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UNIQUE PATTERN OF ET-743 ACTIVITY IN DIFFERENT CELLULAR SYSTEMS WITH DEFINED DEFICIENCIES IN DNA-REPAIR PATHWAYS

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The cytotoxic activity of ecteinascidin 743 (ET-743), a natural product derived from the marine tunicate *Ecteinascidia turbinata* that exhibits potent anti-tumor activity in pre-clinical systems and promising activity in phase I and II clinical trials, was investigated in a number of cell systems with well-defined deficiencies in DNA-repair mechanisms. ET-743 binds to N2 of guanine in the minor groove, but its activity does not appear to be related to DNA-topoisomerase I poisoning as the drug is equally active in wild-type yeast and in yeast with a deletion in the DNA-topoisomerase I gene. Defects in the mismatch repair pathway, usually associated with increased resistance to methylating agents and cisplatin, did not affect the cytotoxic activity of ET-743. However, ET-743 did show decreased activity (from 2- to 8-fold) in nucleotide excision repair (NER)–deficient cell lines compared to NER-proficient cell lines, from either hamsters or humans. Restoration of NER function sensitized cells to ET-743 treatment. The DNA double-strand-break repair pathway was also investigated using human glioblastoma cell lines MO59K and MO59J, respectively, proficient and deficient in DNA-dependent protein kinase (DNA-PK). ET-743 was more effective in cells lacking DNA-PK; moreover, pre-treatment of HCT-116 colon carcinoma cells with wortmannin, a potent inhibitor of DNA-PK, sensitized cells to ET-743. An increase in ET-743 sensitivity was also observed in ataxia telangiectasia–mutated cells. Our data strongly suggest that ET-743 has a unique mechanism of interaction with DNA.

Key words: *marine compound; ET-743; DNA repair; nucleotide excision repair; DNA-protein kinase; ATM*

Ecteinascidin 743 (ET-743) is a natural product derived from the marine tunicate *Ecteinascidia turbinata*, selected for its significant anti-tumor activity in different *in vitro* and *in vivo* models.[1–3] Currently, ET-743 is undergoing phase II clinical trials after having shown activity in phase I clinical studies with responses observed in different human tumors, including soft tissue sarcoma, osteosarcoma, melanoma and breast cancer.[4,5]

The mechanism of action of ET-743 has yet to be fully defined, but DNA appears to be the primary target. Indeed, it has been shown to bind in the minor groove of DNA and to alkylate the N2 position of guanine with some degree of specificity.[6,7] Such ET-743 minor-groove alkylation induces a bend in the DNA helix toward the major groove, a finding not common to the other minor groove–alkylating agents, which bend DNA into the minor groove.[8] No single-strand breaks, double-strand breaks or DNA–protein cross-links could be found by alkaline elution in cells exposed to IC_{50} concentrations of ET-743.[9] However, Takebayashi *et al.*[10] showed that ET-743 was able to induce DNA–topoisomerase I cross-linking but that high concentrations were required to produce the effect.

To provide some insight into the mechanism of action of ET-743, we have evaluated its cytotoxic activity using a panel of different cellular systems characterized by well-defined deficiencies in different mammalian repair pathways. Our data indicate that ET-743 has a unique mechanism of interaction with DNA.

MATERIAL AND METHODS

Cells and drugs

Yeast strains K699 *(MATa trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-11 can1-100)* and the isogenic derivatives CY2823 *(Δrad52)* and CY2278 *(ΔtopI)* (kindly provided by Dr. M. Lopes, Instituto F.I.R.C. di Oncologia Molecolare, Milan, Italy) were grown in YPD plates (1% yeast extract, 2% bacto-peptone, 2% glucose) with or without the drugs.

