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Research Paper

Antitumor activity- and gene expression-based profiling of ecteinascidin Et 743 and phthalascidin Pt 650

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Abstract

Background: Ecteinascidin 743 (Et 743) is a potent antitumor marine alkaloid currently undergoing phase II clinical trials. The synthetic analog phthalascidin (Pt 650), a designed structural analog of Et 743 displays in vitro potency comparable to Et 743. In this study, we used a panel of 36 human cancer cell lines, flow cytometry and oligonucleotide microarrays to analyze further these two compounds in a parallel fashion with regard to both antitumor activity (phenotype) and gene expression (genotype) bases.

Results: The cancer panel experiment established that activity patterns of Et 743 and Pt 650 were essentially the same with their IC_{50} values ranging from pM to low nM. By means of flow cytometric cell cycle analysis using HCT116 cells, they were shown to disrupt S phase progression after a 12-h treatment at 2.0 nM, eventually resulting in the late S and G2/M accumulation at the 24-h time point. Array-based gene expression monitoring also demonstrated that the Et 743 and Pt 650 profiles were highly similar in two distinct cancer cell lines, HCT116 colon and MDA-MB-435 breast. Characteristic changes were observed in subsets of genes involved in DNA damage response, transcription and signal transduction. In HCT116 carrying the wild-type *p53* tumor suppressor gene, the up-regulation of several p53-responsive genes was evident. Furthermore, a subset of genes encoding DNA-binding proteins to specific promoter regions (e.g. the CCAAT box) was down-regulated in both cell lines, suggesting one potential mode of action of this series of antitumor agents.

Conclusion: A combination of gene expression analysis using oligonucleotide microarrays and flow cytometry confirms an earlier finding that Et 743 and Pt 650 have remarkably similar biological activities.

Keywords: Ecteinascidin 743 (Et 743); Phthalascidin (Pt 650); Human cancer cell line panel; Flow cytometry; Oligonucleotide microarray

1. Introduction

Ecteinascidin 743 (Et 743) is an exceedingly potent and rare marine-derived antitumor agent isolated from the tunicate *Ecteinascidia turbinate* [1]. Because of its remarkable preclinical activity against various cancer cell lines in vitro and against several human tumor xenografts in vivo, Et 743 has entered into clinical trials and has progressed to phase II studies in Europe and the United States [2–5]. To compensate for the scarcity of availability from the natural source, Et 743 has recently been synthesized by a process that is being applied for the supply of clinical samples [6]. By using similar synthetic approaches, a number of analogs have also been prepared for biological evaluation, resulting in the discovery of phthalascidin (Pt 650) that is comparable to Et 743 with respect to its in vitro antiproliferative potency (Fig. 1) [7–9].

Although many objective responses have been observed in clinical tumors including sarcoma, melanoma and breast carcinoma, the precise mechanism of action of Et 743 has yet to be fully understood. The compound alkylates the N2 position of guanine in the minor groove of duplex DNA with some sequence specificity for 5′-AGC, 5′-CGG, 5′-GGC, 5′-TGC, etc. [10,11], and thus bends DNA toward the major groove [12]. Poisoning of DNA topoisomerase I (topo I) was also reported by Takebayashi et al. [13] and by our group [7] independently. However, we clearly mentioned in the paper that the topo I inhibition seemed to be only an auxiliary effect incidental

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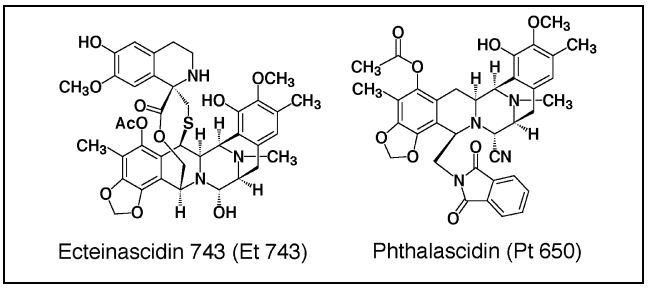

Fig. 1. Structures of ecteinascidin 743 (Et 743) and phthalascidin (Pt 650).

to the primary mechanism of action of Et 743 and Pt 650. This statement was based on the experimental facts that very high drug concentrations (μM range) were required for optimum DNA topo I cross-linking and that camptothecin (another topo I inhibitor)-resistant murine leukemia cells completely retained sensitivity to these two compounds. Consistent with our results, a more recent paper by Erba et al. described that pharmacologically reasonable concentrations of Et 743 (nM range) yielded neither DNA strand break nor DNA–protein cross-linking in sensitive cells [14]. Furthermore, Takebayashi et al. [15] and Damia et al. [16] reported that P388 CPT45 murine leukemia cells and CY2278 yeast cells, not expressing the *topo I* gene in both, were invariably sensitive to Et 743 while showing resistance to camptothecin. Therefore, all these findings indicate that topo I is not the primary target for the antiproliferative activity of Et 743.

Apart from the topo I poisoning, DNA binding of the CCAAT box transcription factor NF-Y was shown to be impaired by Et 743 in a cell-free in vitro experiment albeit at concentrations (10 and 30 μM) much higher than are sufficient for potent cytotoxicity [17]. Employing cell-based assay systems, two parallel studies by Jin et al. [18] and Minuzzo et al. [19] further revealed that Et 743 interfered with induced transcriptional activation in a CCAAT-dependent manner at physiologically relevant nM concentrations. According to their reports, the activation of the MDR1 and HSP70 promoters, both of which contain the CCAAT box, were significantly repressed by Et 743 with minimal inhibitory effects on constitutive expression of the *MDR1* and *HSP70* genes. These data suggest that Et 743 targets induce NF-Y-mediated transcription, distinct from other DNA-interacting anticancer drugs.

Based on the important observations described above, we decided to utilize oligonucleotide microarrays for characterizing transcriptional effects of Et 743 and Pt 650.

