment on LoVo, SW620, CHO parental and UV-sensitive DNA repair deficient cell lines was evaluated by a standard clonogenic assay. Exponentially growing cells were treated for 1 h (LoVo and SW620) and for 24 h (CHO cell lines) with different concentrations of ET-743. After treatment, cells were washed twice with PBS; 800 control and treated cells (LoVo and SW620) and 300 control and treated cells (CHO cell lines) were plated in 30-mm petri dishes with 3 ml fresh medium. Cell viability was checked using erythrosin B. The colonies were allowed to develop for 10–14 days then stained with 1% crystal violet solution in 20% ethanol. The number of colonies was measured using the entry level image system (Immagini & Computer, Bareggio, Milan, Italy). A background correction was made and the smallest control cell colony (50 cells/colony) was taken as the minimum for setting the cut-off point.

2.3. Cell synchronisation

A centrifugal elutriation technique with the Beckman J6-MC elutriation system and JE-50 rotor was used to separate SW620 cells in the different cell cycle phases. With centrifugal elutriation, the sediment rate of cells reflects their size and consequently their position in the cell cycle. After elutriations the G_1, S and G_2M SW620 cells were treated for 1 h with different ET-743 concentrations. After treatment, cells were washed twice with PBS; 800 control and treated cells were plated in 30-mm petri dishes with 3 ml fresh medium. The clonogenic inhibitory effect of ET-743 was evaluated as previously described.

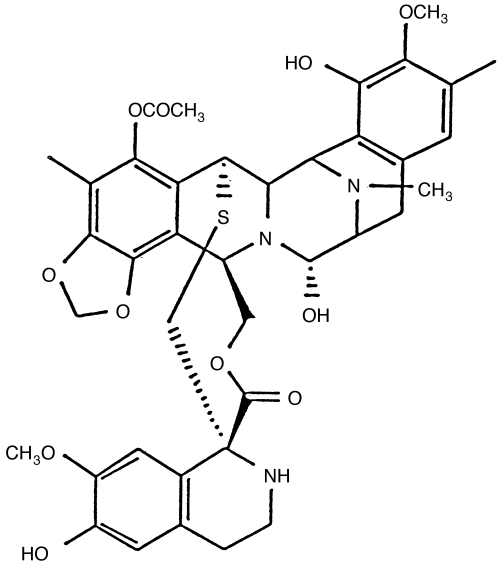

Fig. 1. Ecteinascidin-743 (ET-743) chemical structure.

2.4. Cell cycle studies: BrdUrd/DNA analysis

Exponentially growing LoVo and SW620 cells were treated for 1 h with 80 or 20 nM ET-743, respectively (that caused approximately 40% growth inhibition at 24 h after drug washout). During the last 15 min drug treatment, 20 μM 5-bromo-2-deoxyuridine (BrdUrd) was added to the cells. After treatment, the drug-containing medium was removed, the cells were washed twice with PBS and fresh medium was provided. After 1 h treatment and at 3, 6, 14, 24, 39 and 48 h after drug washout control and treated cells were fixed in 70% ethanol and kept at 4°C before staining. BrdUrd incorporation was not modified by ET-743 treatment, thus allowing a correct estimation of S phase cells.

By applying this protocol, it was possible to obtain a distinct evaluation of the cell cycle perturbations in cells that were in S phase (BrdUrd-positive cells) or in G_1 or in G_2M phases (BrdUrd-negative cells) during 1 h ET-743 treatment.

