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Molecular characterisation of two human cancer cell lines selected *in vitro* for their chemotherapeutic drug resistance to ET-743

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Abstract

ET-743 (Yondelis™, Trabectedin) isolated from the tunicate *Ecteinascidia turbinata*, is being tested in phase II clinical trials in Europe and the United States of America (USA). Studies with different solid tumours have shown antitumour activity in advanced, pre-treated sarcomas as well as in drug-resistant breast and ovarian cancer. The primary mechanism of action for ET-743 has not been fully elucidated and different models have been suggested to explain its molecular mechanism of action. ET-743 binds tightly to the minor groove of DNA and previous data have suggested that ET-743 acts by interfering with RNA transcription. To further investigate the mechanism of *in vitro* drug resistance, we evaluated the gene expression profile in ovarian and chondrosarcoma cell lines selected for resistance to ET-743. We found 70 genes whose expression was modulated in both drug-resistant cell lines when compared with their respective parental drug-sensitive cell lines. This pattern of gene expression seems to be selective for ET-743-resistant cells, since ovarian cancer cells resistant to paclitaxel did not share the same gene expression changes. Data presented in this study reveal different molecular pathways that could be involved in the cellular mechanism of ET-743 resistance.

Keywords: Microarrays; ET-743; Drug-resistant cells

1. Introduction

ET-743 (Yondelis™, Trabectedin) is a marine-derived antitumour agent isolated from the sea squirt *Ecteinascidia turbinata*, a tunicate that grows on the mangrove roots throughout the Caribbean sea [1,2]. ET-743 has shown promising pre-clinical activity against different cancer cell lines *in vitro* and transplantable *in vivo* human tumour xenografts [3–6]. Phase II clinical trials of ET-743 in Europe and United States of America (USA) are ongoing, after encouraging phase I results in which clinical responses were observed in

Abbreviations: DDH, dihydrodiol dehydrogenase gene; cDDP, cis-diamminedichloroplatinum; GLUL, glutamine synthase; GST, glutathione *S*-transferase; NER, nucleotide excision repair; XPG, *Xeroderma pigmentosum* group G; DMSO, dimethylsulphoxide; DX, doxorubicin; LC–MS/MS, liquid chromatography-coupled mass spectrometry; LOXL1, Lysyl oxidase-like 1; HPLC, high-pressure liquid chromatography; IC_{50}, the mean concentration causing 50% growth inhibition compared with control cells; IMPD1, (inosine monophosphate) dehydrogenase 1; MDR, multi-drug resistance; NOTCH3, Notch homologue 3; PYCR, pyrroline 5 carboxylate reductase-1; PKN, protein kinase C-like 1; VBP-1, Von Hippel–Lindau binding protein-1.

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different human tumours, such as soft tissue sarcomas, endometrium, breast and ovarian cancer [7–10]. The mechanism of action for ET-743 is not well understood, but it exhibits a unique activity profile characterised in the National Cancer Institute (NCI) panel of 60 human cancer cell lines [11]. Based on various studies, DNA may be the primary target of ET-743 [2]. In fact, ET-743 can bind to the minor groove of DNA with some degree of sequence specificity, but, unlike the conventional minor groove binding agents, the bond between the drug and the DNA can be reversed upon denaturation [12]. No single-strand breaks, double-strand breaks or DNA protein crosslinks could be found by alkaline elution in cells after ET-743 treatment [1,13], while only at micromolar concentrations (i.e., 100-fold higher than the IC_{50}) is ET-743 able to trap DNA topoisomerase I [14]. At physiologically relevant nanomolar concentrations, ET-743 has been found to be a specific transcriptional interfering agent [15,16]. We have reported that while the DNA mismatch repair system did not affect the cytotoxic activity of ET-743 (in contrast with methylating agents or cisplatin), nucleotide excision repair (NER)-deficient cell lines are less sensitive to ET-743 when compared with the proficient ones [13]. A novel model has been proposed to explain ET-743 activity such that the compound interacts with transcription coupled NER machinery to produce lethal DNA strand breaks. Moreover, XPG gene inactivating mutations have also been correlated with ET-743 activity [17].

In the present study, we used two different human cancer cell lines that are sensitive to ET-743 treatment, ovarian Igrov-1 and the chondrosarcoma cell line CS-1, along with two independent sublines derived from these parent cell lines that display resistance to ET-743 treatment. Employing microarray-based gene expression analysis, we investigated potential gene alterations in these cell lines that might be relevant in ET-743 resistance.

2. Materials and methods

2.1. Cell lines and drug treatments

ET-743 was prepared as a 1 mg/ml (i.e., 1.3 mM) stock solution dissolved in dimethylsulphoxide (DMSO), stored at −20 °C and diluted freshly in medium to the desired final concentration just before use. Cisplatin (cDDP) and doxorubicin (DX) were dissolved in fresh medium just before use. ET-729, a naturally occurring ecteinascidin, was prepared as a 1 mg/ml stock solution dissolved in methanol and diluted immediately before use.