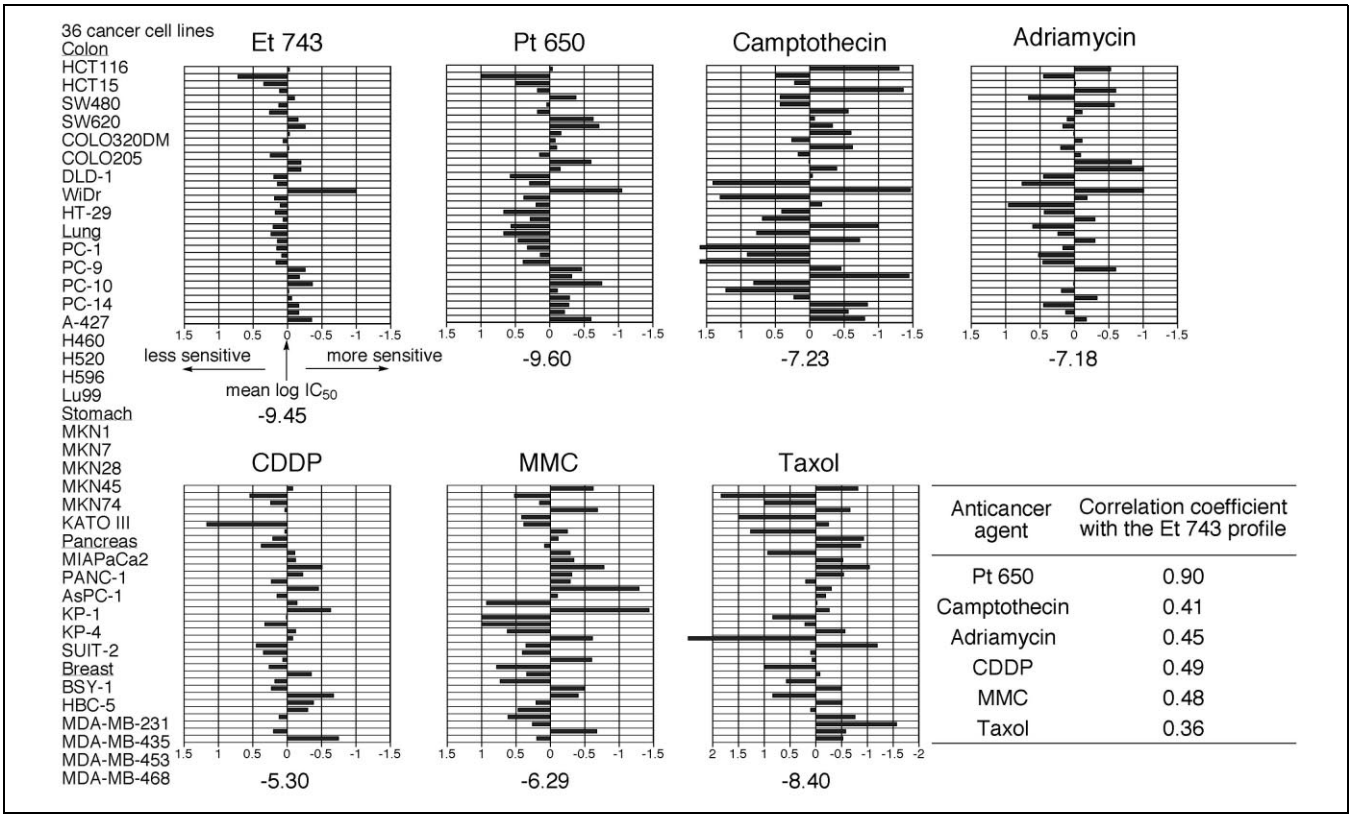

Fig. 2. IC_{50} mean graphs and COMPARE analysis. Based on the MTT assay data, the IC_{50} value of each test drug was determined by the least-squares method. The arithmetic mean of the log IC_{50} values for all 36 cell lines was then calculated for each compound. The delta value of each cell line, defined as the difference between the logarithm of the cell line IC_{50} and the mean, was also calculated. The mean graph was constructed by projecting bars to the right or left of the mean, depending on whether cell sensitivity to a compound was more or less than average. All graphs were illustrated with the length of a bar proportional to the delta value. The Pearson product moment correlation coefficients for each set of delta value pairs between two test compounds were determined by using a statistical program. The correlation data between Et 743 and other drugs are presented here.

Activity-based comparative profiling was also carried out with a panel of 36 human cancer cell lines and flow cytometry to determine appropriate experimental conditions (i.e. drug concentration and exposure time) for microarray analysis. Transcriptional profiling presented herein appears to be a powerful tool in examining the mechanistic basis of this class of transcription-targeting agents.

2. Results and discussion

2.1. Mean graph-based COMPARE analysis

By using a panel of 36 human cancer cell lines, the antiproliferative activities of Et 743 and Pt 650 were evaluated and compared with those of several well-known anticancer agents, i.e. camptothecin (topo I inhibitor), adriamycin (DNA-intercalating topo II inhibitor), cisplatin (CDDP, DNA-alkylating agent), mitomycin C (MMC, DNA-alkylating agent) and taxol (microtubule-stabilizing antimitotic agent). The results of 3-day MTT assays [20] are presented in Fig. 2 as mean graphs for the test drugs. Et 743 and Pt 650 demonstrated prominent growth inhibition potencies in the range of pM to low nM. The means of the logarithm of the IC_{50} values for these two were calculated at -9.45 and -9.60, respectively. Obviously, both compounds were orders of magnitude more potent than the other anticancer agents. Breast cancer cell lines tended, on average, to be more sensitive than colon, lung, stomach and pancreas to Et 743 and Pt 650. Pt 650 has been assessed as NSC701549 by the Developmental Therapeutic Program (DTP) of the NCI, with comparable results (data not shown).

The NCI has developed the COMPARE program with a set of 60 human cancer cell (NCI60) lines, in which mean graph 'fingerprints' are used to characterize the activity profiles of test drugs [21]. It has been shown that agents operating by a similar mechanism of action produce comparable antitumor spectra on the mean graph basis. Thus, the correlation coefficients between Et 743 and the other six compounds were determined by using the sets of delta values from their mean graphs. As expected, a high correlation was observed between Et 743 and Pt 650 ($r = 0.90$). In contrast, all the correlation coefficients between Et 743 and each of the other drugs were less than 0.5. These results suggest that Et 743 and Pt 650 share a primary mechanism different from those utilized by the well-defined anticancer agents, and that only simple DNA alkylation and topo I inhibition cannot account for their activity profiles.

The tumor suppressor protein p53 plays a central role in cell cycle arrest, DNA repair and cellular apoptosis in response to DNA damage [22]. It has been reported that the mutant p53 cell lines tend to be less sensitive than the wild-type p53 lines to a wide variety of DNA-alkylating agents, DNA/RNA antimetabolites, and topo I and II inhibitors [23]. Although the 36 human cancer cell lines used in this study include both cell types (e.g. HCT116 and NCI-H460 keep wild-type p53, while SW620, COLO205, HT-29, MDA-MB-231 and MDA-MB-435 contain mutated p53; [23]), the antiproliferative activities of Et 743 and Pt 650 appear to be unrelated to the p53 status. In a recent paper, Erba et al. also stated this point more evidently based on the observation that Et 743 exhibited very much the same cytotoxicity against isogenic cellular systems with different p53 expression [14]. It is an obvious merit for this series of antitumor agents because p53 is known to be altered in clinical tumors with a high frequency.