2.5. BrdUrd/DNA detection

For detection of BrdUrd incorporation into DNA, the fixed cells were washed with cold PBS and the DNA was denatured with 1 ml of 2N HCl for 20 min at room temperature to allow the anti-BrdUrd monoclonal antibody (MAb) to react with the BrdUrd incorporated in the DNA chain. DNA denaturation was stopped by adding 3 ml 0.1 M sodium tetraborate pH 8.5. After centrifugation, the pellet was incubated with 1 ml 0.5% Tween-20 (Sigma Chemicals Co., St Louis, MO, USA) in PBS and 1% (v/v) normal goat serum (NGS) (Dakopatts, Glostrup, Denmark) for 15 min at room temperature. The incorporated BrdUrd was visualised by incubating the cells with the monoclonal antibody anti-BrdUrd (Becton Dickinson, Sunnyvale, CA, USA) diluted 1:10 in 0.5% (v/v) Tween-20 in PBS for 1 h at room temperature in the dark. After centrifugation the pellet was incubated with 1 ml of 0.5% (v/v) Tween-20 and 1% (v/v) NGS for 15 min at room temperature. Then the cells were incubated with a fluorescent fluorescein isothiocynate (FITC)-conjugated affinipure F(ab′)2 fragment goat anti-mouse IgG (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) diluted 1:50 in 0.5% (v/v) Tween-20 in PBS for 1 h at room temperature in the dark. The cells were then resuspended in 2 ml of a solution containing 2.5 μg/ml of propidium iodide (PI) in PBS and 25 μl RNAse 1 mg/ml in water, and stained overnight at 4°C in the dark. Biparametric BrdUrd/DNA analysis was done on at least 20 000 cells for each sample by the FacSort system (Becton Dickinson). The data are the average of three replications and were analysed using Cell Quest software. Fluorescence pulses were detected using a band pass filter 530±30 nm and 620±35 nm, for green and red fluorescence, respectively, in combination with a dichroic mirror at 570 nm [14]. The percentages of the cell cycle phase distribution were calculated by the method of Krishan and Frei [15].

2.6. Cyclins A-B1/DNA staining

At the end of treatment, and at different time intervals after drug washout, the cells were fixed in 70% (v/v) ethanol and stored at 4°C before staining. The fixed cells were washed in cold PBS and permeabilised with 0.25% (v/v) Triton X-100 (Sigma) in PBS for 5 min in ice. Then were washed in PBS and incubated with 200 μl of MAb anti-human cyclin A, 6E6 clone or cyclin B1, 7A9 clone (Novocastra Laboratories Ltd, Newcastle, UK) at a concentration of 2.5 μg/ml in PBS + 1% (v/v) NGS overnight at 4°C in the dark. A blank sample was prepared by incubation of cells with 200 ml of 1% (v/v) NGS in PBS or with the isotype IgG instead of the cyclin. After removing the cyclin A or B1, the cells were incubated with 200 μl of fluorescein FITC-conjugated affinipure F(ab′)2 fragment goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:100 in 0.5% (v/v) Tween-20 in PBS for 1 h at room temperature in the dark. The cells were finally resuspended in 2 ml of a solution containing 2.5 μg/ml of PI in PBS and 25 μl RNAse 1 mg/ml in water, and stained overnight at 4°C in the dark [16].

2.7. p53 expression

Control and ET-743 treated cells were fixed in 70% (v/v) ethanol and stored at 4°C before staining. The fixed cells were washed in cold PBS and permeabilised with 0.5% Tween 20 (Sigma, St Louis, MO, USA) in PBS for 15 min at room temperature. Then the cells were washed in PBS and incubated with 200 μl of p53 (DO-1) sc-126 monoclonal antibody (Santa Cruz Biotech, Inc., Santa Cruz, CA, USA) diluted 1:10 in PBS + 1% (v/v) NGS for 60 min at 4°C in the dark. A blank sample was prepared by incubation of cells with 200 ml of 1% (v/v) NGS in PBS or with the isotype IgG instead of p53. After removing the p53, the cells were incubated with 200 μl of fluorescein FITC-conjugated affinipure F(ab′)2 fragment goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:100 in 0.5% (v/v) Tween-20 in PBS for 1 h at room temperature in the dark. The cells were finally resuspended in 2 ml of a solution containing 2.5 μg/ml of PI in PBS and 25 μl RNAse 1 mg/ml in water, and stained overnight at 4°C in the dark [17].

2.8. DNA single-strand breaks assay

LoVo and SW620 cells were labelled for 24 h using medium supplemented with 0.05 μCi/ml [^{3}H]-TdR (specific activity 85 Ci/mmol, Amersham Italia S.r.l., Milan, Italy), washed, fresh medium was added and chased for

24 h before exposure to different concentrations of ET-743 for 1 h. Then the cells were kept in ice, washed with PBS and layered on polycarbonate filters, 2 μm pore size and 25 mm diameter (Nucleopore, Costar, Acton, MA, USA). Cells (10^6 cells/sample) were lysed with 5 ml of a lysis solution containing 2% (v/v) sodium dodecyl sulphate (SDS), 0.02 M ethylene diamine tetra acetic acid (EDTA), 0.1 M glycine (pH 10), which was allowed to flow through the filter by gravity. After connecting the outlet of the filter holders to the pumping system, 2 ml of the lysis solution containing 0.5 mg/ml proteinase K (Merck KgaA, Darmstadt, Germany) were added to the reservoir over the polycarbonate filters. DNA was eluted from the filters with a solution containing 20 mM EDTA, 0.1% (v/v) SDS and adjusted to pH 12.2 with tetrapropylammonium hydroxide (Fluka Chemie AG, Buchs, Switzerland). 3-, 6-, 9-, 12- and 15-h fractions were collected and fraction and filters were processed as previously described [18].

