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Cancer Research

Antitumor and Anti-inflammatory Effects of Trabectedin on Human Myxoid Liposarcoma Cells

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Abstract

Inflammatory mediators present in the tumor milieu may promote cancer progression and are considered promising targets of novel biological therapies. We previously reported that the marine antitumor agent trabectedin, approved in Europe in 2007 for soft tissue sarcomas and in 2009 for ovarian cancer, was able to downmodulate the production of selected cytokines/chemokines in immune cells. Patients with myxoid liposarcoma (MLS), a subtype characterized by the expression of the oncogenic transcript *FUS-CHOP*, are highly responsive to trabectedin. The drug had marked antiproliferative effects on MLS cell lines at low nanomolar concentrations. We tested the hypothesis that trabectedin could also affect the inflammatory mediators produced by cancer cells. Here, we show that MLS express several cytokines, chemokines, and growth factors (CCL2, CCL3, CCL5, CXCL8, CXCL12, MIF, VEGF, SPARC) and the inflammatory and matrix-binder protein pentraxin 3 (PTX3), which build up a prominent inflammatory environment. *In vitro* treatment with noncytotoxic concentrations of trabectedin selectively inhibited the production of CCL2, CXCL8, IL-6, VEGF, and PTX3 by MLS primary tumor cultures and/or cell lines. A xenograft mouse model of human MLS showed marked reduction of CCL2, CXCL8, CD68+ infiltrating macrophages, CD31+ tumor vessels, and partial decrease of PTX3 after trabectedin treatment. Similar findings were observed in a patient tumor sample excised after several cycles of therapy, indicating that the results observed *in vitro* might have *in vivo* relevance. In conclusion, trabectedin has dual effects in liposarcoma: in addition to direct growth inhibition, it affects the tumor microenvironment by reducing the production of key inflammatory mediators. *Cancer Res; 70(6); 2235–44. ©2010 AACR.*

Introduction

Trabectedin (ET-743; Yondelis) is the first marine-derived anticancer drug that has reached the market. It was approved in Europe in 2007 for second line treatment of soft tissue sarcomas and in 2009 for relapsed ovarian cancer in combination with pegylated liposomal doxorubicin. Studies are in progress to ascertain its efficacy in other neoplastic diseases, including breast and prostate cancer, as suggested by the early clinical trials. Originally derived from the marine tunicate *Ecteinascidia turbinata*, it is now obtained by a semisynthetic method starting from cyanosaframycin B, an antibiotic that can be produced in large quantities by fermentation of *Pseudomonas fluorescens* (1). Trabectedin is a DNA-binding agent interfering with gene transcription, regulation, and DNA repair machinery (2, 3), and is able to induce cell cycle perturbation with a delayed S phase progression and accumulation of cells in G_2 phase (4). Its mechanism of action is incompletely understood and presents some unique features. Unlike most conventional antitumor agents, it binds the minor groove of DNA at the N2 position of guanines, bending the DNA sharply towards the major groove (3, 5, 6); its cytotoxicity seems to be dependent on the efficiency of transcription-coupled nucleotide excision repair, a deficiency of which makes cells less sensitive to the drug; instead, cells carrying defects of homologous recombination repair, e.g., for *BRCA* gene mutations, are especially sensitive (7–9).

A distinct histologic subtype of soft tissue sarcoma is the myxoid liposarcoma (MLS) which is particularly susceptible to trabectedin (10–12). Second line treatment in patients with MLS was reported to be exceptionally effective with

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long-lasting responses higher than 50% and ~20% of pathologic complete responses (10). Most MLS carry the fusion gene *FUS-CHOP*, caused by the t(12:16) chromosome translocation, considered the pathogenetic lesion of this tumor (13–15). *FUS* encodes an RNA and DNA binding protein. *CHOP* is a stress-induced transcription factor forming DNA binding dimers with CAAT/enhancer binding proteins, acting as a negative regulator. Members of the CAAT/enhancer binding protein family have major roles in adipogenesis and their function can be subverted by FUS-CHOP, with a consequent block in adipogenesis differentiation. The oncogenic potential of the transcript has been proven in *FUS-CHOP* transgenic animals which develop liposarcoma and in transformed mesenchymal progenitors (16, 17). The high susceptibility of MLS raised the question of whether *FUS-CHOP* is a target of trabectedin. A study conducted on MLS cells showed that trabectedin does not affect the transcription of the *FUS-CHOP* chimera, but blocks its transactivating ability by inducing the detachment of FUS-CHOP protein from the promoters of target genes (18).

