RESEARCH ARTICLE

Anti-angiogenic effects of trabectedin (Yondelis; ET-743) on human breast cancer cells

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ABSTRACT. Trabectedin, a tetrahydroisoquinoline alkaloid derived from a Caribbean tunicate *Ecteinascidia turbinata*, has been shown to have antitumor effects. In this study, we assessed the possible anti-angiogenic effects of trabectedin on human umbilical vein endothelial cells (HUVECs) and breast cancer cell lines. An XTT cell viability assay was used to determine cytotoxicity. A scratch assay was used to detect the migration of cells after trabectedin treatment. Angiogenic cytokine profiles of breast cancer cell lines, before and after treatment with trabectedin, were investigated using an angiogenesis antibody array. Changes in mRNA expression levels of VEGF were evaluated using qRT-PCR. Trabectedin inhibited the viability of HUVECs and breast cancer cells in a concentration- and time-dependent manner. The migration of both HUVECs and breast cancer cells was suppressed by trabectedin treatment. Angiogenic cytokines which are known to regulate tumorigenicity and angiogenesis, such as GM-CSF, IGFBP-2, VEGF, and uPA, were inhibited, while several anti-angiogenic cytokines such as TIMP-1 and Serpin E1were induced in breast cancer cells. Furthermore, expression levels of VEGF mRNA were inhibited in all breast cancer cells tested. Although additional studies are needed to elucidate the molecular mechanisms underlying the anti-angiogenic activity of trabectedin, our results suggest that trabectedin may act as a potential anti-angiogenic agent in breast cancer cells.

Key words: trabectedin (Yondelis, ET-743), angiogenic cytokines, VEGF, breast cancer cells, HUVECs

Angiogenesis, the process of new blood vessel formation, plays a central role in development, invasion and metastasis in breast cancer pathogenesis [1, 2]. Tumor cells secrete various growth factors which trigger endothelial cells to form new capillaries. Prevention of the expansion of new blood vessel networks results in reduced tumor size and metastases [3]. Since angiogenesis is essential for tumor vasculature, it is considered an optimal target for anti-cancer strategies. Therefore, many researchers are testing compounds of different origins and mechanisms of action in order to develop anti-angiogenic agents as a treatment for breast cancer, or as an adjunct to standard chemotherapeutic regimens [4-7].

Trabectedin (ET-743; Yondelis) is a tetrahydroisoquinoline alkaloid derived from a Caribbean tunicate *Ecteinascidia turbinata.* It binds to guanine in the minor groove of DNA and affects various transcription factors involved in cell proliferation [8]. It has a potent antitumor activity in different *in vitro* and *in vivo* preclinical models, and its clinical activity is currently being evaluated for the treatment of a variety of cancers, including prostate and breast [9]. We have previously demonstrated that trabectedin induces cytotoxicity and DNA fragmentation, increases ROS production, and mediates apoptosis by selective activation of extrinsic and/or intrinsic pathways in two genotypically different breast cancer cell lines [10]. Based on these findings, the aim of the present study was to investigate the potential anti-angiogenic activities of trabectedin in MCF-7, MDA-MB-231 and MDA-MB-453 breast cancer cells, and human umbilical vein endothelial cells (HUVECs).

METHODS

Cell lines and reagents

Breast cancer cells (MCF-7, MDA-MB-231 and MDA-MB-453) and HUVECs were obtained from Interlab Cell Line Collection (ICLC, Genova, Italy). The cells were grown as adherent monolayers. Breast cancer cells were cultured as described in our previous studies [10, 11]. HUVECs were cultured in DMEM F12 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin in 75 cm^2 polystyrene flasks (Corning Life Sciences, UK) and maintained at 37°C in a humidified atmosphere with 5% CO_2. Cell culture supplies were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Trabectedin was provided by PharmaMar (Madrid, Spain) and was

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prepared as a 2 mM stock solution in dimethyl sulfoxide (DMSO). The DMSO concentration in the assays did not exceed 0.1% and was cytotoxic to neither breast cells nor HUVECs. All other chemicals, unless mentioned, were purchased from Sigma Chemical Co (St. Louis, MO, USA).