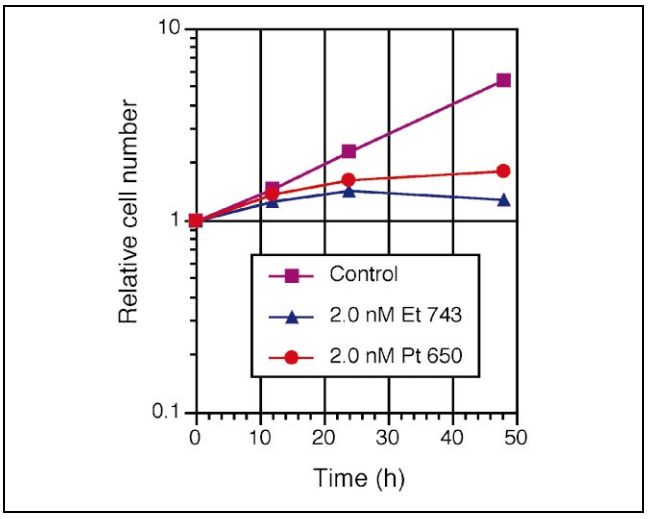

Fig. 3. Time-course profiles of the drug effect on HCT116 cell growth. The experiment was carried out in triplicate with each of Et 743 and Pt 650 at 2.0 nM. The initial cell number was 1.0×10^6 cells per well (2.0 ml). The relative cell numbers at time points of 0, 12, 24 and 48 h are plotted in a semi-logarithmic graph. The plotted values represent the means of three independent data.

2.2. Time-course profiles of the growth inhibition of Et 743 and Pt 650

From the results of 3-day MTT assays, the IC_{50} values of Et 743 and Pt 650 for HCT116 cells were estimated to be 0.32 and 0.23 nM, respectively. Thus, they were further tested at 2.0 nM to examine time-course profiles of their antiproliferative effects on the same cell line. As shown in Fig. 3, no significant differences in cell growth were found at the 12-h time point among cells untreated (control), Et 743-treated and Pt 650-treated. After 24 h of treatment, growth inhibition was evident for both compounds. At time points of 24 and 48 h, cellular apoptosis was detectable in both Et 743- and Pt 650-treated cells by nuclear staining with the Hoechst 33342 dye (data not shown).

2.3. Cell cycle analysis with Et 743 and Pt 650

By using flow cytometry, the effects of Et 743 and Pt

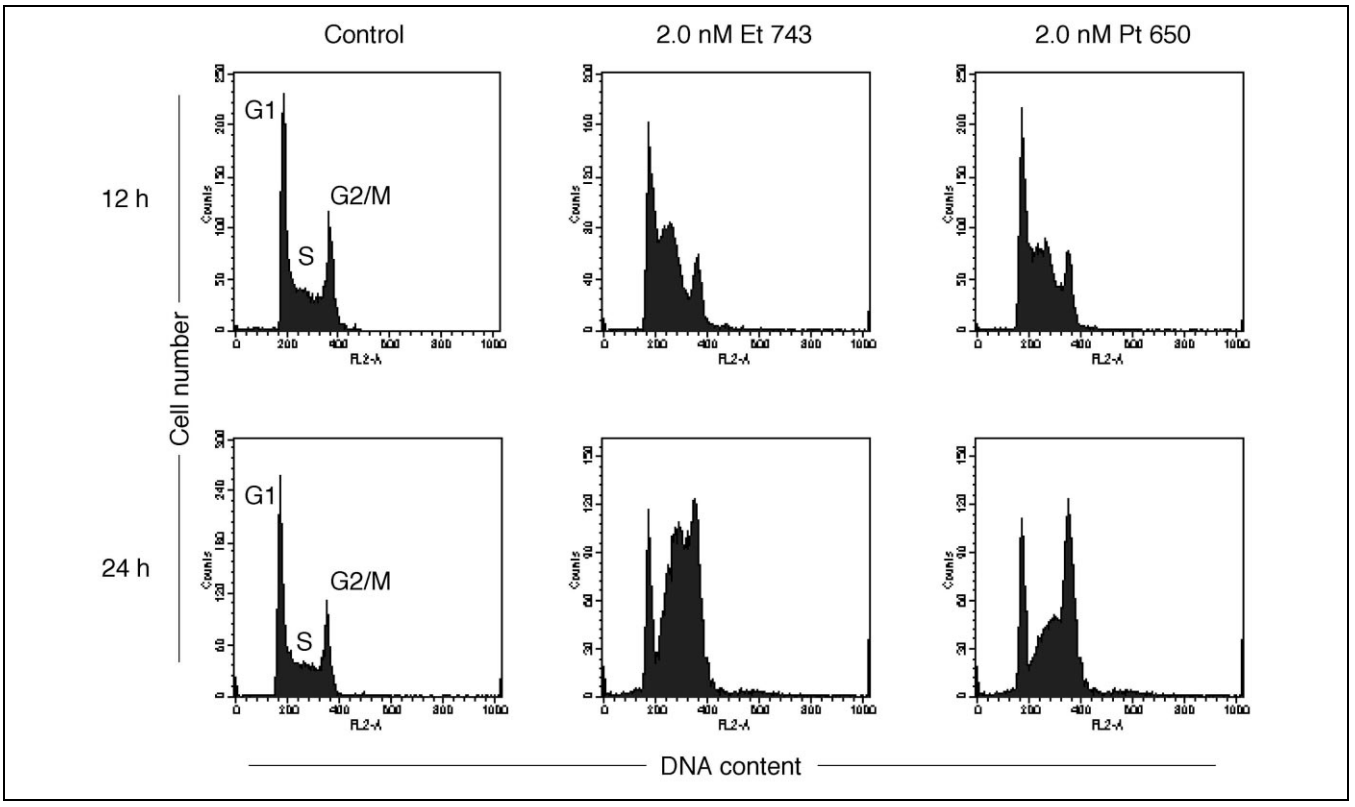

Fig. 4. Flow cytometric cell cycle analysis. The effects of Et 743 and Pt 650 on cell cycle progression were examined according to the procedure described in Section 4. HCT116 cells were treated with 2.0 nM of each compound for 12 or 24 h. DNA histograms from drug-treated and untreated cells are shown here.