Igrov-1 is a human ovarian cancer cell line, while CS-1 is a chondrosarcoma cell line established *in vitro* from a surgically resected human high-grade chondrosarcoma that was not previously exposed to chemotherapy or radiation therapy [18]. The human ovarian Igrov-1 and Igrov/ET/PSC, the 1A9 and 1A9/cl22 as well as the chondrosarcoma CS-1 and CS-1R cancer cell lines were grown as monolayers in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% foetal calf serum v/v (serum inactivated for CS-1 and CS-R), supplemented with 1% glutamine v/v (200 mM) at 37 °C in a humidified 5% carbon dioxide atmosphere. The Igrov/ET/PSC cancer cell line is a subline of Igrov-1 and kindly provided to us by Dr. Eugenio Erba (Mario Negri Institute, Milan, Italy). It was obtained by stepwise increases in ET-743 concentrations from 1 to 100 nM in the presence of 1 μM of PSC-833, a P-gp-reversing drug agent, structurally similar to cyclosporin (Novartis Basel, Switzerland). The ovarian cancer Igrov/ET/PSC cells were 5-fold resistant to ET-743 compared with the parental cell line. Resistance was determined by clonogenic assay and the cell line was found to be stable for at least 20 passages in the absence of drug treatment.

To investigate drug effects on cell growth, cells growing *in vitro* were seeded in 96-well plates as above at a concentration close to 2×10^4 cells/ml, and allowed to attach for at least 48 h. A concentration range of drug was added into six replicate wells. Treatment was performed for 1 h with cDDP and ET-743 and for 2 h with DX. After treatment, cells were washed with ice-cold sterile phosphate-buffer solution (PBS) and allowed to recover in drug-free medium for 72 h. Control untreated cells were incubated with an equivalent volume of fresh medium. Growth inhibition was calculated using the MTT assay (Sigma chemical Co., St. Louis, MO), as a percentage of cell number in drug-free control cultures [19]. The mean concentration causing 50% growth inhibition compared with the control cells (IC_{50}) was determined for each drug from at least three separate experiments.

2.2. Intracellular drug uptake

Intracellular concentrations of ET-743 were assessed by adding 10 ng/ml of ET-729 as an internal standard to the pellets of cells treated with ET-743. Cells were then extracted with 1 ml of 0.1 M hydrochloric acid in methanol. A calibration curve was plotted by adding increasing amounts of ET-743 to untreated cells in order to produce concentrations of 0.5–25 ng/ml for the drug.

After centrifugation at 1400 rev/min for 5 min, the supernatants were removed and evaporated to dryness under a nitrogen stream. Before the injection of 20 μl into the liquid chromatography–coupled mass spectrometry (LC–MS/MS) system, the reconstituted samples passed over a PTFE filter. ET-743 was measured by high-pressure liquid chromatography (HPLC) coupled with electrospray ionisation tandem mass spectrometry using an API 3000 triple quadrupole mass-spectrometer (Applied Biosystems-Sciex, Toronto, Canada) operating in positive ion mode with a standard

TurboIonSpray source. Separation was achieved on a Luna C18 column (Phenomenex, Torrance, CA), consisting of a mobile phase of 0.1% acetic acid v/v in water (solvent A) and acetonitrile (solvent B) with gradient elution using solvent B from 10% to 70% at a flow rate of 200 μl/min.

2.3. Gene expression analyses

Total RNA from sensitive and drug-resistant cells was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD) following the manufacturer's protocol. For each cell line, three independent experiments were performed and the total RNA was pooled together before mRNA purification with oligo$(dt)_{25}$ Dynabeads (Dynal, Oslo, Norway). Microarray slides were purchased from NEN Life Sciences (Micromax Human cDNA System I-Direct MPS102 and MPS122). Both slides contain 2400 human cDNAs, but in the MPS122 system, the cDNAs are spotted in duplicate on each array. Cy3 and Cy5 cDNA (direct) labelling, hybridisation and washing were performed according to the instructions provided by the vendors. Cy3-labelled samples were compared with their Cy5-labelled controls. Labels were swapped in order to validate the data.

2.4. Array quantisation and data processing

Following hybridisation, arrays were scanned using a laser-scanner Axon 4000-B (Axon Instruments, Union City, CA) using set-up photo-multipliers parameters that best defined the signal-intensity-weighted 'average' spot on each array to have a Cy3/Cy5 ratio of 1.0. All arrays experiments were performed in duplicate with dyes swapping and employing separate reverse transcriptions, in order to increase the robustness of our data. Separate images were acquired for Cy3 and Cy5. We carried out data reduction with the program *GenePix 4.1* software and data analysis with the "R" software (http://cran.au.r-project.org/). Each spot was defined by automatic positioning of a grid of circles over the array image. For each fluorescent image, the average pixel intensity within each circle was determined, and a local background was computed for each spot. The background value was equal to the median pixel intensity on the spot, excluding all spots without a well-defined shape and that had a major local background signal in the third quintile of the global background density distribution. Net signal was determined by subtraction of this local background from the average intensity for each spot. Spots deemed unsuitable for accurate quantisation because of array artefacts were manually flagged and excluded from further analysis. Raw data files generated by *GenePix 4.1* were entered into a custom database that maintains web-accessible files in agreement with MIAMI rules [20] (internet address: ftp://ftp.marionegri.it/download/european_journal_of_cancer/ or ftp://ftp.marionegri.it/anonymous/dowload/european_journal_of_cancer/).

The logarithms of the measured fluorescence ratio of reliable spots were analysed using the M vs. A plot [21] and the *Lowess smoothing functions algorithm* [22]. The log-ratio for each gene was normalised by subtracting the correspondent *Lowess* M-value computed.