XTT viability assay

Cell viability was verified using the trypan blue dye exclusion test using a Cellometer automatic cell counter (Nexcelom Inc., Lawrence, MA, USA). Cells were seeded at 1×10^4 cells/well, in a final volume of 200 μL, in 96-well flat-bottomed microtiter plates. After 24 h incubation, cells were exposed to increasing concentrations of trabectedin (10^{-12}-10^{-6} M). Plates were then incubated at 37°C in a 5% CO_2 incubator for 24 and 48 h. 100 μL of XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) (Roche Applied Science, Mannheim, Germany) was added to each well, and cytotoxicity was evaluated as described in our previous study [12].

Scratch assay

The scratch assay was performed according to the protocol of Liang *et al*. [13]. Briefly, cells (2×10^6 cells/well) were plated and allowed to form a confluent mono-layer. The cell-surface was scratched using a p-200 pipette tip. After washing with PBS, the cells were treated with a medium containing IC_{10} values of trabectedin (0.08 nM for MCF-7, 0.06 nM for MDA-MB-231 and 0.01 nM for HUVECs), and 2% FBS bovine serum that allow cell survival, but not cell proliferation. Cells were allowed to fill the scratched area and were monitored over the course of 48 hours after the trabectedin treatment. The distances between the two edges of the scratch were photographed on each well using an inverted microscope at a magnification of 100×, and analyzed quantitatively by measuring the distance between the migrating cell boundaries (Leica Microsystems, Heerbrugg, Switzerland). Four different fields from each sample were considered for quantitative estimation of the distance. Since MDA-MB-453 cells grow in clumps and do not form a confluent monolayer, this assay was not suitable for these cells. Therefore, the scratch assay was not performed for the MDA-MB-453 breast cancer cells.

Profiling of angiogenic cytokines using an angiogenesis antibody array

Angiogenic cytokine profiles of breast cancer cell lines, before and after treatment with trabectedin, were investigated using a Human Angiogenesis Antibody Array (R&D Systems, UK) according to the manufacturer's instruction manual. The supernatants from untreated and trabectedin-treated cells with the IC_{50} values for 48 hours were collected and investigated. The principle of the method involved a membrane that was coated with specific antibodies for each cytokine, forming an array. After blocking the membrane, the sample was added and incubated at room temperature. Cytokine detection was completed by incubation with a biotinylated antibody followed by horseradish peroxidase-conjugated streptavidin. Chemiluminescence detection of signals was processed by the Kodak® Gel Logic 1500 imaging system. The spots were quantified using a computer-assisted system for image analysis (Koadaarray® 2.6 software); normalized intensities were calculated from each array by first subtracting the local background from each spot and then normalizing by the average intensity of the arrays. The data were then corrected for the cell protein content of each well. The relative expression level of each cytokine was calculated according to both spot pixels mean values ± standard deviation (SD) and a confidence index from 0 to 100 assigned to each spot by the Koadarray® algorithms. The spot pixel mean value represents the background-subtracted, total intensity of each spot. The spot intensity is then given by the total of all the background-subtracted values within the spot area. Pixels determined by Koadarray® to be part of an artefact are excluded. The confidence index is based on several parameters, including spot shape, intensity and homogeneity. Changes in angiogenic cytokine expression after exposure to trabectedin were expressed as a -fold decrease.

RNA isolation and real time quantitative PCR (qRT-PCR) assay

The total cellular RNA was extracted from the untreated and trabectedin-treated (with the IC_{50} values) cells ($\sim 1 \times 10^5$) using Trizol (Qiagen). The cells were lysed in 1 mL Trizol, incubated at room temperature for 5 min, and then 200 mL chloroform added to the lysate. This was then incubated for 3 min, and centrifuged for 15 min at 12,000 g at 4°C. The aqueous layer was removed, mixed with an equal volume of isopropanol, and incubated for 1 hr at 4°C. The purified RNA was precipitated by centrifugation at 12,000 g for 15 min and finally dissolved in 50 μL diethylpyrocarbonate (DEPC)-treated water. The integrity and quality of the isolated RNA was determined by running the RNAs on agarose gel electrophoresis.