650 on cell cycle progression were investigated. HCT116 cells were exposed to 2.0 nM of each compound for 12 or 24 h. As DNA histograms in Fig. 4 show, cell cycle perturbation in the S phase was evident for both compounds at the 12-h time point leading after 24 h to a conspicuous accumulation of the cells in the late S and G2/M phases. These observations are consistent with those presented by Erba et al. using 20 and 80 nM of Et 743 for SW620 and

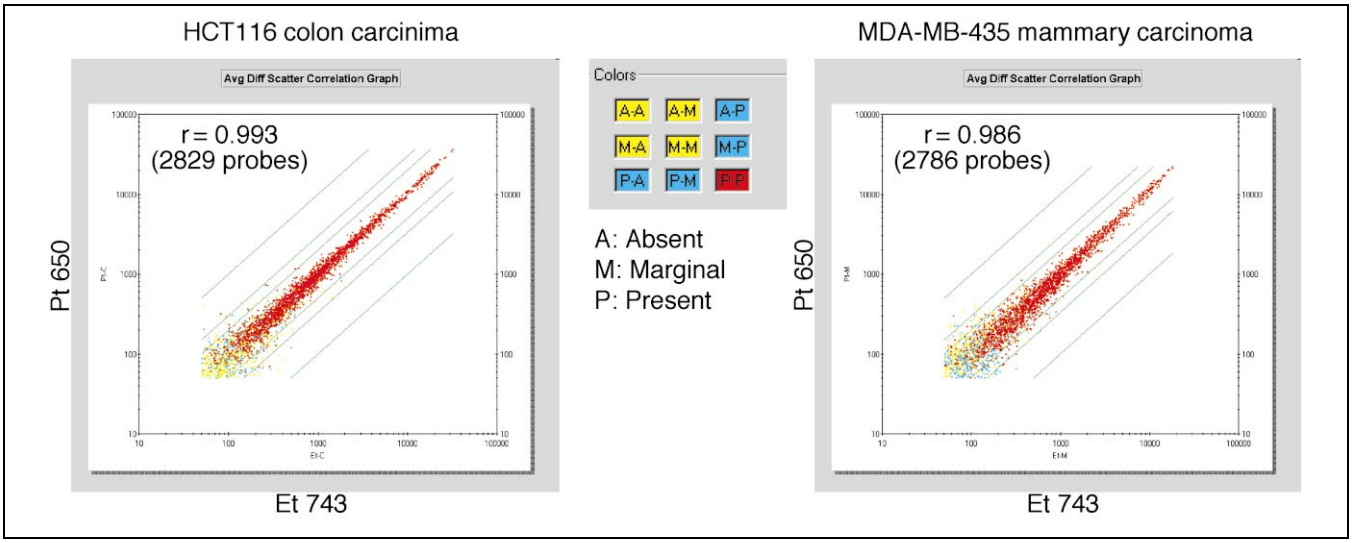

Fig. 5. Average difference scatter correlation graphs. The transcription profiles of Et 743 and Pt 650 were compared in two cancer cell lines HCT116 colon and MDA-MB-435 breast. Average difference change values for all gene probes were calculated as the means of two independent values from duplicate array experiments. The means more than 50 for both compounds before antibody amplification are plotted in scatter correlation graphs for HCT116 and MDA-MB-435. These were also used after eliminating the data sets of the genes absent (designated A) in both control and drug-treated cells, yielding the Pearson product moment correlation coefficients between Et 743 and Pt 650.

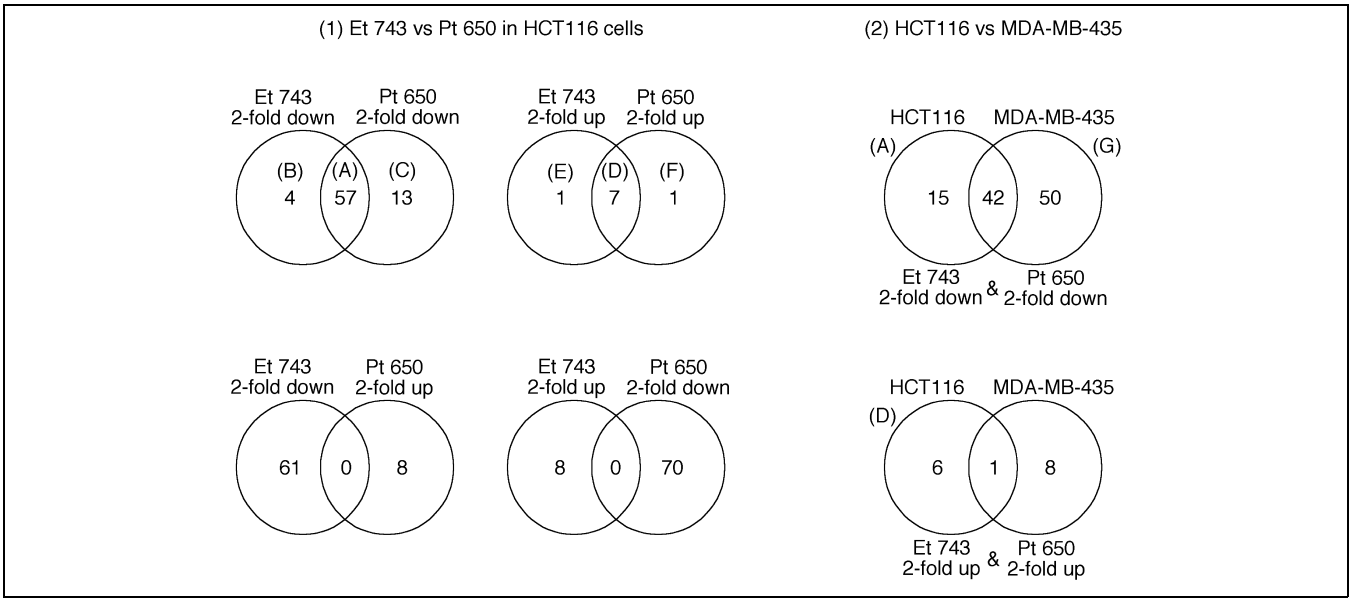

Fig. 6. Venn diagrams comparing sets of up- and down-regulated genes. In HCT116 colon and MDA-MB-435 breast cancer cells, induced or repressed genes with each of Et 743 and Pt 650 were determined based on the following criteria of the Affymetrix software algorithm: (i) fold change, ≥ 2.0 or ≤ -2.0; (ii) differential call, I (increase) or D (decrease); (iii) absence call, genes except those displaying A (absent) in both control and drug-treated cells; (iv) genes satisfying all of (i)–(iii) in the data set either before or after antibody amplification; and (v) genes satisfying all of (i)–(iv) in both data sets taken in duplicate.

LoVo cells, respectively [14] and by Takebayashi et al. [15] using 10 nM of Et 743 for HCT116 cells.

2.4. *Comparison of Et 743 and Pt 650 profiles on the gene expression basis*

In order to profile comparatively Et 743 and Pt 650, we investigated mRNA levels for more than 5000 different genes in sensitive cancer cell lines by using Affymetrix HuGeneFL oligonucleotide arrays. Based on the results of the phenotypic examinations described above, we fixed the experimental parameters for microarray analysis as follows: (i) cancer cell lines, HCT116 colon with wild-type p53 and MDA-MB-435 breast with mutated p53 and (ii) drug concentration and exposure time, 2.0 nM and 12 h for both compounds. The choice of these two cell lines was guided by their differences with respect to p53 status. The gene product of *p53* is known to act as a transcription factor induced by DNA damage, and thus its status, wild-type or mutated, can be influential in transcription profiles of DNA-interacting agents like Et 743 and Pt 650. In fact, Et 743 treatment at 20 nM for 6 h was found to elevate the levels of the p53 protein in LoVo cells carrying wild-type p53, but not in SW620 cells possessing mutated p53 [14]. The conditions for drug treatment (2.0 nM drug for 12 h) were chosen to avoid the complications of terminal expression changes resulting from apoptosis and to detect primarily the early effects of the test drugs on transcription.