In order to evaluate which genes have modified their expression levels, an intensity-dependent *Z-score* calculation was performed for each microarray dataset [23]. In particular, those genes that had at least a *Z-score* value >1 and <-1 were selected for further analysis; i.e., genes that lie over or below a standard deviation from the mean of a supposed local normal distribution.

Furthermore, in order to reveal correlations between the genes selected with the *Z-score* criteria, we performed a clustering procedure. In particular, we have used the agglomerative hierarchical clustering algorithm of the *Cluster* software, with the choice of adopting an average linkage clustering rule. The *Cluster* software implements hierarchical clustering methods that are described in Eisen and colleagues [24]. The endpoint of the clustering process is a dendrogram, in which short branches connect very similar elements, and longer branches join elements with diminishing degrees of similarity. In the cluster-ordered data table each graded cell colour (pure red through black to pure green) represent the mean-adjusted *Z-score* value of each gene.

2.5. Real-time RT-PCR

Total RNA was purified from cell culture with the Trizol protocol (Invitrogen, Life technologies, Inc.) followed by DNAse treatment (Celbio, Milan, Italy). Two hundred nanograms were reverse-transcribed in 20 μl with TaqMan Reverse Transcription Kit (Applied Biosystems, UK) and 2 μl further amplified by Real-time polymerase chain reacter (PCR) using the Syber green-based amplification protocol. As internal controls for data normalisation, actin for chondrosarcoma model and tubulin for the ovarian cancer model were used. Primers were designed using the Primer Express 2 software (Applied Biosystem) and their sequences are reported in Table 1.

3. Results

We initially tested the *in vitro* sensitivity of each cell line towards ET-743. The data reported in Table 2 are the mean of at least three different experiments, each consisting of six replicates. Ovarian cancer cell line 1A9 and its sub-clone, 1A9/cl22, selected for *in vitro* non-MDR-related resistance to paclitaxel, were used as controls [25]. Cell lines selected for resistance to

Table 1
List of primers used in real-time reverse transcriptase-polymerase chain reaction (RT-PCR) to confirm differences in gene expression analysis

Gene name (Accession number)	Forward primer	Reverse primer
VBP-1 (U96759)	agcttcagttcctcctaccgataa	gtcttcctccagggaatcaagat
RHO E (S82240)	acatgcctagcagaccagaactct	tcttcgctttgtcctttcgtaagt
RAC-β (M77198)	tccgaggtcgacacaaggtact	gatggaactgggcggtaaattc
LOXL1 (L21186)	cgtggtgagatgcaacattca	tcccggagatcaggattgg
MM-1 (S76730)	cggtggatgcggtacagaa	gccccgtgtcgttgagagt
IMPDH1 (J05272)	tgagtggtccacagatttgca	ccctcctctctgcctggaa
GLUL (Y00387)	tggccaggagaagaagggtta	cgccggtttcattgagaaga
PYCR (M77836)	caggccagctcaaggacaac	catgcaaggcatggatggt
NOTCH3 (U97669)	cccccaagaggcaagtgtt	cccaagatctaagaactgacga
PKN (D26181)	cggaccacgggtgacatatc	ccgagtcagtgccgaggtt
Gravin (AB003476)	gaggaggcagtatgcaccaaa	gcagccgctgttagagtgaat
DHD (M86609)	gagcagcgcatcagacagaac	tctgaagtcaactggaattcaaa
Tubulin (X02344)	acccagacaggatcatgaa	cgtagagagcttcgttatcaatg
Actin beta (NM_001101)	tcacccacactgtgcccatctac	cagcggaaccgcccattgccaat

I.R., is the IC_{50} ratio after 72 h of recovery in drug-free medium between the resistant and sensitive cells. Data are the mean of three different experiments, each consisting of six replicates.

Table 2
The mean concentration causing 50% growth inhibition compared with control cells (IC_{50}) values reported for the three different cell lines and their resistant sub-clones after different drug exposure

	CS-1	CS-1R	I.R.	Igrov-1	Igrov/ET/PSC	I.R.	1A9	1A9/cl 22	I.R.
ET-743 (nM)	23.84	112.42	**4.71**	18.9	**102.33**	**5.4**	3.74	**4.22**	**1.12**
cDDP	36	77	**2.15**	99.4	81.45	**0.8**	nt	nt	
Dx (mM)	1	1	1	2.2	2.4	**1**	nt	nt	
Paclitaxel	nt	nt		nt	nt		9.3	66.7	**7**

For each selected gene, the GenBank Accession No. is given.

ET-743, the chondrosarcoma CS-1R and the ovarian Igrov/ET/PSC cell lines, share roughly the same fold of resistance when compared with their parental counterpart CS-1 and Igrov-1 cell lines (I.R. 4.7 and 5.4, respectively) with an IC_{50} value after 72 h of recovery in drug-free medium within the range of 20 nM for the sensitive and 100 nM for the resistant cells (i.e., 18.9 and 102.3 nM for Igrov and Igrov/PSC/ET and 23.8 and 112.4 nM for CS-1 and CS-1R cells, respectively). Neither cell line was cross-resistant towards doxorubicin; CS-1R was slightly resistant to cDDP (I.R. 2.15), but this effect was not observable in the Igrov-1 pair of cell lines. 1A9/cl22 cells displayed 7-fold more resistance to paclitaxel than 1A9 parental cells, with no cross-resistance to ET-743 (I.R.1.12, Table 2).