One μg of the total RNA was converted to cDNA using the Quantitect reverse transcription kit (Qiagen, USA). qRT-PCR was performed on VEGF gene. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a positive control using a Real-Time™ qPCR Primer Assay (SABioscience, Frederick, MD) on the Light Cycler 480 instrument (Roche Applied Science, Mannheim, Germany). The PCR reaction mixture was prepared at a final volume of 25 μL, containing 12.5 μL of RT2 SYBR Green qPCR Master Mix, 10.5 μL of DNAase-RNaseFree water, 1.0 μL of gene-specific 10 μM PCR primer pair stock, and finally 1.0 μL of diluted cDNA sample for each primer (SABioscience). Universal cycling conditions (10 min at 95°C, 15 s at 95°C, 1 min 60°C for 40 cycles) were employed. The melting protocol consisted of 95°C for 1 minute and a continuous fluorescence reading from 65°C to 95°C at 30 acquisitions per degree and a 1°C increase per second. Data were normalized to the endogenous control GAPDH. Each replicate cycle threshold (CT) was normalized to the average CT of the endogenous control on a sample basis. The comparative CT method was used to calculate the relative quantification of gene expression. The following formula was used to calculate the relative amount of the transcripts in the extract-treated samples and the control group, both of which were normalized to the endogenous control. $\Delta\Delta CT = \Delta CT$ (extract treated) $-\Delta CT$ (control) for RNA samples. ΔCT is the $\log^2$ difference in CT between the target gene and endogenous

Table 1
IC_{10} and IC_{50} values for trabectedin in tested cell lines after 48 h of trabectedin treatment.

Cell Line	IC_{10} (nM)	IC_{50} (nM)
MCF-7	0.08 ± 0.5	4.6 ± 1.2
MDA-MB-231	0.06 ± 1.2	3.6 ± 1.4
MDA-MB-453	0.04 ± 1.4	2.8 ± 0.6
HUVEC	0.01 ± 0.8	0.5 ± 0.4

controls, and is determined by subtracting the average CT of controls from each replicate. The fold-change for each treated sample relative to the control sample = $2^{-\Delta\Delta CT}$.

Statistical analysis

Experiments were carried out in triplicate and results were expressed as mean ± S.D. Data analysis was performed and graphs prepared using Graphpad Prism 5.0 software (La Jolla, CA, USA). The difference between two groups was analyzed by a two-tailed Student's test, and that between three or more groups was analyzed using one way analysis of variance test (ANOVA) followed by Dunnett's t-test. Values with $p<0.05$ (*) were considered to be statistically significant.

RESULTS

Concentration- and time-dependent inhibition of viability in trabectedin-treated breast cancer cells and HUVECs

We investigated the effect of trabectedin on the viability of MCF-7, MDA-MB-231 and MDA-MB-453 breast cancer cells to determine the inhibitory concentration 10% (IC_{10}) and 50% (IC_{50}) values. Cells were exposed to increasing concentrations of trabectedin (10^{-12} to 10^{-6} M) for 24 and 48 h, and then the XTT cell viability assay was performed. Trabectedin decreased cell viability in a time- and concentration-dependent manner in all breast cancer cells ($p<0.05$*) (*figures 1A-C*).

The effect of trabectedin was also tested in HUVECs that have proved to be a useful tool for angiogenesis studies. Capillary endothelial cells proliferate in response to an angiogenic stimulus during neovascularization [3]. Trabectedin decreased the viability of HUVECs in a time- and concentration-dependent manner ($p<0.05$*) (*figure 1D*). As shown in *figure 1D*, there were 36%, 45% and 73% decreases in the viability of HUVECs exposed to 10^{-12}, 10^{-9} and 10^{-7} M trabectedin, respectively, when compared to untreated controls at 48 h (*$p<0.05$). The IC_{10} and IC_{50} values for trabectedin in HUVECs after 48 h of trabectedin treatment are shown in *table 1*.

Subtoxic concentrations (IC_{10}) of trabectedin were selected for further functional assays in order to ensure that the inhibitory effects of trabectedin on invasion and migration are not as a result of cell death.