Illustrated in Fig. 5 are average difference scatter correlation graphs for both HCT116 and MDA-MB-435 cell lines, facilitating the comparison of the Et 743 and Pt 650 profiles. After eliminating data points that were unreliable due to low signal intensity or high background, these graphs were prepared by using the GeneChip software (Affymetrix). For each gene, the mRNA expression level based on the average difference change value in Et 743 treatment is given on the x-axis and the level of the same gene in Pt 650 treatment is plotted on the y-axis. Obviously, the Et 743 and Pt 650 profiles are highly similar in both cell lines. The Pearson product moment correlation coefficients between these two data sets were calculated at 0.993 utilizing 2829 gene probes in HCT116 and at 0.986 utilizing 2786 gene probes in MDA-MB-435. The probe selection criteria for the statistical calculation are described in the legend to Fig. 5.

Shown in Fig. 6 are Venn diagrams representing the number of genes that changed expression levels at least two-fold in each of the Et 743 and Pt 650 data sets. The criteria for the gene selection are described in the Fig. 6 legend. Table 1 provides a list of the up- and down-regulated genes by drug treatment. Some important points in the comparison of the Et 743 and Pt 650 profiles are summarized below. First, the down-regulation of transcripts is predominant over the up-regulation in both HCT116 and MDA-MB-435. This observation appears to have some relation to those in recent reports arguing that Et 743 exerts negative effects on activated transcription not via widespread Pol II inhibition but via functional interference in some specific gene promoters like the CCAAT box [18,19]. Second, in the data set for HCT116, there is quite significant overlap between down-regulated genes in Et 743- and Pt 650-treated cells and also between up-regulated genes in Et 743- and Pt 650-treated ones. In contrast,

Table 1
Gene expression changes caused by Et 743 and Pt 650

Down-regulated genes

Accession no.	Fold change HCT116		Gene definition	Putative function	Fold change MDA-MB-435	
(A)	Et 743	Pt 650			Et 743	Pt 650
HG2167-HT2237	-3.2	-3.4	protein kinase Ht31, cAMP-dependent	signal transduction	-3.7	-5
HG3075-HT3236	-2.1	-2.7	focal adhesion kinase - also represents: L13616	signal transduction	-3.7	-5.9
U26424	-4.6	-8.6	Ste20-like kinase (MST2)	signal transduction	-7.4	-5.3
U33936	-2.7	-2.6	adenosine kinase	signal transduction	-3	-2.9
U50196	-2.8	-2.3	adenosine kinase	signal transduction	-2.5	-2.5
U88666	-2.2	-2.3	serine kinase SRPK2	signal transduction	-2.2	-3.7
L77886	-7.9	-8.4	protein tyrosine phosphatase	signal transduction	-8.2	-9.3
U46116	-2.2	-2.9	receptor tyrosine phosphatase gamma (PTPRG)	signal transduction	-7	-5.1
U40038	-4.3	-2.9	GTP-binding protein alpha q subunit (GNAQ)	signal transduction	-2.8	-4.1
U50078	-6.2	-4	guanine nucleotide exchange factor p532	signal transduction	-5.2	-5
U59919	-3.3	-2	Smg GDS-associated protein SMAP	signal transduction	-2.1	-2.7
HG511-HT511	-2.2	-4.4	ras inhibitor Inf	signal transduction	-4.2	-5.5
D26070	-2.1	-3.4	type 1 inositol 1,4,5-trisphosphate receptor	signal transduction	-3.6	-2.6
M97675	-3.2	-3	transmembrane receptor ror1	signal transduction	-2.2	-1.7
S62539	-2.5	-3.2	insulin receptor substrate-1	signal transduction	-1.7	-2.6
D50857	-3.5	-3.1	DOCK180	signal transduction	-3.8	-3.4
U68019	-6.2	-3.9	mad protein homolog (hMAD-3)	signal transduction	-2.4	-7.2
U22055	-2.3	-2.4	100 kDa coactivator	transcription	-2.4	-2.1
U37146	-2	-3.2	silencing mediator of retinoid and thyroid hormone action (SMRT)	transcription	-2.2	-4.3
M74099	-4	-4.1	CCAAT displacement protein	transcription	-7.6	-7.1
HG3342-HT3519	-2	-2.3	Id1 - also represents: S78825	transcription	-1.2	-1.4
S78825	-3.6	-3.7	Id1 (Id1-b)=transcription regulator helix-loop-helix protein	transcription	AA	AA
L32832	-2.2	-2.3	zinc finger homeodomain protein ATBF1-A	transcription	-2.4	-2.4
AF005887	-2.5	-4.9	ATF family member ATF6	transcription, ER stress response	-2.7	-3.5
M83233	-3.6	-4.2	transcription factor HTF4A	transcription	-2.4	-4
M29971	-2.5	-4.3	6-O-methylguanine-DNA methyltransferase (MGMT)	DNA repair	AA	AA
U40622	-4.8	-3.9	XRCC4	DNA repair	-7	-6.1
L38616	-9.9	-8.7	brain and reproductive organ-expressed protein (BRE)	DNA damage response	-2.4	-1.7
X06745	-2.2	-3.8	DNA polymerase alpha-subunit	DNA replication	-2.5	-4.4
U28749	-2.2	-2.1	high-mobility group phosphoprotein isoform I-C (HMGIC)	chromatin structure	-1.8	-3
U49957	-5.7	-3.8	LIM protein (LPP), preferred fusion partner gene of HMGIC	cellular signaling, tumorigenesis	-5.1	-4.2
S79639	-3	-2.1	EXT1=putative tumour suppressor	tumour suppressor	-2.9	-4.5
U84388	-4.4	-3.7	death domain containing protein CRADD	apoptosis	-4.9	-4.4
U28042	-2	-2.7	DEAD box RNA helicase-like protein	ribosome assembly	-4.4	-3.9
Z34918	-3.1	-3.7	translation initiation factor eIF-4gamma	translation	-4.3	-4.3
D16688	-3	-2.4	LTG9/MLLT3	leukemogenesis	-4.6	-4.7
L13744	-2	-2.2	AF-9	leukemogenesis	-1.8	-1.7
L07956	-3.6	-3.9	1,4-alpha-glucan branching enzyme (HGBE)	glycogen metabolism	-3.3	-4.3
U43944	-2.2	-2.5	breast cancer cytosolic NADP(+)-dependent malic enzyme	metabolism	-2.3	-3.7
L40904	-3.2	-3.1	peroxisome proliferator activated receptor gamma	nuclear receptor	AA	AA
L38608	-2.2	-2.4	CD6 ligand ALCAM	cell adhesion	AA	AA
M83822	-4.9	-6.4	beige-like protein (BGL)	membrane traffic	-6.1	-5.6
U91931	-4.5	-3.6	AP-3 complex beta3A subunit	vesicle endocytosis	-4.8	-8.9
U30999	-2.2	-2.3	memc	melanoma metastasis	AA	AA
U83115	-2.1	-2.4	non-lens beta gamma-crystallin like protein (AIM1)	melanoma tumorigenicity	-1.3	-1.5
S72904	-2.2	-2.4	APK1 antigen=MAb KI recognized	tumor antigen	-5.6	-3.4
S79219	-2.3	-2.4	metastasis-associated gene	metastasis	-2.5	-2.1
D85433	-5.6	-7.6	MURR1	unknown	-3.6	-3.4
D63480	-7.4	-6.7	human KIAA0146 gene	unknown	-7.5	-6
D79994	-2	-2	human KIAA0172 gene	unknown	-1.5	-1.6
D87446	-2.1	-2.2	human KIAA0257 gene	unknown	-2.9	-4.3
L40393	-2.4	-3.7	human clone S171	unknown	-2.3	-2.6
U79260	-3.5	-3.3	human clone 23745.	unknown	-2.8	-2.6
U90902	-5.2	-5.8	human clone 23612	unknown	-3.5	-12.6
U90916	-2.9	-2.6	human clone 23815	unknown	AA	AA
U95740	-3.3	-4.3	362G6.1 gene (unknown protein CIT987SK_362G6_1)	unknown	AA	AA
X99050	-2.4	-2.7	orf gene extracted from human mRNA for 63 kDa protein	unknown	-5.3	-7.5
(B)						
M23379	-2	-1.8	GTPase-activating protein ras p21 (RASA)	signal transduction	-1.9	-4
L76937	-2	-1.5	unnamed protein gene extracted from Werner syndrome gene	DNA metabolism	-2	-2.3
U97188	-2.1	-1.9	putative RNA binding protein KOC (koc)	RNA metabolism	-1.3	-2.7
X59841	-2.1	-1.8	PBX3	development, differentiation	-2.6	-3.4
(C)						
L33801	-1.6	-2.1	protein kinase	signal transduction	-3.4	-6
U51336	-1.8	-3.3	inositol 1,3,4-trisphosphate 5/6-kinase	signal transduction	-1.7	-2.5
M68941	-1.3	-2.7	protein tyrosine phosphatase	signal transduction	-1.7	-2.6
U37352	-1.3	-2	protein phosphatase 2A Balpha1 regulatory subunit	signal transduction	-1.7	-3.3
L12535	-1.5	-2.1	ras suppressor RSU-1/RSP-1	signal transduction	-2.1	-2.6
Y11306	-1.6	-2.1	hTcf-4 gene extracted from human mRNA for beta catenin/TCF-4	transcription	-2.2	-2.9
L78833	-1.4	-2	BRCA1 gene extracted from human BRCA1, Rho7 and vatl genes	transcription, DNA repair	-1.2	-2.8
U66615	-1.6	-3.9	SWI/SNF complex 155 KDa subunit (BAF155)	chromatin remodeling	-1.2	-2
U72936	-1.2	-2	putative DNA dependent ATPase and helicase (ATRX)	chromatin remodeling	-2.5	-4.1
M14745	-1.8	-2.6	bcl-2	anti-apoptosis	-2.3	-2
D86982	-1.7	-2.1	human KIAA0229 gene	unknown	-2.2	-4.4
U96629	-1.7	-5.3	2A8.2 gene (unknown protein CIT987SK_2A8_1)	unknown	-2.2	-3.1
Z93784	-1.3	-2.1	human DNA sequence from PAC 398C22	unknown	-2.1	-3.1