2.9. DNA protein cross-links assay

Partial labelling of DNA strands was ensured by incubating exponentially growing cells with 0.05 μCi/ml [^{3}H]-TdR added to the medium for 48 h. Cells were then washed, fresh medium was added and chased for 24 h before a 1 h treatment with ET-743. DNA–protein cross-links were detected by alkaline elution at pH 12.2 by the method of Khon and colleagues [18]. Drug-induced DNA–protein cross-links were expressed as rad equivalents with respect to the cross-links produced by 3000 rads in the set without drug treatment.

3. Results

Fig. 2 shows the clonogenic inhibitory effect of 1 h treatment with ET-743 on LoVo and SW620 cells. One hour exposure caused a significant inhibition in the clonogenicity that was higher in SW620 than in LoVo cells.

Figs. 3 and 4 show the cell cycle phase perturbations induced by ET-743. In these figures the upper panel shows the biparametric BrdUrd/DNA cytograms of control and ET-743-treated cells, while the lower panel shows the percentage of cells in the different cell cycle phases evaluated at different time intervals after drug washout. After drug washout, those cells in S phase (BrdUrd-positive cells) during drug treatment, progressed through this phase of the cell cycle more slowly than control cells. At 14 h after drug washout, cells in the G_1 phase (BrdUrd-negative cells) during ET-743 treatment were blocked in S phase. Although this block was found in both cell lines, it was more evident for SW620 cells than for LoVo cells. At 24 h after drug washout the majority of the BrdUrd-positive and BrdUrd-negative cells were blocked in the G_2M phase. At 48 h in the SW620 cells this G_2M block was still evident either for the BrdUrd-positive and BrdUrd-negative cells, while the LoVo BrdUrd-positive cells had started to recover from this block.

By monitoring LoVo and SW620 cells for several days in drug-free medium after 1 h treatment with ET-743, we observed that the cell cycle perturbations were no longer evident after 120 h for LoVo and 168 h for SW620 cells (data not shown). This reversibility might be related to the repair of ET-743-induced DNA lesions.

The biparametric analyses of cyclins/DNA were determined to more precisely characterise the cell cycle phase perturbations induced by ET-743. As expected, the levels of cyclin A increased at 14 h after drug washout when the ET-743-treated cells were accumulated in the S phase compared with control cells (data not shown). When cyclin B1/DNA analyses were performed at different time intervals after drug washout, the level of cyclin B1 increased at the time of the G_2M block in both LoVo and SW620 cells (data not shown). The results of these experiments indicate that cyclin A and B1 levels increased concomitantly with the accumulation of cells, in the S and G_2M phases, respectively. As we did not notice any changes in the levels of these cyclins before the cell cycle perturbation the increase in the cyclin levels appears to be more a consequence, rather than the cause, of the changes in the cell cycle distribution.

We then asked the question of whether the drug was equally potent in the different cell cycle phases or exhibited some degree of specificity for a particular phase of the cell cycle. SW620 cells synchronised by elutriation in G_1, S or the G_2M phase were exposed to ET-743 and then the cytotoxicity was evaluated by a clonogenic assay. SW620 cells in G_1 were more sensitive