By mass spectrometry analysis, we compared the intracellular ET-743 uptake in both sensitive and resistant cells after 1, 2, or 6 h of ET-743 treatment. Data reported in Fig. 1 clearly show that in both the CS-1R and in the Igrov/ET/PSC cells, the intracellular ET-743 uptake is comparable to that measured in their drug-sensitive cell counterparts. These data have been confirmed at ET-743 concentrations close to the IC_{50} values measured in the different cell lines.

Having found that the ET-743 resistance in these cells is not due to a different intracellular drug content, we performed a microarray analysis using the micromax NEN slides onto which 2400 different cDNAs had been previously spotted. Analysis was performed by comparing RNA extracted from sensitive and resistant cells in at least duplicate samples along with dye swapping to avoid false-positive results.

Of the selected genes with a Z-score value >1 or <−1, we found 692 genes whose expression was different between the Igrov/ET/PSC and Igrov-1 (345 upregulated and 347 downregulated in the resistant compared with the parental cells) and 312 whose expression were different between the CS-1R and the CS-1 cells (129 upregulated and 183 downregulated in the resistant compared with the parental cells) (Fig. 2). The Venn diagram in Fig. 2 shows that of the selected genes with differential expression, 70 were shared between the two cell pairs (21 upregulated and 49 downregulated in the resistant compared with the parental cells). We decided to focus our attention on these genes. Visualised Z-scores using the TreeView software, after applying hierarchical clustering following the Eisen protocol, are reported in Fig. 3.

The rows represent the different genes, while the columns correspond to the different cell types. Two distinct branches can be observed: the subset of genes downregulated in the resistant cells compared with the sensitive (green squares) and the subset of genes whose expression was upregulated in the resistant compared with their parental sensitive cells (red squares). Raw data are free-share available at the Fttp server (ftp://ftp.marionegri.it/download/european_journal_of_cancer/ or ftp://ftp.mar-

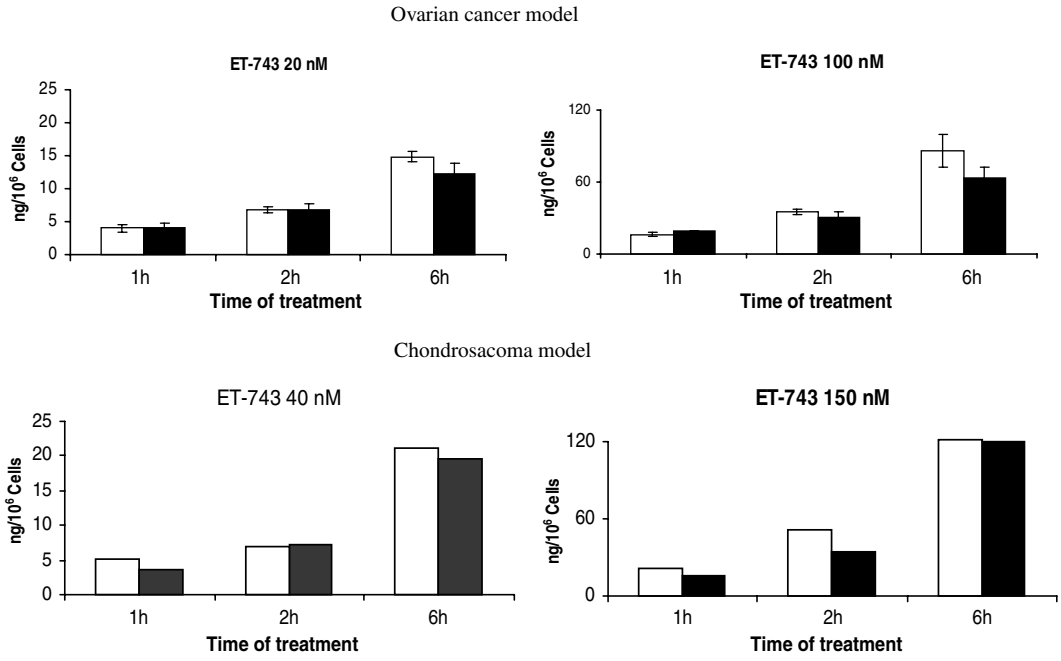

Fig. 1. Intracellular ET-743 uptake after different doses and times of incubation measured by mass spectrometric analysis. Upper panel, ovarian carcinoma cells system. Lower panel, chondrosarcoma cells system. □, ET-743-sensitive cells and ■, ET-743-resistant cells. Bars are ± standard deviation (SD).