AA: mRNA levels were determined to be "A (absent)" in both control and treated cells.
: genes changed more than three-fold by each of Et 743 and Pt 650 in both two cell lines.

Table 1 (*Continued*)

Up-regulated genes

Accession no.	Fold change HCT116		Gene definition	Putative function	Fold change MDA-MB-435	
(D)	Et 743	Pt 650			Et 743	Pt 650
U09579	4.3	3.2	p21/waf1/cip1	cell cycle arrest	3.3	4.3
U72649	2.8	2.1	BTG2	cell cycle arrest	1.4	1.3
M60974	2.1	2	GADD45	cell cycle arrest, DNA repair	1.4	1.1
AB000584	2.2	2.6	TGF-beta superfamily protein	growth factor inhibitor	AA	AA
L13391	2	2.3	helix-loop-helix basic phosphoprotein (RGS2/G0S8)	signal transduction	AA	AA
X83490	3.3	2	Fas/Apo-1	apoptosis	1	1
M83667	2.4	2.1	NF-IL6-beta	transcription	1.4	1.3
(E)						
HG4322-HT4592	2	1.7	beta-tubulin	cytoskeleton	1.1	1
(F)						
V00599	1.4	2	human mRNA fragment encoding beta-tubulin	cytoskeleton	1.2	2

Down-regulated genes encoding DNA binding proteins to specific promoter regions (found in the group G)

Accession no.	Fold change MDA-MB-435		Gene definition	Binding sequence	Fold change HCT116	
(G)	Et 743	Pt 650			Et 743	Pt 650
Z74792	-2.6	-3.6	CCAAT transcription binding factor subunit gamma (CBF-C/NF-YC)	CCAAT	-1.6	-1.5
M74099	-7.6	-7.1	CCAAT displacement protein	CCAAT	-4	-4.1
AF005887	-2.7	-3.5	ATF family member ATF6	CCAAT-N(9)-CCACG	-2.5	-4.9
D31716	-2.1	-2.6	GC box-bindig protein (Sp1 family member BTEB)	GGGCGG	AA	AA
L04282	-2	-2.5	CACCC box-binding protein	CACCC	-1.4	-1.7
L05515	-3.1	-4	cAMP response element-binding protein (CRE-BP1/ATF2)	TGACGTCA	AA	AA
M83233	-2.4	-4	transcription factor HTF4A	CAGCTG or CACCTG	-3.6	-4.2
Y11306	-2.2	-2.9	hTcf-4 gene extracted from human mRNA for beta catenin/TCF-4	(A/T)(A/T)CAAAG(G/C)	-1.6	-2.1

AA: mRNA levels were determined to be "A (absents)" in both control and treated cells.
: genes changed more than three-fold by each of Et 743 and Pt 650 in both two cell lines.