In patients with MLS undergoing trabectedin therapy, it is not infrequent to observe clinical responses after several cycles of chemotherapy, unlike what happens with most cytotoxic drugs. In addition, radioimaging (magnetic resonance imaging and computed tomography scans) show that clinical responses are associated with a decrease in tumor density that precedes tumor shrinkage (10, 19). It is therefore suspected that trabectedin plays biological effects on the tumor microenvironment other than direct cytotoxicity to cancer cells.

On the basis of our previous findings that trabectedin reduces the production of some cytokines/chemokines in immune cells (20), we postulated the hypothesis that a reduction of inflammatory mediators at the tumor site might contribute to its antitumor efficacy. It is now established that a persistent cancer-related inflammation promotes tumor cell proliferation, survival, and spread (21–23). Genetic events causing neoplasia actively support the construction of an inflammatory milieu. Examples of oncogene-driven activation of the inflammatory cascade include the oncogene *RET* in thyroid papillary carcinoma, and *RAS, BRAF,* or mutated tumor suppressor genes in different tumors (22, 24, 25). Little is known about the downstream target genes activated by the oncogenic transcript *FUS-CHOP*, particularly inflammatory genes, with the exception of IL-6, and recently, IL-8 (26, 27).

In this work, we investigated the spectrum of inflammatory mediators present in the microenvironment of MLS and the effect of trabectedin treatment *in vitro* and *in vivo*. We show that MLS cancer cells build up a prominent inflammatory microenvironment. Trabectedin significantly reduced the expression and production of CCL2, CXCL8, IL-6, VEGF, and the proinflammatory protein and matrix binder pentraxin 3 (PTX3). Mice transplanted with human MLS showed lower infiltration of CD68 macrophages and lower numbers of vessels after therapy. This anti-inflammatory effect of trabectedin might contribute to its antitumor efficacy in MLS.

Materials and Methods

Drug. Trabectedin (PharmaMar, Spain) was dissolved in DMSO to 1 mmol/L, and kept at −20°C.

MLS cell lines. MLS cell lines, 402.91 and 1765, the fibrosarcoma cell line HT1080 and its subline transfected with FUS-CHOP, were kindly provided by Dr. Pierre Aman (University of Gothenburg, Sweden). Cells were grown in RPMI 1640 with 10% FCS (Lonza), Ultraglutamine 1 200 mmol/L (Lonza), and Pen/Strep. The liposarcoma cell line SW872, lacking FUS-CHOP (kindly provided by Prof. Bussolati, University of Parma, Parma, Italy), was grown in complete DMEM + 10% FCS. Tumor cell lines were routinely verified by morphology, growth curve analysis, and tested for *Mycoplasma*. Primary cultures of human MLS were obtained from surgical specimens. Tumors were minced and enzymatically digested with 500 units/mL of collagenase II and 2 units/mL of DNase for 1 h at 37°C. Cell suspension was filtered and the cells were seeded in ISCOV-1 medium + 10% FCS, L-glutamine 200 mmol/L, and Pen/Strep.

Establishment of MLS xenografts. Fragments of human MLS tumor biopsies were established as xenografts through serial transplantation in nude mice for a limited number of passages. The presence of fusion transcript type corresponding to the original tumors was assessed by fluorescence *in situ* hybridization analysis at each passage. Mice were treated with trabectedin (0.15 mg/kg, q7dx3) starting when tumors were palpable. Tumors for immunohistochemistry (n = 3) were excised 48 h after the third dose. Five to seven sections were analyzed from each tumor.