The Chinese hamster ovary (CHO) parental cell line CHO-AA8 and the UV-sensitive DNA repair–deficient mutant cell lines CHO-UV23, CHO-UV61 and CHO-UV96 (hereafter named UV23, UV61 and UV96)[11] were kindly provided by Dr. M. Stefanini (CNR IGBE, Pavia, Italy). The UV96 cell line was transfected by the calcium phosphate technique using 10 μg of a CMV-ERCC1 plasmid encoding for ERCC1 or with 10 μg of CMV empty vector. The CMV-ERCC1 plasmid was obtained by subcloning the HindIII and BamHI fragments from the pE12-12 plasmid (kindly supplied by Dr. J.H. Hoejimakers, Erasmus University, Rotterdam, the Netherlands) in bluescript plasmid, yielding BLER plasmid; the Not-Apa fragment of BLER was subsequently cloned in CMV plasmid (Invitrogen, La Jolla, CA), yielding CMV-ERRC1 plasmid. Different clones were obtained; 2 clones, ERA2 and ERA5, were selected for further study, expressing, respectively, the CMV empty vector and CMV-ERCC1. Clones were selected on 800 μg/ml of G418. All CHO cells were maintained in Ham-F10 medium (GIBCO, Paisley, UK) supplemented with 10% FCS and cultured at 37°C with 5% CO_2. The normal human fibroblast cell line (C3PV) and the xeroderma pigmentosum group A (XPA) fibroblast cell line (XP25RO) were kindly provided by Dr. M. Stefanini and maintained in Ham-F10 medium supplemented with 20% heat-inactivated FCS at 37°C with 5% CO_2.

Human glioblastoma cell lines MO59K and MO59J, respectively, proficient and deficient in DNA-dependent protein kinase (DNA-PK), were obtained from Dr. M.J. Allalunis-Turner (Cross Cancer Institute, Edmonton, Canada), maintained in Ham-F10:D-MEM (1:1) and supplemented with 1 mM sodium pyruvate and 10% FCS at 37°C with 5% CO_2.

Epstein-Barr virus (EBV)–transformed lymphoblastoid cell lines IARC1663 (normal) and AT11 (ataxia telangiectasia homozygote) were kindly provided by Dr. J. Hill (IARC, Lyon,

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France) and cultured in RPMI-1640 medium supplemented with 20% heat-inactivated FCS.

The hMLH1-deficient human colorectal adenocarcinoma cell line HCT-116 and the subline into which a wild-type copy hMLH1 on chromosome 3 was introduced by microcell fusion (HCT-116+ch3) were obtained from Dr. G. Marra (University of Zurich, Zurich, Switzerland). Both cell lines were maintained in Iscove's modified Dulbecco medium (Sigma, St. Louis, MO), supplemented with 100 mM L-glutamine and 10% FCS. The chromosome-complemented cell line was grown in medium containing 400 μg/ml G418 (Sigma). Experiments were performed in medium without G418.

ET-743 (Pharma Mar, Tres Cantos, Spain) stock solution was made in ethanol and stored at –20°C, and further dilutions were done in medium just before use. Wortmannin and camptothecin (Sigma) were stored in DMSO at –20°C and diluted in medium immediately before use.

Cytotoxicity assays

The cytotoxic effect of ET-743 and camptothecin on the different yeast strains, pre-grown in liquid YPD medium, was evaluated by spotting 20 μl aliquots of serially diluted cell cultures in treated (camptothecin and ET-743) or untreated plates. Cell viability was scored after 3 days at 28°C.

The effect of ET-743 on MO59K and MO59J glioblastoma cells, on IARC1663 and AT11 lymphoblastoid cells and on C3PV and XP25RO fibroblasts was evaluated by a standard growth-inhibition test. Briefly, exponentially growing cells were treated for 2 hr (C3PV and XP25RO cells) or 24 hr (MO59K, MO59J, IARC1663 and AT11 cells) with different concentrations of ET-743. After treatment, cells were washed in PBS and fresh drug-free medium was added. At 72 hr after drug washout, cells were counted with a Coulter (Hialeah, FL) counter. Data are expressed as percentages of untreated controls (mean ± SE of 3 different experiments done in quintuplicate).