Up- and down-regulated genes were selected based on the same criteria as described in the legend to Fig. 6. The genes are listed according to the categories of the Fig. 6 Venn diagrams. Fold change values were calculated as the means of two independent values from duplicate array experiments. The data after antibody amplification were generally used for the calculation. Only in case non-linear saturated amplification was evident by comparing changes in average difference values for some genes with those for control probes, their fold change values were determined employing the data before antibody amplification.

there is no overlap between down-regulated genes and up-regulated ones with either Et 743 or Pt 650 treatment. These correspond well to the correlation data based on the average difference scatter plot. Third, there is also significant overlap between down-regulated genes in HCT116 and MDA-MB-435. Et 743 and Pt 650 repressed in common at least two-fold the expression of 57 and 92 genes for HCT116 and MDA-MB-435, respectively. Of these, 42 genes are shared by both cell lines. Additionally, 10 of the 50 (= 92−42) genes repressed only in MDA-MB-435 and four of the 15 (= 57−42) genes repressed only in HCT116 were down-regulated in the other cell line by either Et 743 or Pt 650 (data not shown). The comparable transcriptional repression in both cell lines, derived from completely unrelated clinical specimens, indicates the presence of a universal mechanistic basis for both compounds. Of the total 15 up-regulated genes, however, only one gene (*p21/waf1/cip1*) is common for HCT116 and MDA-MB-435, suggesting that the induction of the genes be mainly caused by independent mechanisms in these two cell lines. As mentioned below, most of the genes induced in HCT116 have been actually reported to be responsive to functional p53, which is defective in MDA-MB-435. Taken altogether, the gene expression-based profiling, along with the antitumor activity-based profiling, strongly suggests that Et 743 and Pt 650 operate by the same mode of action potentially linked to specific transcriptional repression.

2.5. Gene expression changes caused by Et 743 and Pt 650

The results summarized in Table 1 disclose that Et 743 and Pt 650 affect the expression of a variety of genes, including subsets of genes involved in DNA damage response, transcription and signal transduction. It is impressive that only 2.0 nM of each compound, a pharmacologically relevant and also physiologically achievable concentration, can exert such significant effects on the transcription of whole cells. Both compounds have been shown to be capable of interacting covalently, but reversibly, with duplex DNA. The present study further revealed that they markedly disrupted S phase progression in HCT116 cells after 12 h of treatment. Consistent with these phenotypic observations, the transcript levels of brain and reproductive organ-expressed protein (BRE) and DNA polymerase α-subunit were decreased in both cell lines. It has been found that the expression of the *BRE* gene is repressed in response to DNA damage [24]. Moreover, six of the nine genes up-regulated in HCT116 (see Table 1, groups (D), (E) and (F)), *GADD45*, *p21/waf1/cip1*, *BTG2*, a *TGF-β* superfamily member, *RGS2/G0S8* and *Fas/Apo-1*, have been reported to be p53-regulated genes induced in an early or intermediate response to p53 activation [25]. Of these, only *p21/waf1/cip1* was induced in MDA-MB-435-containing mutated p53. Thus, DNA damage caused by Et 743 and Pt 650 appears to activate p53 regulatory pathways in cells with functional

p53. However, it should be stated that the p53-dependent transcriptional changes are not the primary mechanistic basis for this class of antitumor agents because HCT116 and MDA-MB-435 cells were shown to be equally sensitive to each compound in our cell-based assays. Although cell cycle perturbation was evident in HCT116 after 12 h of treatment, changes in the expression levels of cell cycle-regulatory genes were generally inconspicuous at this time point except for some relevant genes such as *DNA polymerase α-subunit* and *p21/waf1/cip1*.

Characteristic down-regulation was observed in a subset of genes encoding DNA-binding proteins to specific promoter regions (see Table 1, group (G)). It is of particular interest that all of their binding motifs contain DNA triplets with a central guanine on either the template or antisense strand. They are certainly Et 743-targeted (also probably Pt 650-targeted) sequences. For instance, the CCAAT sequence holds a TGG site on the complementary strand, which has been shown to be one of the preferred sequences for Et 743 alkylation [10]. It is intriguing that the gene products binding to the drug-targeted DNA sequences were repressed in the mRNA expression by drug treatment. As mentioned above, Et 743 has been shown to have a specific negative effect on transcriptional activation by targeting the CCAAT box transcription factor NF-Y. It has been also proposed that the Sp1-dependent transcription requiring the GC box might be another potential target for Et 743 action [18,19,26]. With relation to these reports, the present study disclosed that the mRNA levels of NF-Y subunit C (CBF-C/NF-YC) and GC box-binding protein BTEB were decreased in both Et 743- and Pt 650-treated MDA-MB-435 cells. Furthermore, transcriptional repression of CCAAT displacement protein (CDP/*cut*) and ATF6, both of which are functionally linked to the CCAAT box, was prominent in both HCT116 and MDA-MB-435. CDP/*cut* has properties of a potent transcriptional repressor associated with a corepressor complex through interactions with histone deacetylases [27]. This protein has also been found to be within a transcriptional activating complex(es) through interactions with histone acetyltransferase coactivators CBP and PCAF [28]. ATF6 has been implicated in the endoplasmic reticulum stress response pathway as an activator of stress-induced transcription with synergistic interactions with NF-Y and YY1 [29]. A recent paper showed that ATF6 could bind to CCACG only when CCAAT exactly 9 bp upstream of CCACG was bound to NF-Y [30].

The profound transcriptional repression of these genes implies a unique mechanism of Et 743 and Pt 650 in their interaction with DNA. It is unlikely that 2.0 nM of each compound can completely eject NY-F from the high-affinity CCAAT box. This consideration is in accord with the previous observations by Bonfanti et al. [17] that inhibition of NF-Y by Et 743 in vitro required much higher drug concentrations (μM range) and that nuclear extracts from cells treated with Et 743 retained normal NF-Y complex formation. Et 743 has a tetrahydroisoquinoline subunit (subunit C) in its structure, which appears to protrude from the minor groove when the drug is bound to duplex DNA [11]. Pt 650 possesses a phthalimide moiety at the same position. Our previous structure–activity relationship studies clarified that the part of the subunit C was crucially important for the exceedingly potent antitumor activity of this series [7,8]. Depending on the structure of this part, both compounds may affect the fine architecture around some specific promoter regions like the CCAAT box, resulting in significant alterations in DNA–protein and/or protein–protein interactions relevant to transcriptional control. Given that distorted DNA structure causes malfunctioning of some important proteins nearby DNA, the down-regulation of their mRNA expression might be a reasonable cellular response based on putative feedback regulatory mechanisms. In this regard, one can note the repression of not only the genes listed in group (G) but also genes encoding coactivator 100-kDa subunit, corepressor SMRT, chromatin-associated proteins HMGIC and SWI/SNF 155-kDa subunit, and DNA repair proteins MGMT, XRCC4 and BRCA1. The negative effects on these DNA repair genes raise the possibility that Et 743 and Pt 650 are also influential in the complex formation of DNA and repair proteins, relating to a recent finding that Et 743 activity is decreased in nucleotide excision repair-deficient cell lines [16].