Cell cycle analysis, growth inhibition, cytotoxic effect, and apoptosis. Exponentially growing cells were treated for 1 h, or as specified, with different concentrations of trabectedin, then washed with PBS and fresh drug-free medium was added. The growth-inhibitory effect of trabectedin was evaluated at different times after drug washout. The effect of trabectedin on clonogenicity was evaluated as previously described (28). Cell cycle analysis was performed by flow cytometry on cells fixed in 70% cold ethanol and kept at 4°C before DNA staining. To detect bromodeoxyuridine (BrdUrd) incorporated into DNA, fixed cells were incubated with anti-BrdUrd monoclonal antibody (Becton Dickinson) and DNA detected by propidium iodide (4). To analyze apoptotic death after trabectedin treatment, mRNA levels were tested with TaqMan Human Apoptosis Array (Applied Biosystems), following the instructions of the manufacturer.

Measurement of inflammatory mediators. Tumor cell lines were starved (without FCS) for 18 h and treated with different doses of trabectedin for 4 h. Cells were washed and stimulated or not with tumor necrosis factor α (TNFα; 20 ng/mL). Total RNA extraction was done with Trizol. cDNA was synthesized by random priming from 1 μg of total RNA with GeneAmp RNA PCR kit (Applied Biosystems). mRNA levels of 91 genes related to inflammation were tested with custom TaqMan Human Inflammation Array (Applied Biosystems). Real-time PCR was performed using SYBR Green dye. Sequences of specific primer pairs (Invitrogen) were

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the following: h*CCL2*—For, AAGATCTCAGTCCAGAGGCTCG; Rev, CACAGATCTCCTTGGCCACAA; h*CXCL8*—For, CCAGGAAGAAACCACCGGA; Rev, GAAATCAGGAAGGCTGCCAAG; h*PTX3*—For, CGAAATAGACAATGGACTCCATCC; Rev, CAGGCGCACGGCGT; h-*Actin*—For, TCACCCACACTGTGCCCATCTACGA; Rev, CAGCGGAACCGCTCATTGCCAATGG.

For each gene, mRNA was normalized to β-actin mRNA (ΔCt). Results are shown as arbitrary units ($2^{-\Delta Ct} \times 10{,}000$). Fold change ($2^{-\Delta\Delta Ct}$) of trabectedin-treated cells is calculated compared with the untreated cells.

Protein levels were measured by ELISA in cell supernatants collected at 24 h culture after drug treatment (4 h) and washout. Under these conditions, cell viability was checked and was usually >87% for all drug concentrations shown. IL-1β, IL-6, IL-10, CCL2, CXCL8, TNFα, and VEGF were determined by the BioPlex Protein Array Luminex 100 System (Bio-Rad). Data acquisition and analysis were done using BioPlex Manager software. PTX3 detection was measured by ELISA as previously described (29).

Immunohistochemistry. Antibodies used for immunohistochemistry of surgical MLS specimens were antihuman CD31 (clone JC70A; Dako), antimouse CD31 (clone MEC13.3; PharMingen), antimurine CD68 (clone FA-11; HyCult Biotechnology), antihuman CD68 (clone KP1; Dako), antihuman CCL2 (clone 5D3-F7; Alexis Biochemicals), antihuman CXCL8 (clone 6217; R&D Systems), antihuman PTX3 (polyclonal), and antimurine PTX3 (clone MNB1; Alexis Biochemicals).