The effect of ET-743 on the AA8 parental cell line and on the UV-sensitive DNA repair–deficient cell lines was evaluated by a standard clonogenic assay. Exponentially growing cells were treated with ET-743 for 24 hr; at the end of treatment, cells were washed in PBS and plated (300 cells) in 30 mm tissue culture dishes supplemented with 3 ml fresh medium. Colonies were allowed to grow for 8 to 10 days. HCT-116 cells were seeded at 125 cells/ml in 6-well Petri dishes and, after 48 hr, treated with wortmannin (35.5 μM) for 2 hr; then, different concentrations of ET-743 were added to the wells; after 1 hr, cells were washed in warm PBS and wortmannin-containing medium or drug-free medium was added for a further 24 hr, after which cells were washed in PBS and drug-free medium was added. Colonies were allowed to form for 8 to 10 days. All colonies were stained with 1% crystal violet solution and counted using the Entry Level image system (Immagini & Computer; Bareggio, Milan, Italy). A background correction was made, and the smallest control cell colony was taken as the minimum for setting the cut-off point.

ET-743 IC_{50} values were obtained by interpolation of the dose-response curves in the different cell lines.

Protein extraction and Western blotting analysis

Proteins were extracted from exponentially growing cells according to procedures described previously.[12] For each sample, 40

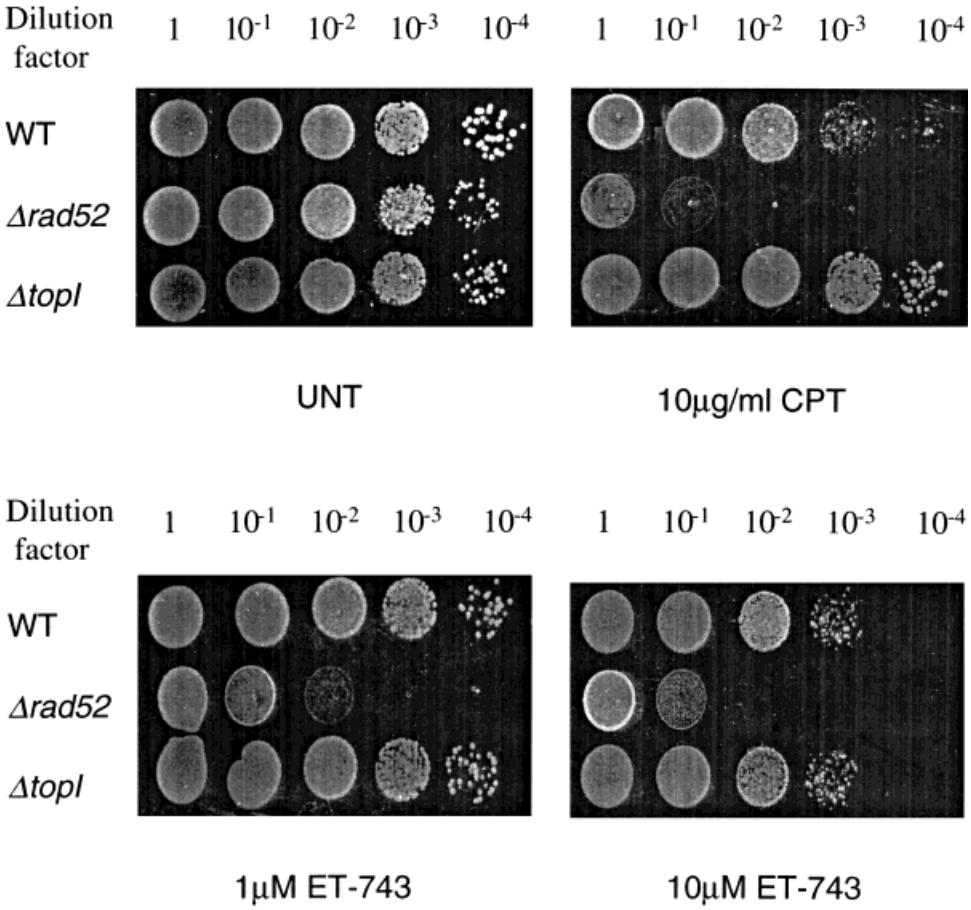

FIGURE 1 – Cytotoxic activity of ET-743 and camptothecin on K699 (wild-type, WT), CY2823 *(ΔRad 52)* and CY2278 *(ΔtopI)* strains, assessed as described in Material and Methods.