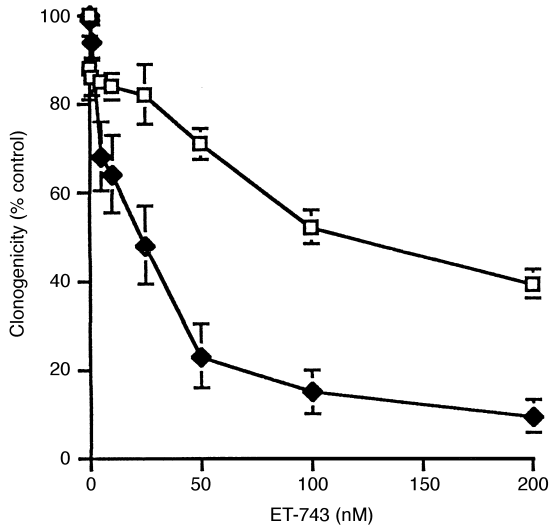

Fig. 2. Effect of ET-743 on the clonogenicity of LoVo and SW620 cells. Each point is the mean of six replicates; bars represent standard deviations. □, LoVo cells; ◆, SW 620 cells.

to ET-743 than the S phase cells and much more sensitive than the G_2M phase cells, whose clonogenicity was apparently barely affected up to 10 nM ET-743 (Fig. 5).

p53 status has been reported as a possibly relevant factor for the cytotoxicity of several DNA-damaging agents. Generally, cells expressing wild-type (wt) p53 are more sensitive than cells not expressing functional p53. ET-743 was able to increase p53 levels very remarkably already at 6 h in LoVo cells which express wt p53 whereas, as expected, no p53 was seen in SW620 cells (data not shown).

The observation that SW620 cells, which do not express wt p53, were more sensitive to ET-743 than LoVo cells suggested that the drug might be more effective against cells with mutated p53, even if the different sensitivity could be due to many other biological differences existing between the two colon carcinoma cell lines. In order to evaluate more precisely the relationship between p53 status and the cytotoxic effect of ET-743 we used isogenic cellular systems with different p53 expression, MEF p53 +/+, MEF p53 −/−, A2780 and A2780/CX3 cells. No statistically significant differences in ET-743 cytotoxicity were found when the treatment was carried out in cells with different p53 status, the IC_{50} being 2.5 nM for both MEF p53 +/+ and MEF p53 −/− and approximately 15 nM for A2780 and A2780/CX3 cells.

We then attempted to evaluate if ET-743 caused DNA damage by assessing DNA breaks and DNA–protein cross-links in SW620 and LoVo cells exposed to cytotoxic concentrations of ET-743 by using alkaline elution methods. The results of a typical experiment are shown in Fig. 6. It can be seen that ET-743 caused no detectable DNA breaks as no increase in the elution rate