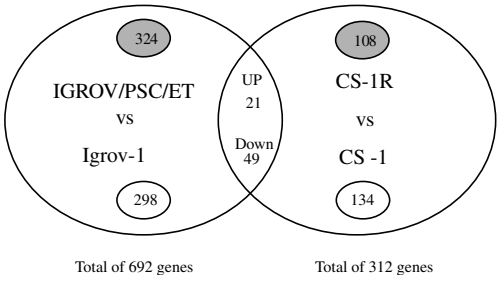

Fig. 2. Venn diagram of genes found modulated in the ET-743-resistant clones compared with their parental sensitive cells. Only genes that showed a *Z* score value of at least >1 or <−1 in the resistant compared with their sensitive counterpart were included in the analysis. The diagram shows that 21 and 49 genes were upregulated and downregulated, respectively, in both of the resistant clones. These genes are denoted as common genes. The total number of genes regulated in either the ovarian or chondrosarcoma resistant cell systems are shown in the lower part of the circle, whereas genes that passed the above cut-off in only one cell system are shown in the small circles (shaded, upregulated; open, downregulated).

ionegri.it/anonymous/dowload/european_journal_of_cancer/). By using the Source software, freeshare available online (http://genome-www5.stanford.edu/cgibin/source/sourceSearch), we functionally classified the selected 70 genes reported in Fig. 3. In Table 3, the complete classification of such genes, with their *Z-score* values in both of the two ET-743-resistant cell systems, is reported. Different classes have been identified, the most prominent of which is the class of downregulated genes coding for proteins involved in the regulation of the cellular architecture (such as *RhoE*, *Rac*-β, *type IV collagens* and *gamma actin*) and of cellular metabolism (i.e., the upregulated *dihydrodiol dehydrogenase*, *glutamine synthase*, as well as the downregulated *IMP dehydrogenase type 1*). Genes belonging to the signal transduction pathways (i.e., the upregulated *Rho GDP dissociation Inhibitor 2*, *XAP-4* mRNA for GDP-dissociation inhibitor as well as the downregulated *NOTCH* and novel protein kinase *PKN* genes) and some transcriptional factor regulators like *MM1*, upregulated, or the *Von Hippel–Lindau binding protein-1*, downregulated, were also found modified at the transcription level in the resistant compared with the sensitive cells.

To further support the validity of our microarray data analysis, we confirmed by real-time RT-PCR the differences in the gene expression profile for 12 of these selected genes. In Fig. 4, panel A, the *Z score* array values are compared with the relative fold changes obtained by real-time RT-PCR analysis. Data are the mean of at least three independent experiments, each consisting of three replicates. With the notable exception of the Von Hippel–Lindau binding protein (VBP-1) in the Igrov-1-Igrov/ET/PSC cell system, the differences in gene expression between sensitive and resistant cells obtained by microarray analysis were similar to the fold changes observed by real-time RT-PCR for all the selected genes. To determine whether the pattern of gene expression observed in ET-743-resistant cells was related to the mechanism of ET-743 drug resistance, we compared these results with those obtained in the 1A9/cl22 cell lines that lack resistance to ET-743, but are resistant to paclitaxel. Data reported in Fig. 4, panel B, show that