Effect of trabectedin on cell migration

Next, we performed an *in vitro* scratch assay and an *in vitro* 'wound-healing' assay, to assess the activity of trabectedin on the migration of MCF-7, MDA-MB-231 breast cancer cells and HUVECs. As shown in *figure 2*, microscopic analysis evaluating cell capacity to migrate and fill the empty areas at different times showed that, in the absence of trabectedin, substantial numbers of cells migrating within the wound area were detectable after 24-48 h, and that treatment with trabectedin significantly inhibited this migration in all cell lines tested ($p<0.05$*).

Changes in the secretion of angiogenic cytokines by trabectedin in breast cancer cells

To increase our understanding of the molecular mechanism of trabectedin-induced migration changes in breast cancer cells, we examined the expression of angiogenic cytokines after trabectedin treatment (*table 2*). Angiogenesis cytokine array results revealed that granulocyte-macrophage colony stimulating factor (GM-CSF), insulin-like growth factor binding protein 2 (IGFBP-2), vascular endothelial growth factor (VEGF) and u-plasminogen activator (uPA) were the angiogenic cytokines inhibited by trabectedin, while tissue inhibitor of metalloproteinases 1 (TIMP-1) and plasminogen activator inhibitor, (serpin E1) were the anti-angiogenic cytokines that were induced by trabectedin in all breast cancer cells tested.

There were significant increases in serpin E1 and TIMP-1 levels of 3.5- and 4.2- fold in MCF-7 cells exposed to trabectedin for 48 h. Tissue factor, dipeptidyl peptidase 4 (DPPIV/CD26), GM-CSF, insulin-like growth factor binding protein 1 (IGFBP-1), IGFBP-2, interleukin 8 (CXCL8/IL-8), urokinase type plasminogen activator (uPA), heparin-binding EGF-like growth factor (HB-EGF) and VEGF secretions were significantly decreased 8.4-, 6.2-, 2.1-, 4.8-, 3.2-, 4.6-, 6.2-, 2.2- and 6.8-fold, in MCF-7 cells ($p<0.05$) (*table 2*). In MDA-MB-231 cells, serpin E1 and TIMP-1 levels were significantly increased by 5.2- and 4.6-fold by trabectedin treatment, while tissue factor/factor III, DPPIV/CD26, chemokine (C-X-C Motif) ligand 16 (CXCL 16), activin A, IGFBP-2, GM-CSF, HB-EGF, matrix metalloproteinase 8 (MMP-8), matrix metalloproteinase 9 (MMP-9), interleukin 1 receptor beta (IL-1β), CXCL8/IL-8, uPA and VEGF levels were significantly decreased by 3.4-, 4.2-, 4.4-, 2.4-, 2.6-, 2.8-, 3.6-, 3.1-, 2.9-, 3.2-, 2.6-, 2.2- and 6.4-fold ($p<0.05$) (*table 2*). In MDA-MB-453 cells, there were significant increases in angiopoietin-1, endoglin, platelet factor 4 (PF4), serpin E1, TIMP-1, TIMP-4 and vasohibin levels of 2.1-, 2.6-, 3.5-, -2.8, 3.0-, 2.2- and 2.5-fold by trabectedin treatment. Angiogenin, CXCL16, GM-CSF, IGFBP-2, MCP-1, MMP-8, MMP-9, thrombospondin-1 and VEGF levels were significantly decreased by 2.0-, 3.5-, 2.5-, 4.7-, 3.5-, 6.2-, 2.1-, 6.0-, 8.4-fold in MDA-MB-453 cells by trabectedin treatment ($p<0.05$) (*table 2*).