μg of protein were electrophoresed by 10% SDS-PAGE and transferred to a nitrocellulose filter. Filters were hybridized with antibodies against ERCC1 (kindly provided by Dr. R.D. Wood, Imperial Cancer Research Fund, London, UK) and actin (from Santa Cruz Biotechnologies, Santa Cruz, CA), which were subsequently detected with the ECL system (Amersham, Aylesbury, UK).

RESULTS

Effect of ET-743 on yeast lacking a functional DNA-topoisomerase I gene

The cytotoxic effect of ET-743 was evaluated in yeast carrying a deletion of the DNA-topoisomerase I *(TOPI)* gene. As shown in Figure 1, ET-743 (10 μM) was clearly equally cytotoxic on wild-type and *TOPI*-deletion yeast strains. On the contrary, in the same experimental condition, a typical topoisomerase I inhibitor, camptothecin, was much less active on the *TOPI*-deletion strain than on the wild-type strain. Conversely, cells carrying a deletion in the *RAD52* gene, which impairs recombinational repair, showed enhanced sensitivity to both ET-743 and camptothecin.

Effect of ET-743 on nucleotide excision repair (NER)-proficient and NER-deficient cell lines

The cytotoxic effect of ET-743 was evaluated in 3 UV-sensitive strains derived from CHO-AA8 cell lines: one lacking a functional *ERCC3/XPB* gene (UV23), one lacking a functional *ERCC1* gene (UV96) and one lacking a functional *ERCC6/CSB* gene (UV61) by a standard clonogenic assay.

As shown in Table I, ET-743 was surprisingly less active in all UV-sensitive NER-deficient cell lines, with a statistically 2- to 8-fold increase in its IC_{50} value compared to the AA8 parental cell line. On the contrary, DDP, a cross-linking alkylating agent, and tallimustine, a minor groove–alkylating agent, showed enhanced activity in the same cell lines compared to the parental cell line. In UV96, DDP and tallimustine were, respectively, approximately 75 and 2 times more active than in the parental AA8 cell line.

To define better the role of NER in the recognition and/or repair of DNA lesions induced by ET-743, we restored NER activity in the UV96 cell lines by transfecting the human *ERCC1* gene. Two clones were selected, ERA2 and ERA5, respectively, transfected with a pCMV empty vector and a pCMV-ERCC1 vector. ERA5, which over-expresses ERCC1 protein (Fig. 2*b*) was less sensitive to DDP treatment than the UV96 cell lines, with a sensitivity similar to that observed in the AA8 parental cell line (data not shown). Restoration of NER activity sensitized cells to ET-743 at a level comparable to that observed in AA8 cell lines (Fig. 2*a*). As expected, ERA2 displayed sensitivity to ET-743 treatment similar to that of UV96.

To further corroborate these data, we tested the cytotoxic activity of ET-743 in human fibroblasts from patients suffering from XPA and normal control fibroblasts. Again, ET-743 was significantly less active ($p < 0.01$) in XPA fibroblasts compared to normal fibroblasts (Fig. 3).

Effect of ET-743 on mismatch repair (MMR)–proficient and MMR-deficient cell lines

The cytotoxic activity of ET-743 was evaluated in HCT-116 cells and HCT-116+chr-3 cells, a subline obtained after chromosome 3 transfer, respectively deficient and proficient in MMR. No difference in the ET-743 cytotoxic profile was observed between the 2 lines (data not shown), with IC_{50} values of 0.15 ± 0.03 and

TABLE I – IC_{50} VALUES OF ET-743 AND OTHER ALKYLATING AGENTS IN CHO NER-PROFICIENT (AA8) AND NER-DEFICIENT (UV23, UV61 AND UV96) CELL LINES[1]