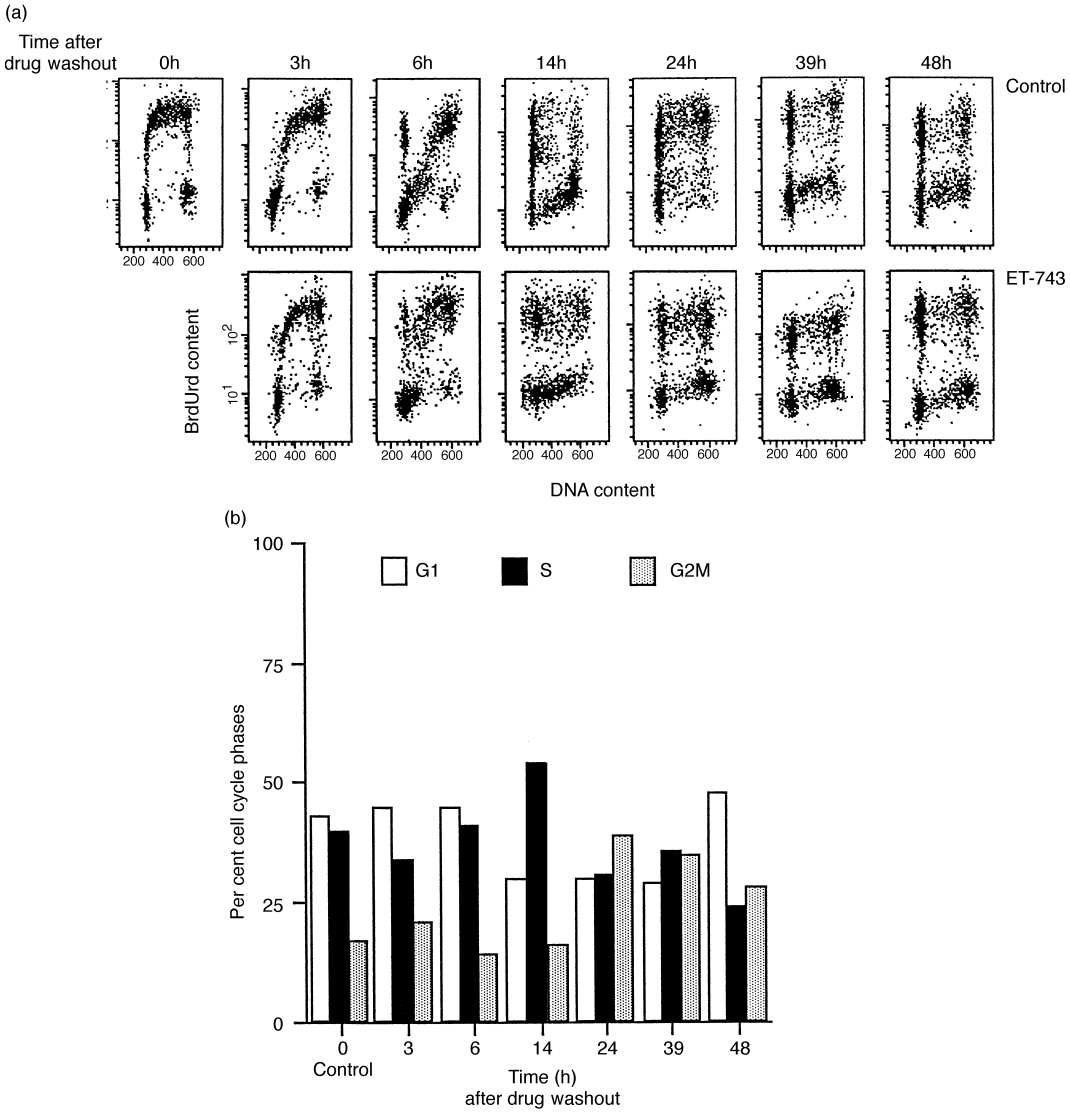

Fig. 3. Effects of 1 h ET-743 exposure on the cell cycle phase distribution of LoVo cells evaluated at 0, 3, 6, 14, 24, 39 and 48 h after drug washout. During the last 15 min of treatment with 80 nM ET-743, 20 μM BrdUrd was added to the cells, then the cells were washed with phosphate-buffered solution (PBS) and drug-free medium was provided. (a) Biparametric BrdUrd/DNA analysis of control cells and ET-743-treated cells. (b) Percentage of cells in the different cell cycle phases evaluated at different time intervals after drug washout. BrdUrd, 5-bromo-2-deoxyuridine.