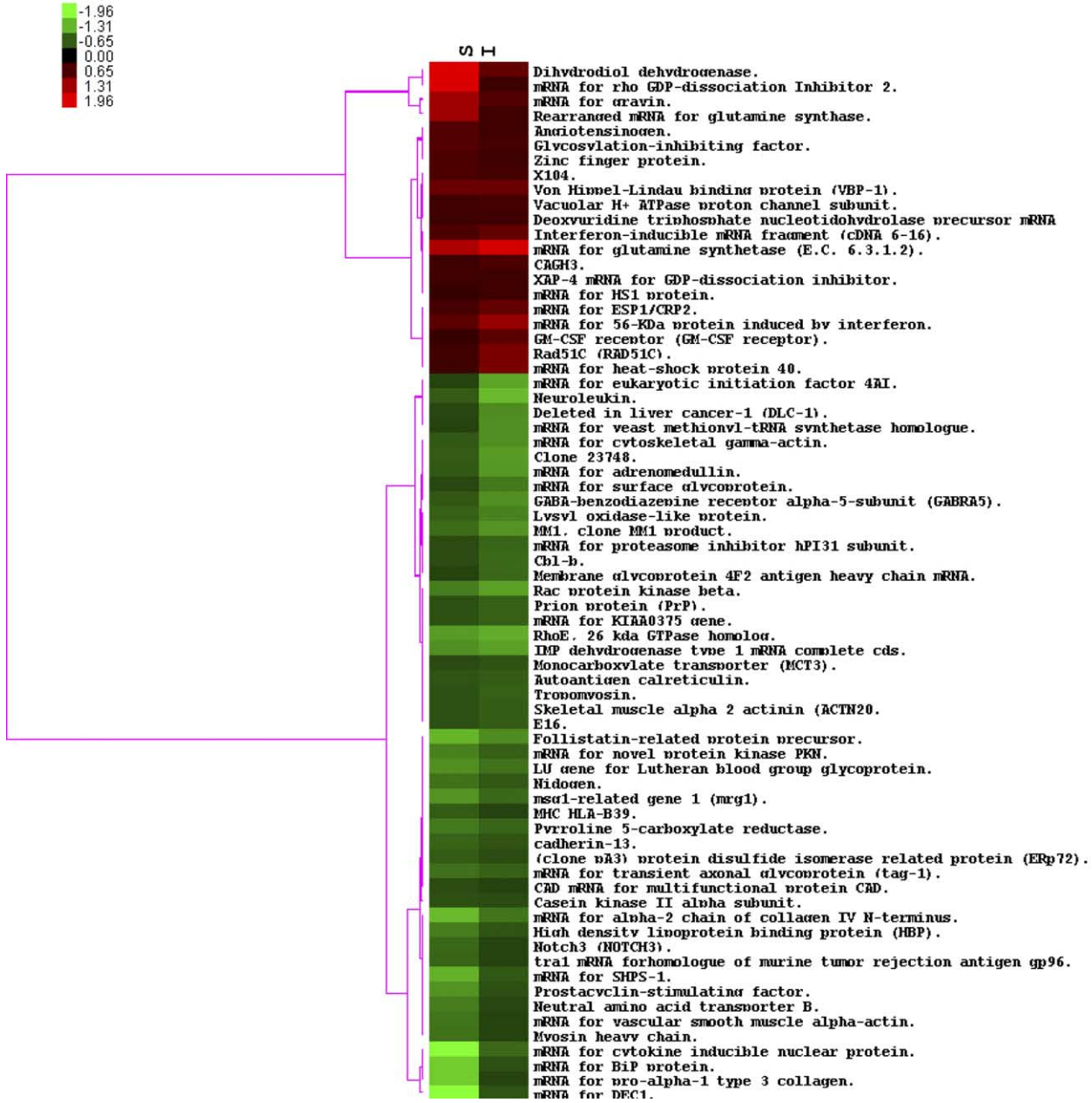

Fig. 3. A coloured representation of the data table, with the rows (genes) and columns (cell lines) in cluster order. The dendrogram represents the hierarchical relationships among the 70 common genes. The dendrogram indicates the degree of similarity between their expression profiles. The colour in each cell of this table reflects the mean-adjusted expression Z-*score* level of the gene in at least two experiments. The colour scale used to represent the expression ratios is shown. **S**, CS-1R vs CS-1. **I**, Igrov/ET/PSC vs Igrov-1.

apart from the *VBP-1* gene, changes in gene expression analysis observed in the ET-743-resistant cellular systems were absent or opposite in the paclitaxel-resistant cells.

4. Discussion

The search for naturally occurring marine compounds as potential new anticancer agents has yielded promising candidates [26,27]. The tetrahydroisoquinoline alkaloid ET-743 is among the most advanced in terms of clinical development [28]. Phase I and II clinical trials have shown efficacy of ET-743 in treating different solid human malignancies, in particular towards ovarian cancer and sarcomas that often respond poorly to standard chemotherapies [29–32]. However, as with other chemotherapeutics, the propensity of tumour cells to develop drug resistance poses a significant challenge for administering ET-743 over an extended period of time

Table 3
A summary of the genes shared in common by the two ET-743 cell systems and classified according to the Source software

ID	Name	*Z* score sarcomi	*Z* score Igrov
Structural and structural regulator codifing genes			
L27476	X104.	1.32	1.22
AB003476	**mRNA for gravin**	**2.89**	**1.56**
S82240	**RhoE, 26 kDa GTPase hom**	**−2.39**	**−2.66**
M77198	**Rac protein kinase beta**	**−1.97**	**−2.49**
X04098	mRNA for cyt. γ actin	−1.32	−2.22
AF035119	DLC-1.	−1.07	−2.14
Z50022	Glycoprotein.	−1.10	−1.88
X05562	Alpha-2 chain of collagen IV	−2.88	−1.81
X67734	Tag-1	−1.77	−1.49
M19267	Tropomyosin	−1.22	−1.45
M86406	Skeletal muscle alpha 2 actinin (ACTN20)	−1.56	−1.26
L34058	Cadherin 13	−1.56	−1.26
X13839	Vascular smooth muscle alpha-action	−1.81	−1.03
M35250	Myosin heavy chain	−1.73	−1.03
X14420	mRNA for pro alpha-1 type 3 collagen	−3.11	−1.03
Metabolism-related codifing genes			
M86609	**Dihydrodiol dehydrogenase**	**10.38**	**2.14**
Y00387	**mRNA for glutamine synthetase (EC 6.3.1.2)**	**3.14**	**3.73**
X59834	Rearranged mRNA for glutamine synthetase	2.80	1.09
U90223	Deoxyuridine triphosphate nucleotidohydrolase precursor	1.07	1.08
K03515	**Neuroleukin**	**−1.42**	**−2.84**
J05272	**IMP dehydrogenase type 1**	**−2.20**	**−2.45**
D14874	**mRNA for adrenomedullin**	**−1.38**	**−2.36**
X94754	Methionyl-tRNA synthetase homologue	−1.04	−2.20
M77836	**Pyrroline 5-carboxylate reductase**	**−1.90**	**−1.55**
M64098	High-density lipoprotein binding protein (HBP)	−1.94	−1.29
S75725	Prostacyclin-stimulating factor	−2.27	−1.23
L21186	**Lysyl oxidase-like protein**	**−1.47**	**−1.99**
J05016	Disulphide isomerase	−1.44	−1.16
Signal transduction			
X69549	mRNA for rho GDP-dissocitation Inh.2	4.70	1.07
K02215	Angiotensinogen	1.50	1.13
X02492	Interf.-ind. mRNA fragment	1.32	1.77
X79353	XAP-4 for GDP-diss. inhib.	1.08	1.18
X57347	mRNA for HS1 protein	1.05	1.13
D26181	**mRNA for novel protein kinase PKN**	**−2.02**	**−1.49**
U97669	Notch3	−1.61	−1.05
Transcriptional factors			
U96759	**Von Hippel–Lindau binding protein (VBP-1)**	**1.90**	**1.86**
X83703	mRNA for cytokine inducible nuclear protein	−3.85	−1.64
U65093	msg1-related gene 1 (mrg1)	−2.29	−1.64
AB004066	mRNA for DEC1	−5.23	−1.26
S76730	**MM1, clone MM1 product**	**−1.68**	**−2.28**
DNA/RNA-associated proteins			
D42123	mRNA for ESP1/C	1.21	1.84
U68536	Zinc finger protein	1.39	1.14
D13748	mRNA for eukaryotic initiation factor 4AI	−1.02	−2.50
U26710	Cbl-b	−1.12	−1.64
Post-translational modifications			
AB002373	mRNA for KIAA0375 gene	−1.19	−1.45
M80244	E16	−1.22	−1.40
D86043	SHPS-1	−2.71	−1.36
M30269	Nidogen	−1.74	−1.35
U81800	Monocarboxylate transporter (MCT3)	−1.14	−1.27
L42024	MHC HLA-B39	−1.43	−1.00
Proteasome			
D88378	Proteasome inhibitor hPI31 subunit	−1.15	−1.56