Drug	Cell lines			
	AA8	UV23	UV61	UV96
ET-743 (nM)	0.82 ± 0.15	5.4 ± 1.3[2] (6.5)	2.02 ± 0.41[2] (2.5)	6.9 ± 2.5[2] (8.4)
DDP (μM)	42 ± 2	10 ± 2[3] (0.23)	8 ± 3[3] (0.19)	0.56 ± 0.1[3] (0.013)
Tallimustine (μM)	503 ± 27	381 ± 14[2] (0.76)	490 ± 41 (0.97)	258 ± 27[3] (0.51)

[1]Each value is the mean ± SE of at least 3 experiments. Numbers in parentheses represent fold increase in resistance compared to the parental cell line, AA8.–[2]$p < 0.05$.–[3]$p < 0.01$.

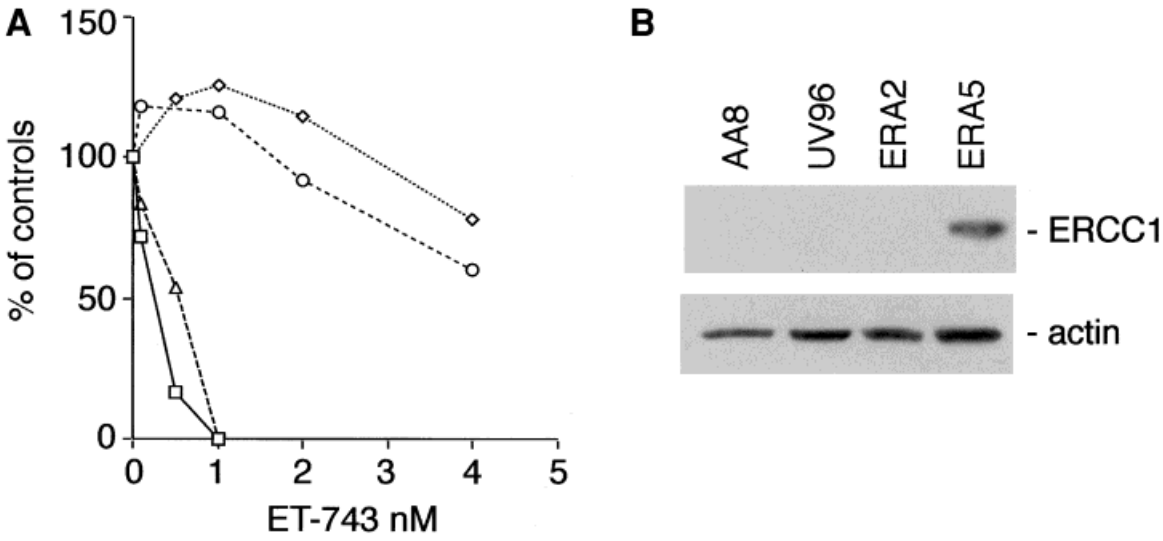

FIGURE 2 – *(a)* Survival curves of AA8 (squares), UV96 (diamonds), ERA2 (triangles) and ERA5 (circles) after treatment with ET-743. *(b)* Western blot analysis of the different clones. Protein extracts were obtained from exponentially growing cells, and blots were hybridized with antibody recognizing human ERCC1 and actin.

0.16 ± 0.01 nM in HCT-116 and HCT-116+chr3 cells, respectively.

Effect of ET-743 on DNA-PK-proficient and -deficient systems

MO59K and MO59J are glioblastoma cell lines derived from the same patient characterized, respectively, by the presence or absence of the DNA-PK catalytic subunit. As shown in Figure 4, MO59J cells were much more sensitive to ET-743 treatment than MO59K cells, with a 5-fold decrease in ET-743 IC_{50} values (0.041 ± 0.004 *vs.* 0.2 ± 0.02 nM) ($p < 0.05$).