Inhibition of VEGF mRNA expression levels in breast cancer cells

The expression levels of VEGF, which is found to be significantly decreased in angiogenesis array analyses, were investigated using qRT-PCR after exposure to trabectedin (with the IC_{50} values) for 24 and 48 h. Trabectedin reduced VEGF mRNA levels 2.0-fold in MCF-7, 3.0-fold in

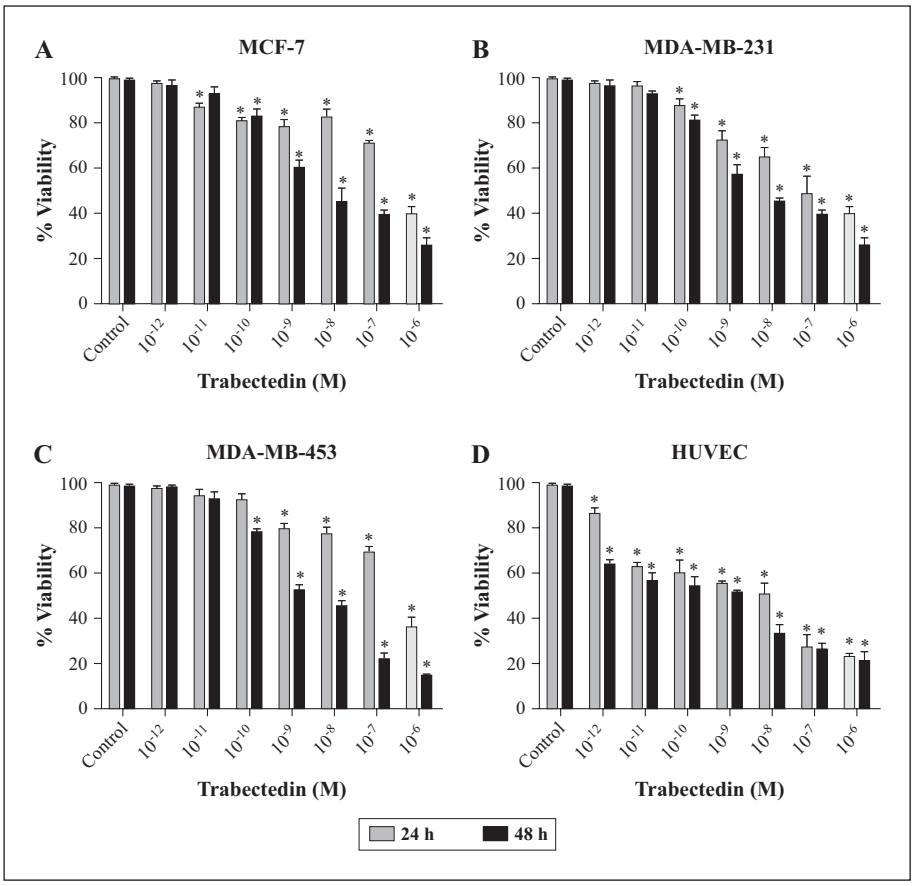

Figure 1
Inhibition of viability in trabectedin-treated **(A)** MCF-7,**(B)** MDA-MB-231,**(C)** MDA-MB-453 breast cancer cells and **(D)** HUVECs at 48 h. Cytotoxicity was determined using the XTT cell viability test. The results are expressed as the mean of three different experiments (± SD) (*P<0.05 compared to untreated control).

MDA-MB-231 and 4.5-fold in MDA-MB-453 cells at 24 h, and 3.8-fold in MCF-7, 4.9-fold in MDA-MB-231 and 7.0-fold in MDA-MB-453 cells at 48 h as compared to untreated control (p<0.05*) (*figure 3*).

DISCUSSION

Despite advances in the detection and treatment of breast cancer, it is still the most common, and remains the leading cause of cancer-related deaths in women [14]. It is therefore of the utmost importance that the search for new chemotherapeutic agents to treat breast cancer patients that have not responded to conventional therapies such as surgery, radiation, chemotherapy and hormone therapy continues.

Trabectedin is a marine-derived, anti-cancer drug that presents certain unique features. Unlike conventional anti-tumor agents, it binds the minor groove of DNA, bending the DNA towards the major groove. However, its mechanism of action is not entirely understood. Previously, we demonstrated that trabectedin exerted anti-proliferative and pro-apoptotic effects in breast cancer cells [10]. In the present study, we investigated the anti-angiogenic effects of trabectedin in MCF-7, MDA-MB-453 and MDA-MB-231 breast cancer cells.