Another possible explanation for the characteristic down-regulation is that Et 743 and Pt 650 may prevent specifically activated transcription in these cancer cells. In fact, Et 743 has been shown to interfere with the activation of the MDR1 and HSP70 promoters without affecting the constitutive expression of the corresponding genes [18,19]. Although we examined drug effects on the constitutive transcription of more than 5000 endogenous genes, there might be some highly activated genes in the transcription levels under cancerous conditions. With relation to this speculation, Et 743 and Pt 650 down-regulated the expression of a subset of signal transduction genes encoding kinases, phosphatases and G protein signaling molecules, many of which serve as positive mediators for cell proliferation. The possibility that both compounds can exert negative effects on the promoter activity for these genes is not excluded. Further extensive promoter analyses for the genes listed here may afford a clue for a valid conclusion(s).

3. Significance

Herein described is antitumor activity- and gene expression-based profiling of Et 743 and Pt 650, demonstrating an intelligible example for integrated studies on phenotype and genotype analyses. This is the first report to reveal the global expression profiles of more than 5000 human genes

in Et 743- and Pt 650-treated cancer cells. Our results may provide potential clinical response markers for pharmacodynamic drug assessment on the basis of gene expression changes in tumors.

The remarkable similarities between Et 743 and Pt 650 with regard to (i) the profile of antiproliferative activity with many cell lines, (ii) the profile of gene expression changes and (iii) the effect on cell cycle progression would appear to imply that any biochemical effect produced by one of these agents but not the other may not represent the principal or biologically most profound mode of action of this class. The microarray data reported here are especially compelling.

4. Materials and methods

4.1. Drugs and cell culture

Et 743 and Pt 650 (Fig. 1) were synthesized as described [6,7]. Camptothecin, adriamycin, MMC and taxol were purchased from Calbiochem, and CDDP was from Sigma. All compounds were dissolved in dimethyl sulfoxide (DMSO) and added to cells with less than 1% DMSO in the final drug dilution with a culture medium. Nine colorectal (HCT116, HCT15, SW480, SW620, COLO320DM, COLO205, DLD-1, WiDr and HT-29), nine lung (PC-1, PC-9, PC-10, PC-14, A-427, NCI-H460, NCI-H520, NCI-H596 and Lu99), six gastric (MKN1, MKN7, MKN28, MKN45, MKN74 and KATO III), six pancreatic (MIAPaCa2, PANC-1, AsPC-1, KP-1, KP-4 and SUIT-2) and six breast (BSY-1, HBC-5, MDA-MB-231, MDA-MB-435, MDA-MB-453 and MDA-MB-468) cancer cell lines were all cultured in RPMI1640 (Sigma) containing 10% heat-inactivated fetal bovine serum (Life Technologies), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

4.2. Cell growth inhibition assay and COMPARE analysis based on the mean graph profiles

Exponentially growing cells were seeded at 1.0×10^3–1.0×10^4 cells per well (100 μl) in 96-well microtiter plates: 1.0×10^3 cells for NCI-H460; 1.3×10^3 cells for HCT116 and DLD-1; 2.0×10^3 cells for COLO320DM, WiDr, PC-9, PC-14, KP-1, KP-4, SUIT-2, MDA-MB-231, MDA-MB-435, MDA-MB-453, BSY-1 and HBC-5; 2.5×10^3 cells for HCT15, SW620, HT-29, PC-10, A-427, NCI-H596, Lu99, MIAPaCa2, PANC-1, AsPC-1, MDA-MB-468 and MKN28; 5.0×10^3 cells for SW480, COLO205, NCI-H520, MKN1, MKN7, MKN45, MKN74 and KATO III; and 1.0×10^4 cells for PC-1. After pre-culture for 1 day, serially increasing concentrations of test drugs in the medium were added to the plates. Following additional 3-day incubation, the antiproliferative activity was measured by the MTT colorimetric assay [20], giving the drug concentrations required for 50% growth inhibition (IC_{50} values). The activity patterns of the compounds were further compared according to the NCI cancer screening method COMPARE program [21]. Based on their mean-graph profiles, correlation coefficients between Et 743 and other drugs were calculated using a statistical program.

4.3. Monitoring the time-course of antiproliferative activity

HCT116 cells were seeded at 4.0×10^5 cells per well (2.0 ml) in six-well plates and pre-cultured for 1 day. Et 743 and Pt 650 were each added to the plates at the final concentration of 2.0 nM, and then incubation was continued for 12, 24 or 48 h. At these time points, the number of viable cells was assessed by counting the cells with a Coulter Counter instrument.

4.4. Flow cytometric cell cycle analysis

HCT116 cells were plated at 5.0×10^6 cells in 10-cm diameter dishes with 10 ml fresh medium and allowed to adhere for 1 day. Et 743 and Pt 650 were each added to the dishes at the final concentration of 2.0 nM, and then incubation was continued for 12 or 24 h. For flow cytometric analysis, the cells were fixed in 70% ethanol, treated with RNase (1.0 mg/ml), and stained with propidium iodide (50 μg/ml). The DNA content was quantitated by detecting red fluorescence with a flow cytometer (FACSCalibur, Becton-Dickinson).

4.5. High-density oligonucleotide array analysis

HCT116 and MDA-MB-435 cells were plated in 10-cm diameter dishes with 10 ml fresh medium at 5.0×10^6 and 3.0×10^6 cells per dish, respectively. After 24-h pre-incubation, the cells were treated for 12 h with 2.0 nM of each of Et 743 and Pt 650 or with 0.015% DMSO (as control). The following microarray experiments were all carried out in duplicate. Details for probe preparation and data analysis are available from Affymetrix (Santa Clara, CA, USA). Briefly, total RNA was extracted with the TRIZOL reagent (Life Technologies), and further purified with an RNeasy column (Qiagen). Double-strand cDNA was prepared from 10 μg of total RNA by using the SuperScript Choice System (Life Technologies) and T7-$d(T)_{24}$ primer. The cDNA product was purified by phenol/chloroform extraction. In vitro transcription was performed with the RNA Transcript Labeling Kit containing biotinylated UTP and CTP (Enzo Diagnostics). After purification with an RNeasy column, the cRNA was fragmented and then hybridized onto Affymetrix GeneChip HuGeneFL arrays for 16 h at 45°C. According to the EukGE-WS2 protocol, the arrays were washed and stained with streptavidin–phycoerythrin and biotinylated goat antistreptavidin on an Affymetrix fluidics station. Fluorescence intensities were captured with a Hewlett Packard confocal laser scanner. All quantitative data were processed using the Affymetrix GeneChip software [31].

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