(*continued on next page*)

Table 3 (*continued*)

ID	Name	*Z* score sarcomi	*Z* score Igrov
DNA Repair			
AF029669	Rad51C (RAD51C)	1.17	2.13
Cytokines and growth factors			
X03557	56-KDa prot.ind. by interf.	1.60	2.65
L10612	Glycos.-inhib. fact.	1.47	1.22
Transport			
M62762	Vacuolar H + ATPase proton	1.16	1.19
U53347	Neutral amino acid tran	−1.97	−1.07
Receptors			
M73832	GM-CSF receptor	1.03	1.66
L08485	GABAr	−1.35	−2.19
Protein secretion and chaperones			
D49547	heat-shock protein 40	1.15	2.17
X87949	mRNA for BiP protein	−3.11	−1.23
X15187	tra 1	−1.57	−1.02
Not yet classified			
U80747	CAGH3	1.13	1.39
U79294	Clone 23748	−1.42	−2.41
U06863	Follistatin-related protein precursor	−2.80	−2.11
X83425	LU gene	−2.23	−1.72
J02939	Membrane glycoprotein 4F2 antigen	−1.05	−1.58
M13899	Prion protein (PrP)	−1.23	−1.51
M84739	Autoantigen calreticulin	−1.31	−1.43
Transferases			
J02853	Casein kinase II alpha subunit	−1.23	−1.13
D78586	CAD	−1.16	−1.01

(http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch). Genes whose expression was confirmed by real-time RT-PCR are indicated in bold.

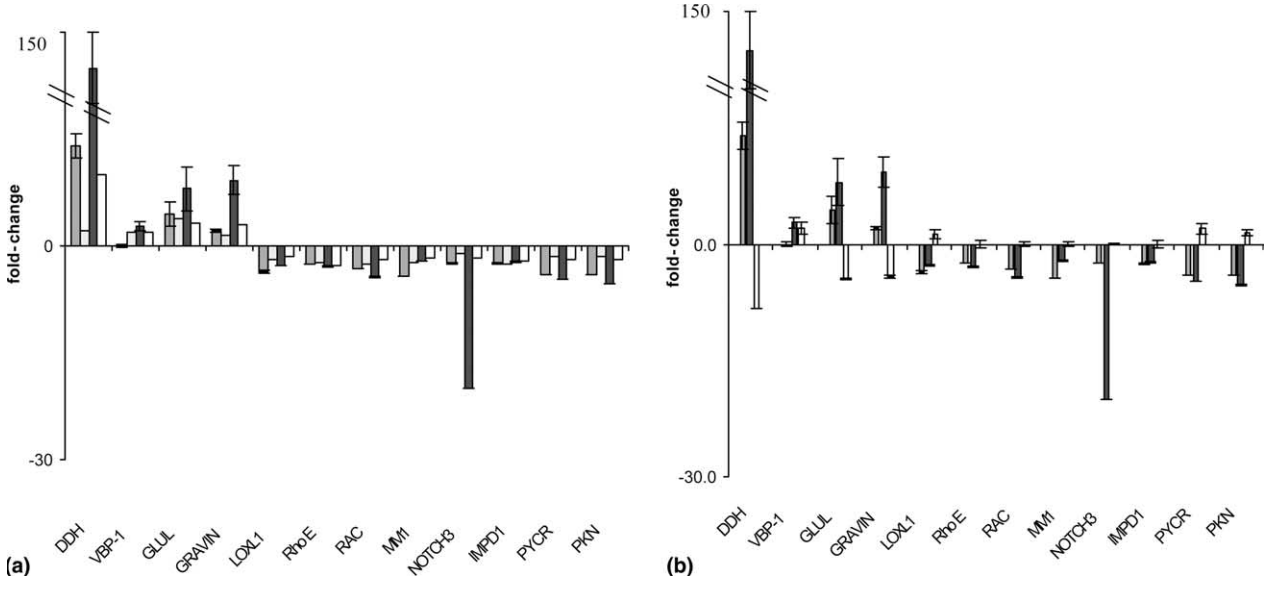

Fig. 4. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of certain genes found upregulated or downregulated by an array analysis. Panel A: For the two cell systems, the comparison between the array data ((▒), for Igrov/ET/PSC vs Igrov-1 and □ for CS-1R vs CS-1, respectively) and the real-time RT-PCR results ((■), for Igrov/ET/PSC vs Igrov-1 and ■ CS-1R vs CS-1, respectively) is reported. Panel B: Summarising table of real-time PCR results tested on Igrov/ET/PSC vs. Igrov–1 (■), CS-1R vs CS-1 (■) and 1A9 vs. 1A9/cl22 (□). Bars are ± S.D.

in cancer treatment. Identifying mechanisms of ET-743 drug resistance may shed light on pathways of drug resistance to other cancer drugs. Therefore, gene expression changes were compared for ovarian cancer cell lines, Igrov-1 and CS-1, with respect to that of their related drug-resistant sub-clones, Igrov-1/PSC/ET and CS-1R. Both ET-743-resistant cell clones showed a similar degree of resistance, an ideal situation to compare possible common mechanisms of resistance.