Angiogenesis, the formation and growth of new blood vessels, is crucial for the proliferation and spread of cancer cells. As a result, angiogenesis has become an important target for anti-cancer therapies in a variety of malignant breast tumors. Tumor cells trigger epithelial cells to form new capillaries by secreting various angiogenic cytokines. We found that trabectedin inhibited certain cell functions, such as cell proliferation and migration. Moreover, trabectedin reduced the levels of several cytokines that are known to regulate tumorigenity and angiogenesis, while inducing some anti-angiogenic cytokines in breast cancer cells. Thus, one of the mechanisms that might account for this effect of trabectedin on breast cancer cell proliferation and migration may be its inhibitory impact on angiogenic cytokines. The effect of trabectedin on angiogenic cytokines were investigated in immune cells [monocytes, macrophages, tumor-associated macrophages (TAM)] and freshly isolated ovarian tumor cells, and it was found that it reduced the production of CCL2 and IL-6. In myxoid liposarcoma primary tumor cultures, it has been shown that treatment with noncytotoxic concentrations of trabectedin inhibits the production of CCL2, CXCL8, IL-6, VEGF, and PTX3 in *in vitro* and xenograft models [15]. This is the first study investigating the effect of trabectedin on angiogenic cytokines in breast cancer cells.

The serine protease inhibitor superfamily (serpins) members regulate cell adhesion, motility, apoptosis, and angiogenesis and are critically involved in mammary gland development. Serpin E1 interacts with vitronectin and uPA/uPAR complexes, and has dramatic effects on the ability of cells to attach, migrate and detach from their substratum. Moreover, as mentioned above, the inhibitory action of serpin E1 could favor tumor progression by preventing excessive degradation of the ECM by uPA,

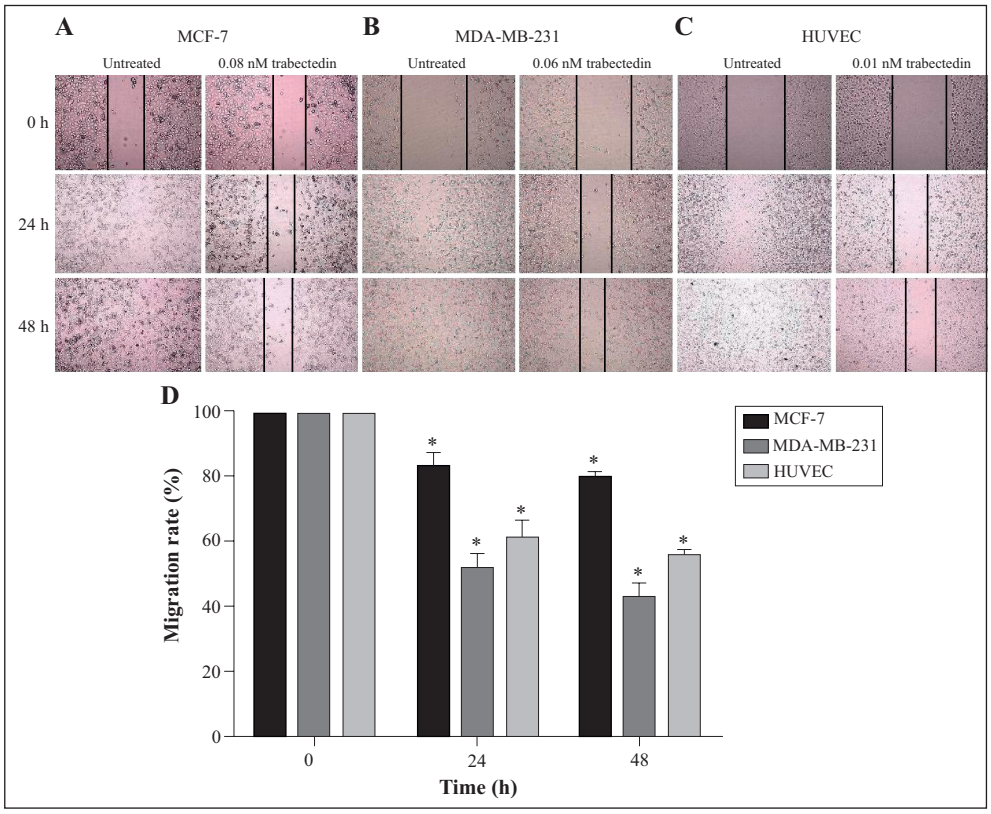

Figure 2

Effects of trabectedin on the cell migration of **(A)** MCF-7,**(B)** MDA-MB-231 and **(C)** HUVECs. Cell migration was analyzed by the scratch assay. Cells were treated with the IC10 values for trabectedin for 24 and 48 h. (D) Scratched areas were measured and normalized relative to the control values that were assumed at 100%. The data from four independent experiments were collected and the mean values were plotted with ±SD. The graph showed that the cell migration rates of the trabectedin-treated cells were significantly reduced in comparison with the untreated control cells (*P<0.05).