To better evaluate the role of DNA-PK in the cellular response to ET-743, HCT-116 cells were pre-treated with wortmannin, a well-known inhibitor of the PI3-kinase family with some degree of specificity toward DNA-PK, at doses able to completely inhibit DNA-PK activity,[13] before ET-743 treatment. A clear shift to the left of the dose-response curve was observed in cells treated with the combination of wortmannin and ET-743 compared to ET-743 treatment alone, with a 2-fold decrease in IC_{50} value (Fig. 4*b*).

Effect of ET-743 on ataxia telangiectasia–mutated (ATM) cell lines

To understand the role, if any, of the ATM gene in the mechanism of action of ET-743, we compared its cytotoxic effect in lymphoblastoid cell lines carrying a normal or mutated ATM gene. ATM-defective cells were statistically ($p < 0.05$) more sensitive to ET-743 treatment than wild-type ATM cells (Fig. 5).

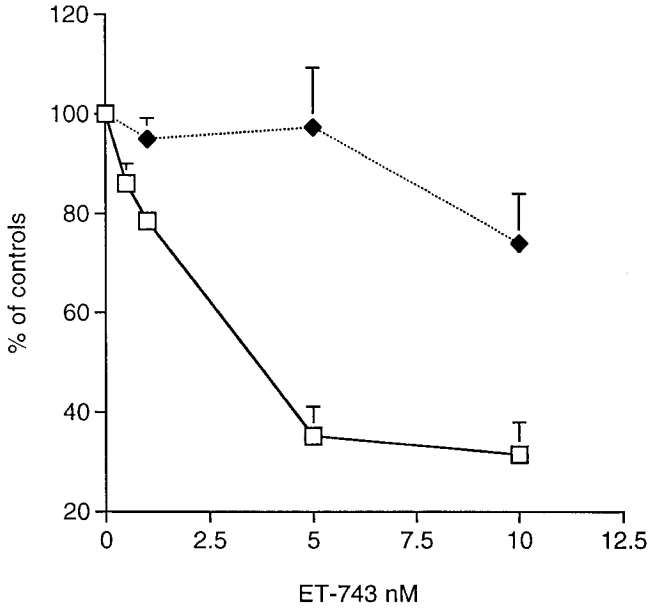

Figure 3 – Cell growth inhibition of normal fibroblasts (squares) and fibroblasts from patients suffering from XPA (diamonds) after treatment with ET-743.

DISCUSSION

Our studies were designed to clarify the mechanism of action of ET-743 using cellular systems with well-defined defects in different repair pathways.

ET-743 induces DNA-topoisomerase I cross-linking, though only at very high concentrations.[10] However, our finding that cell survival in the presence of 10 μM ET-743 is not affected by the presence of a functional *TOPI* gene indicate that DNA-topoisomerase I is not the primary target of ET-743.

ET-743 binds in the minor groove of DNA and alkylates the N2 of guanine, but in comparison to the other minor groove–alkylating agents, such as CC1065 or tallimustine, which covalently bind to N3 of adenine in AT-rich regions and bend DNA into the minor groove, ET-743 binds in GC-rich regions and bends DNA into the major groove.[8] These differences in DNA bending and directionality have been suggested to be responsible for the different anti-tumor activity and toxicity observed between these 2 classes of compound. CHO mutant cell lines with specific defects in NER mechanisms have been shown to be useful for investigating the mechanisms of action of anti-cancer agents.[14] Indeed, they display unusually high sensitivity to cross-linking agents such as DDP and melphalan and slight but statistically significantly increased sensitivity (2-fold) to the minor groove alkylators tallimustine and CC1065.[14] On the contrary, our results show decreased sensitivity (2- to 8-fold) in NER-deficient hamster systems (*e.g.*, the CHO-UV-sensitive NER-deficient cell lines) and in a human NER-deficient system (fibroblasts of patients suffering from XPA).