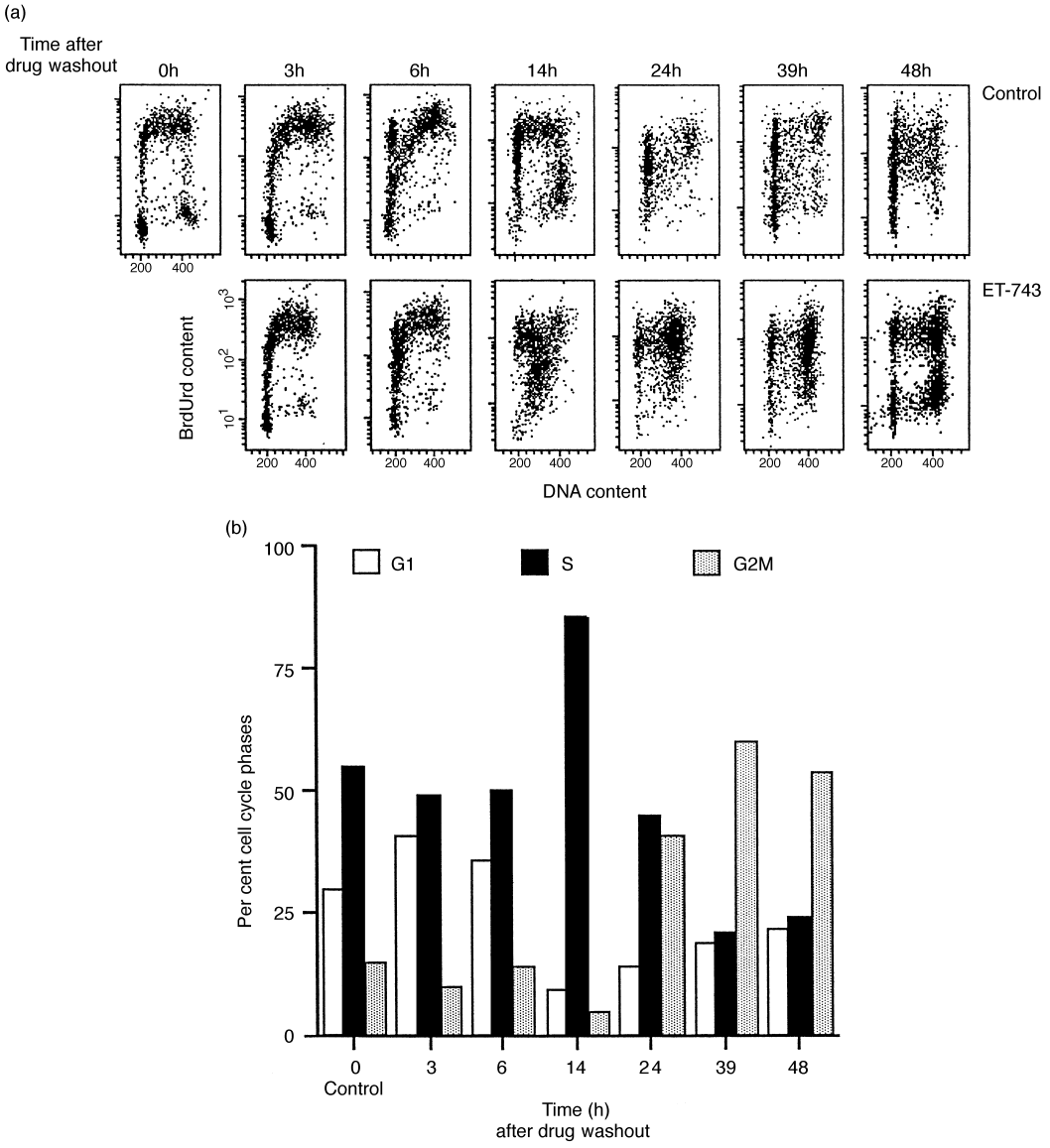

Fig. 4. Effects of 1 h ET-743 exposure on the cell cycle phase distribution of SW620 cells evaluated at 0, 3, 6, 14, 24, 39 and 48 h after drug washout. During the last 15 min of treatment with 20 nM ET-743, 20 μM BrdUrd BrdUrd, 5-bromo-2-deoxyuridine was added to the cells, then the cells were washed with phosphate buffed solution (PBS) and drug-free medium was provided. (a) Biparametric BrdUrd/DNA analysis of control and ET-743-treated cells. (b) Percentage of cells in the different cell cycle phases evaluated at different time intervals after drug washout.

of DNA was observed (Fig. 6a). In contrast, VP16 (etoposide) and radiotherapy (Rx), used as positive controls, caused a faster elution rate of the DNA. As shown in Fig. 6(b), ET-743 induced no detectable DNA–protein cross-links, as demonstrated by the fact that the curve of drug-treated samples overlapped the curve of the untreated samples. Again VP16 caused a significant level of DNA–protein cross-links, as might be expected, knowing that the drug is a DNA–topoisomerase II inhibitor.

Table 1 shows a comparison of the ET-743 IC_{50} obtained in the CHO-AA8 cell line and in ultraviolet (UV)-sensitive nucleotide excision repair (NER)-deficient cell lines; UV23, lacking a functional xeroderma pigmentosum complementation group B gene (XPB); UV61, lacking a functional cockayne's syndrome group B gene (CSB); and UV96, lacking a functional excision repair cross-complementing group 1 gene (ERCC1). ET-743 was less active ($P < 0.05$) in UV23 and UV96 cell lines, while no statistically significant difference was observed between CHO-AA8 and UV61 cell lines.

Table 1
IC_{50} of ET-743 in the CHO-AA8 parental cell line and UV-sensitive DNA repair deficient mutant cell lines, UV23, UV61 and UV96

	CHO-AA8	UV23	UV61	UV96
ET-743 nM IC_{50}	0.82±0.1	5.4±1.3*	2.02±0.4	6.9±2.5*

UV, ultraviolet.
*$P < 0.05$.