sustaining mitogenic cell stimuli initiated by uPA/uPAR complex and thus promoting tumor angiogenesis [16, 17]. Trabectedin treatment significantly induced serpin E1 levels and decreased uPA levels in all breast cancer cells tested.

GM-CSF was originally identified as a hematopoietic growth factor, but it is also produced by a variety of nonhematopoietic cells, including fibroblasts, endothelial cells, keratinocyte and tumor cells [18-21]. It has been found that overexpression of GM-CSF in tumor cells results in increased cell proliferation and migration *in vitro*, while *in vivo* expression by tumor cells contributes to increased malignancy and angiogenesis [22-25]. GM-CSF levels were significantly decreased by trabectedin treatment in all breast cancer cells.

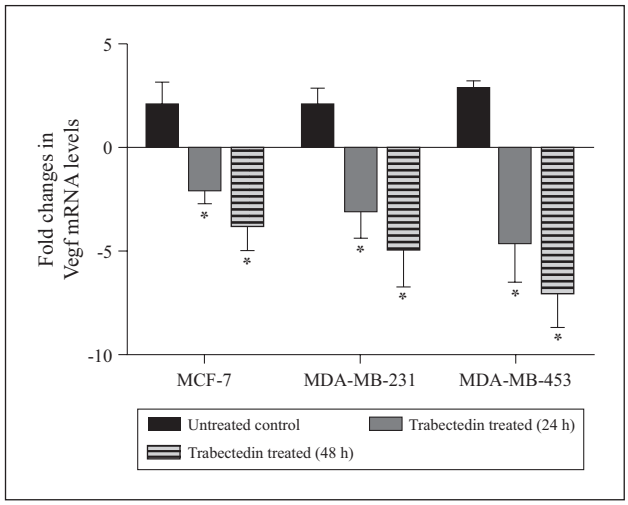

Figure 3

Inhibition of VEGF mRNA levels in trabectedin-treated breast cancer cells (*P<0.05). The mRNA expression levels of VEGF were investigated using qRT-PCR after exposure to trabectedin (MCF-7: 4.6 nM, MDA-MB-231: 3.6 nM, MDA-MB-453: 2.8 nM) for 24 and 48 h.

IGFBP-2 is the other cytokine that was decreased by trabectedin treatment in all breast cancer cells. The insulin-like growth factor binding proteins (IGFBPs) are a family of six proteins that bind with high affinity to IGF-I and IGF-II, thereby modulating the prosurvival and mitogenic effects of IGF [26]. IGFBP-2 is found to be overexpressed during breast cancer progression [27]. Recent studies have suggested that not only does IGFBP-2 have a direct, proliferative effect on tumor growth, it is also associated with increased cellular invasion and migration [28].

The TIMPs inhibit active MMPs by forming non-covalent complexes with them and are thus considered to inhibit carcinoma invasion and metastasis. TIMP-1 is capable of inhibiting the activities of all known MMPs except MT-MMPs, and plays a key role in maintaining the balance between ECM deposition and degradation [29]. Trabectedin induced TIMP-1 in all breast cancer cells and also significantly decreased the MMP-8 and MMP-9 levels in MDA-MB-231 and MDA-MB-453 cells.