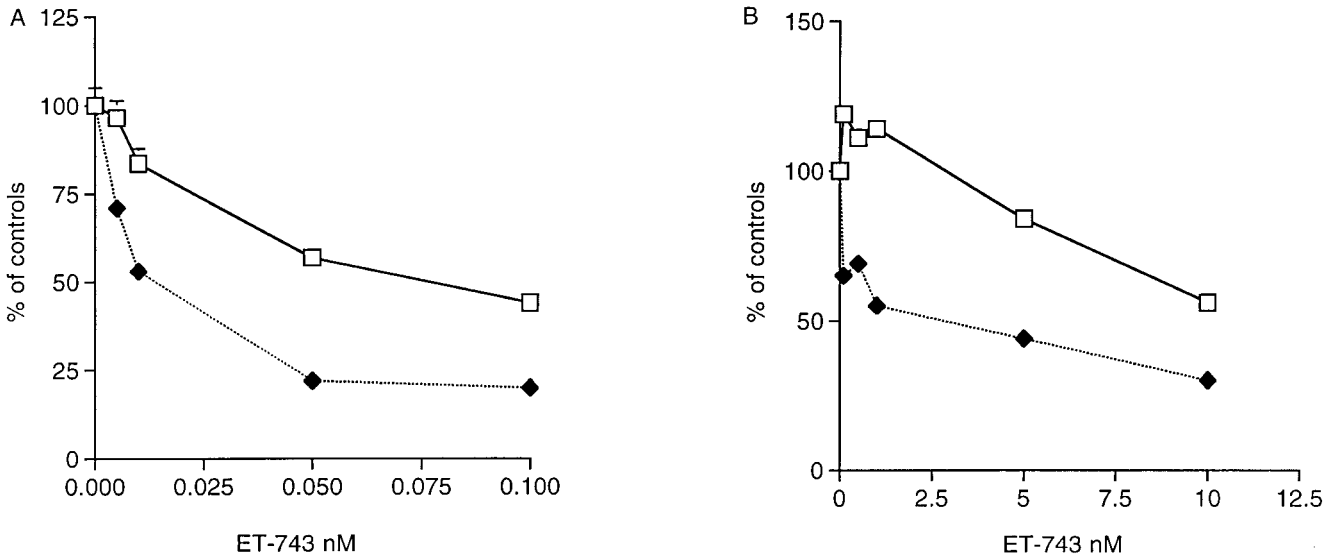

Figure 4 – *(a)* Survival curves of MO59K (DNA-PK–proficient glioblastoma cell line) (squares) and MO59J (DNA-PK–deficient glioblastoma cell line) (diamonds) after treatment with ET-743. *(b)* Survival curves of HCT-116 cells after treatment with ET-743 in the absence (squares) or presence (diamonds) of wortmannin. Experimental conditions detailed in Material and Methods.

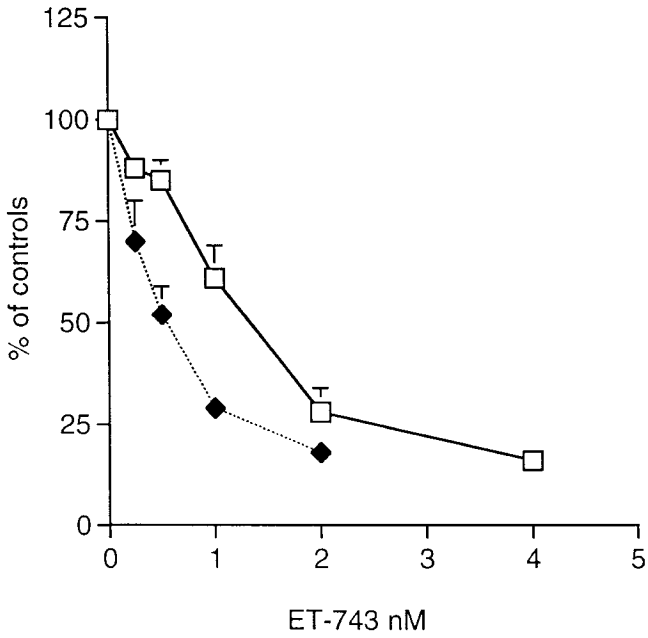

FIGURE 5 – Survival curves of the lymphoblastoid cell lines IARC1663 (ATM^+ cells) (squares) and AT-11 (ATM^- cells) (diamonds) after treatment with ET-743.