4. Discussion

Many anticancer agents currently used therapeutically act by causing DNA damage either directly by reacting with DNA or indirectly by poisoning DNA-processing enzymes as in the case of the inhibitors of DNA-topoisomerase enzymes. The basis of selectivity is largely unknown, but it may not rely only on the type of DNA damage, but on many other cellular mechanisms involved in the recognition of the damage, DNA repair as well as several kinds of responses which follow the drug-induced cellular stress.

We know that ET-743 binds in the minor groove of DNA and subsequently alkylates the N_2 of guanine. This has been shown by Pommier and colleagues [10] who demonstrated a change in the electrophoretic mobility of small oligonucleotides reacted with ET-743 and denatured with SDS, and by Moore and colleagues [19] who provided direct evidence of this reaction by using high-field nuclear magnetic resonance (NMR). Recently Martinez and associates [20] and Takebayashi and colleagues [12] showed that ET-743 can poison DNA-topoisomerase I *in vitro* at very high ET-743 concentrations (μM range). As clearly stated by Martinez and associates in their paper, the high concentration required suggested that the effect on DNA-topoisomerase I was "only an anxillary effect incidental to the primary mechanism of action". In the present paper, using pharmacologically reasonable concentrations of ET-743 (i.e. concentrations in the nM range, achieved in plasma of patients who received the drug in phase I studies) [21,22], we failed to observe any DNA breakage or DNA–protein cross-links in sensitive cells.

That topoisomerase I is not the crucial target of ET-743 is also suggested by recent results we have obtained in *Saccharomyces cerevisiae* yeast cells not expressing the topoisomerase I gene. In fact, the deletion of the topoisomerase I gene while conferring resistance to camptotecins does not change the cytotoxic effect of ET-743 (data not shown). Taken together, these data indicate that the primary mechanism of action of ET-743 is unlikely to be related to topoisomerase I poisoning.

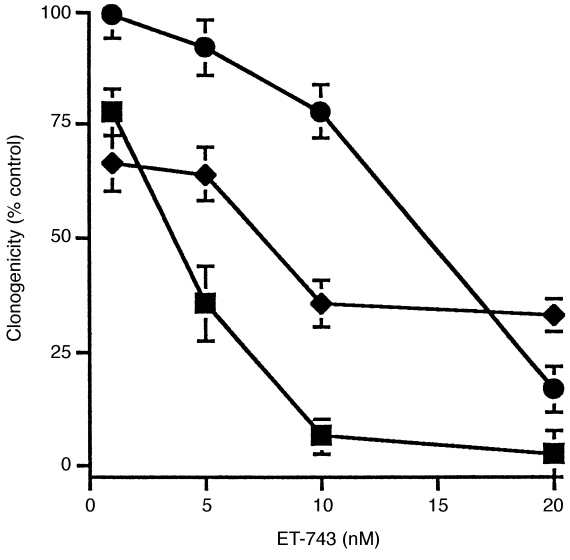

Fig. 5. Effect of ET-743 on the clonogenicity of synchronised SW620 cells treated for 1 h with different ET-743 concentrations. SW620 cells were synchronised in the G_1, S or G_2M phases of the cell cycle by elutriation. Each point is the mean of six replicates; bars represent standard deviations. ■, G_1 phase cells; ◆, S phase cells; ●, G_2M phase cells.