Multi-drug resistance (MDR) is a phenomenon displayed by many tumour types that allow them to withstand increasingly higher doses of cytotoxic drugs, particularly those of natural origin. Although extensive studies addressing mechanistic aspects of MDR in cancer cells have yielded valuable insights, MDR is still considered a significant obstacle for patients receiving chemotherapy [33–35]. However, as we previously reported, drug efflux alterations do not account for ET-743 drug resistance in the cell lines studied [36]. These ET-743-resistant cell lines are not cross-resistant to Dx, a drug typically susceptible to the MDR phenotype. The second reason is the finding that similar ET-743 intracellular levels occur in the sensitive and ET-743-resistant cells. Finally, we have previously reported that cell surface expression of P-glycoprotein and multi-drug resistance-related protein (MRP) is similar in CS-1 and CS-1R cells [18].

Previous studies have shown that different cancer cells lacking the full complement of NER enzymes are significantly less sensitive to ET-743. In particular, the absence of either the ERCC1 or ERCC3 repair enzymes yielded an ET-743-resistance phenotype. This is generally associated with collateral sensitivity to cDDP [17]. In our experimental conditions, only CS-1R showed a 2-fold of resistance to cDDP, while Igrov-1 was as sensitive as its resistant subclone. Since we were interested in identifying those molecular mechanisms responsible for ET-743 resistance shared in common by the two cell systems, we did not further investigate the NER status of the cell lines. Alternatively, we explored mechanisms that might be shared by the two cell systems for mediating ET-743 drug resistance.

Microarray analysis on a panel of 2400 cDNAs revealed multiple effects of prolonged exposure to ET-743. A subset of 70 genes were found to be consistently upregulated or downregulated between the two ET-743-resistant tumour cell lines. Changes in the gene expression profile of genes coding for transcription factors, cytoskeleton reorganisation enzymes, signal transduction proteins, and enzymes involved in cellular metabolism were similar between ovarian and chondrosarcoma models. When compared against an ovarian cancer cell line made resistant to a different natural compound, paclitaxel, these differences were not well correlated, thus supporting our hypothesis that observed changes were likely to be specific to ET-743 drug resistance rather than an aspect of a non-specific cellular defence mechanism(s).

The finding that genes involved in cytoskeleton dynamics such as *gamma actin*, *Rho E*, *Rac* or other small GTPases are strongly downregulated in the resistant compared with the sensitive clones is novel for ovarian cancer, but is in agreement with morphological observations previously reported by us for CS-1/CS-1R cells [18].

Short-term or long-term exposure to ET-743 clearly affects the actin and microtubule networks within the cytoskeleton, and may be important for tumour cell attachment to the extracellular matrix, cell motility and invasion [18,37]. Consistent with observations previously reported by us, we found that in the two cell systems analysed, the *alpha 2 chain of collagen IV* gene is downregulated in the resistant compared with the sensitive cell lines [18]. Since extracellular collagen matrix can exert dramatic effects on cell shape and cell proliferation, collagen expression and cytoskeleton dynamics may be connected to the mechanism of ET-743 drug resistance [38].

Among the other identified genes, most of them have never been previously related to ET-743 drug action. Interestingly, we found the *MM-1* gene downregulated in both of the two resistance cell models, a recently identified c-myc binding protein that antagonises the inhibitory effects of c-myc on p73α (but not p73β) *trans*-activating functions. MM1 binding to c-myc leads to stimulation of p73α-mediated transcription from some p53/p73-responsive promoters like BAX [39]. In our model, one could speculate that ET-743 resistance could be partially explained by the increased ability of c-myc protein to inhibit p73α growth suppression effects.

Another possible relevant gene was the *dihydrodiol dehydrogenase* gene (DDH), which was reported to be upregulated in ovarian cancer cell lines selected for *in vitro* cDDP resistance [40]. Clones overexpressing DDH gene following transfection acquire resistance to cDDP. We tested the same hypothesis in chondrosarcoma cells, but the data did not support a role for DDH alone in modulating resistance to ET-743 (data not shown), suggesting that overexpression of DDH may be a downstream event. As previously observed for other metabolic enzymes (i.e., GST), DDH plays a role in drug resistance as a part of a stimulated metabolic network. Thus, overexpression of DDH may be required, but not sufficient to account for the reduced toxicity of ET-743.

In conclusion, the microarray-based strategy presented herein may help in devising new molecular pathways for identifying the onset of chemoresistance in cancer cell lines and in cancer patients receiving ET-743-based chemotherapy. In particular, the identification of genes playing a potential role in ET-743 resistance in different cellular models could help in elucidating the mechanism of action of this promising anticancer agent.

Conflict of interest statement

None declared.

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