Although we do not have a clear-cut explanation for these results, the cause might be analogous to what has been proposed for MMR deficiency in resistance to some anti-cancer agents; *i.e.*, the resistance to DNA-damaging agents, mainly methylating agents and DDP in MMR-deficient cell lines[15–17] is associated with the futile cycles that attempt to repair the base-pairing DNA anomalies induced by these agents. Such repair is, however, directed to the newly synthesized DNA strand, leaving the lesions on the parental strand unexcised, resulting in the formation of gaps and breaks that ultimately induce cell death. NER-deficient cell lines would be more resistant to ET-743 treatment because they are unable to recognize and process the DNA damage induced by the drug and, therefore, are able to survive. On the contrary, in NER-proficient cells, the recognition and processing of ET-743-induced DNA damage would activate death signals. Restoring NER activity by transfection of the *ERCC1* gene in UV96 cells, which lack a functional ERCC1, indeed sensitized cells to ET-743. The fact that ET-743 adducts are recognized and incised by the UvrABC repair proteins, though with an unexpected pattern of recognition and a higher degree of incision observed in the DNA sequences weakly reactive toward ET-743 than the highly reactive sequences,[18] is consistent with the former hypothesis. However, it is possible that the ET-743-induced changes in DNA structure modify the recognition by proteins involved in NER. In this regard, ET-743 is able not only to block the *in vitro* interaction of the transcription factor NF-Y with its cognate binding site[19] but also to affect the regulation of transcription by inhibiting the normal formation of complexes between NF-Y and the CAAT box.[20]

A defect in DNA-PK activity causes sensitivity to ionizing radiation[21] and to alkylating agents, such as chlorambucil,[22] in relation to a defect in repairing DNA double-strand breaks.[23] Although we were unable to detect any single- or double-strand breaks by alkaline elution in cells treated with cytotoxic ET-743 concentrations,[9] our results show a 2-fold increased sensitivity in cells lacking DNA-PK activity compared to DNA-PK–proficient cells. Moreover, in an experimental setting in which DNA-PK activity was almost completely blocked by wortmannin treatment, a 2-fold decrease in the ET-743 IC_{50} value was observed. This increased sensitivity to ET-743 in a system lacking functional DNA-PK activity might be related to the role of DNA-PK systems in DNA damage-signaling pathways. DNA-PK is a member of the PI3-kinase family; and together with other members of the same family, such as ATM (the protein deficient in the human neurodegenerative and cancer predisposition condition ataxia telangiectasia) and the human AT-related (ATR/FRAP-related) protein, it has been implicated in controlling transcription, the cell cycle and/or genomic instability in organisms ranging from yeast to humans.[23–25] DNA-PK has been implicated in the DNA damage signal-transduction pathway, being able to phosphorylate *in vitro* p53[26] and *in vivo* Replication protein A.[27] A similar degree of ET-743-increased sensitivity (2-fold) has been observed in ATM null cells. Although it is well documented that ATM^- cells are hypersensitive to γ-radiation and some anti-tumor agents, *e.g.*, bleomycin, DDP and topoisomerase II inhibitors,[28–30] the exact mechanims underlying these enhanced sensitivities (specific defect in some DNA-repair pathways or defects in checkpoints) remain under study. Indeed, the role of ATM as a central signaling protein in the cellular response to DNA damage and the connection between its checkpoint function and DNA repair are just beginning to be understood. It might well be that ET-743 interferes with both roles of ATM (checkpoint and repair); this would account for the enhanced sensitivity observed in AT^- cells.

The unusual pattern of sensitivity to ET-743 observed in cells defective in NER emphasizes the fact that ET-743 represents a new class of anti-cancer agent able to interact with DNA in a way different from that observed with other alkylating agents, and this might justify its striking anti-tumor activity in pre-clinical models and in clinical trials against tumors that are not sensitive to the available DNA-interacting anti-cancer drugs.

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