Angiogenesis requires stimulation of vascular endothelial cells through the release of angiogenic peptides, of which VEGF is the most potent. VEGF is expressed by most tumor types, including breast cancer, and it is suggested

Table 2
Angiogenic cytokine profile of human breast cancer cells after trabectedin treatment. The relative expression level of each cytokine was calculated according to both spot pixels mean values ± SD and a confidence index from 0 to 100 assigned to each spot by the Koadarray® algorithms ($p<0.05$) (ns: non-significant as compared to untreated controls)

		Fold-change		
Abbreviation	**Cytokine name**	**MCF-7**	**MDA-MB-231**	**MDA-MB-453**
VEGF	Vascular endothelial growth factor	- 6.8 ± 0.8	-6.4 ± 0.8	-8.4 ± 0.8
TIMP-1	Tissue inhibitor of metalloproteinases 1	+ 4.2 ± 1.2	+4.6 ± 1.6	+3.0 ± 1.6
TIMP-4	Tissue inhibitor of metalloproteinases 4	ns	ns	+2.2 ± 1.8
GM-CSF	Granulocyte macrophage colony stimulating factor	-2.1 ± 0.4	-2.8 ± 0.8	-2.5 ± 1.2
IGFBP-1	Insulin like growth factor binding protein 1	- 4.8 ± 2.4	ns	ns
IGFBP-2	Insulin like growth factor binding protein 2	- 3.2 ± 1.0	-2.6 ± 0.8	-4.7 ± 0.8
uPA	Urokinase type plasminogen activator	- 6.2 ± 2.2	-2.2± 0.6	+2.2 ± 1.6
Serpin E1/PAI-1	Plasminogen activator inhibitor	+ 3.5 ± 0.8	+ 5.2 ± 1.8	+2.8 ± 1.2
DPPIV/CD26	Dipeptidyl peptidase 4	- 6.2 ± 1.4	- 4.2 ± 1.2	ns
CXCL8/IL-8	Interleukin 8	- 4.6 ± 1.6	-2.6± 0.8	ns
CXCL16	Chemokine (C-X-C Motif) ligand 16	ns	-4.4 ± 1.4	-3.5 ± 1.4
HB-EGF	Heparin binding EGF like growth factor	- 2.2 ± 1.6	-3.6 ± 1.4	ns
TF	Tissue factor/Factor III	- 8.4 ± 1.2	- 3.4 ± 1.4	ns
PF4	Platelet Factor 4	ns	ns	+3.5 ± 1.8
MMP-8	Matrix metalloproteinase 8	ns	-3.1 ± 2.1	-6.2 ± 2.4
MMP-9	Matrix metalloproteinase 9	ns	-2.9 ± 2.6	-2.1 ± 2.6
	Activin A	ns	-2.4 ± 1.2	ns
IL-1β	Interleukin 1 receptor beta	ns	-3.2 ± 1.2	ns
ANG	Angiogenin	ns	ns	- 2.0 ± 1.4
AGP1	Angiopoietin-1	ns	ns	+2.1 ± 0.2
END	Endoglin	ns	ns	+2.6 ± 2.1
MCP-1/CCL2	Chemokine (C-C Motif) Ligand 2	ns	ns	-3.5 ± 1.6
THBS1	Thrombospondin-1	ns	ns	-6.0 ± 0.2
VASH1	Vasohibin	ns	ns	+2.5 ± 1.2

that the VEGF content of tumor cells correlates with the prognosis of patients with breast cancer [30]. The presence of its receptors (VEGFR1 and VEGFR2) both on endothelial cells and tumor cells suggests that VEGF may play additional roles in tumor pathology such as involvement in autocrine-dependent tumor cell proliferation and invasion [31]. Trabectedin significantly inhibited the mRNA and protein expression levels of VEGF in all breast cancer cells tested.

Inhibition of a single molecule or cell signaling pathway is unlikely to be an effective treatment of breast cancer; therefore the current paradigm for breast cancer treatment is to either combine drugs or to find novel drugs that modulate multiple targets. Trabectedin has multiple effects in breast cancer cells: in addition to its direct growth inhibitory and apoptotic effects, it affects the production of key angiogenic cytokines. Reduction of these key angiogenic cytokines may represent one of the most important mechanisms of its antitumor effect. Although additional studies are needed to elucidate the molecular mechanisms underlying the anti-angiogenic activity of trabectedin, our results suggest that it could be acting as an anti-angiogenic agent which might explain its anti-tumour activity.

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