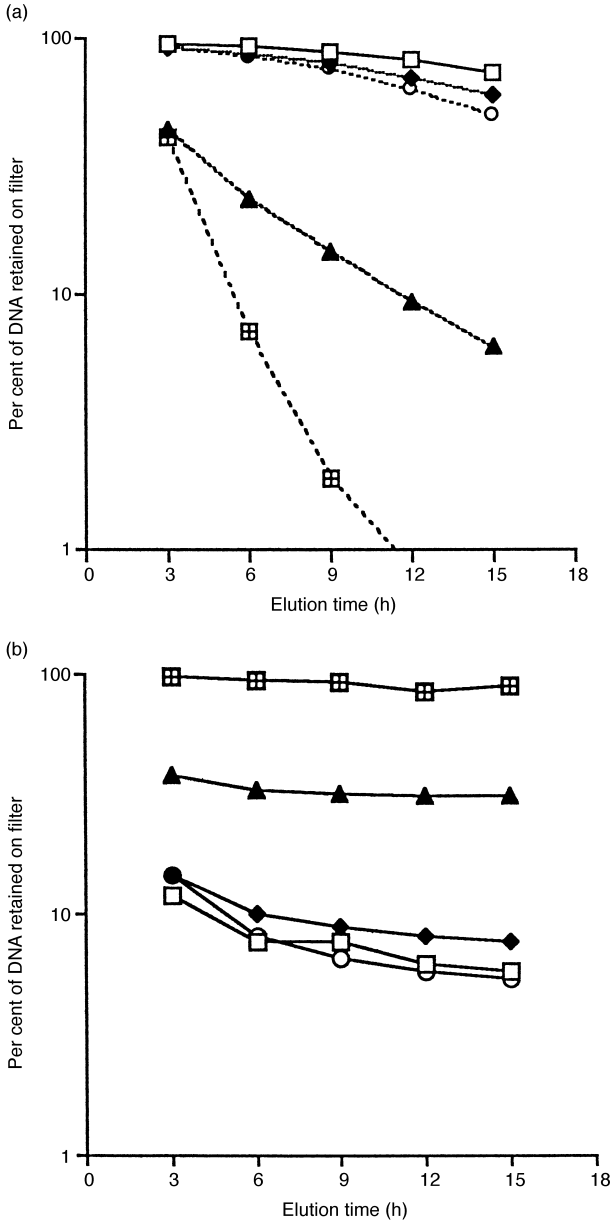

Fig. 6. Formation of DNA breaks (a) and DNA protein cross-links (b) on SW620 cells exposed for 1 h to different ET-743 concentrations. (a) □, control cells; ◆, 20 nM ET-743; ○, 80 nM ET-743; ▲, 10 μM VP16; ⊞, 600 rads. (b) □, control cells; (b) ◆, 20 nM ET-743; ○, 80 nM ET-743; ▲, 10 μM VP16; ⊞, 3000 rads.

Like many other different DNA-interacting drugs, ET-743 causes strong perturbation of the cell cycle with a delay of cells progressing from G_1 to G_2, an inhibition of DNA synthesis and a marked blockade in G_2M which does not appear to be p53-dependent, as it could be observed in both cells expressing or those not expressing a functional p53.

What is quite unique with this drug is the much higher sensitivity of G_1 cells. We do not have an explanation for this finding, but to the best of our knowledge such phase specificity has never been described before for other DNA-interacting agents. In addition, the somehow paradoxical data obtained in NER-deficient cells (i.e. NER-deficient cells become less sensitive to ET-743) might point out a potential difference in the mechanism of interaction of ET-743 with DNA compared with other compounds [23]. Zewail-Foote and Hurley [9] have recently demonstrated that ET-743 binds in the minor groove of DNA forcing a widening of this groove and causing a bending towards the major groove of DNA. This differs from previous observations for other minor groove binders, such as CC-1065, or Tallimustine (FCE 24517) [24,25] which bind in the minor groove and bend DNA into the minor groove or for that seen for cisplatin [26] which occupies the major groove and bends DNA into the major groove.

Separate data by Minuzzo and colleagues [27] and Jin and associates [28] indicate the possibility that ET-743 affects the regulation of transcription by inhibiting the normal formation of complexes between the transcription factor nuclear factor y (NFY) and the CAAT box. It is interesting to note that the ability of ET-743 to alter the regulation of transcription is observed in cells at concentrations close to the cytotoxic ones (i.e. in the nM range). Since NFY activates the transcription of genes involved in cell cycle regulation it may be that the perturbations of the cell cycle induced by ET-743 are partially due to the inhibition of this transcription factor.

The peculiar changes of DNA structure induced by ET-743 might modify the recognition of the protein involved in the NER too. The finding that cells deficient in NER mechanisms are less sensitive to ET-743 than the control proficient cells was unexpected. Several other drugs which cause DNA alkylations were in fact observed to behave in the opposite way (i.e. increased sensitivity of NER-deficient cells) [23]. This finding is somehow analogous to that of the resistance to cisplatin in mismatch-repair-deficient cells [29,30]. In both cases, it might be hypothesised that the activation of the DNA repair mechanism which does not achieve the removal of lethal DNA damage is counterproductive for the cells because it activates a cascade of signals leading the cell to death.

In conclusion, these studies highlight that ET-743 is a novel drug whose mechanism of action is different from that of other DNA-interacting anticancer drugs and this can explain its striking activity observed at preclinical and clinical levels against tumours which are not responsive to conventional anticancer drugs.

Acknowledgements

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