Patients with MLS. Eighteen patients were admitted at the National Cancer Institute (Milan Italy) with persistent disease after standard chemotherapy and were eligible to trabectedin. In all patients, the diagnosis of MLS was confirmed by histology and cytogenetic analyses (presence of FUS-CHOP transcript). Blood samples were collected immediately

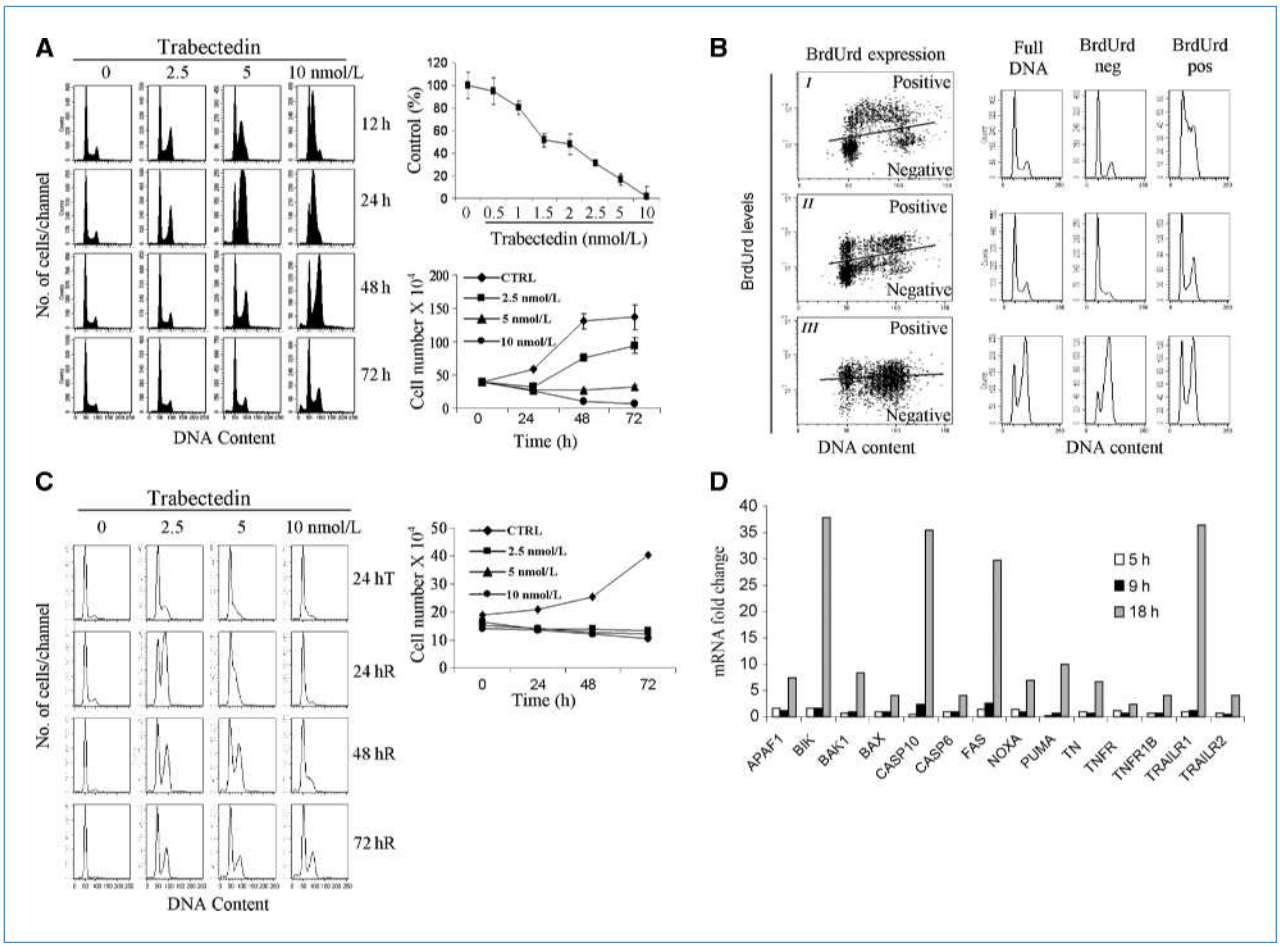

Figure 1. Cell cycle perturbation and growth inhibition in MLS cells. A, cells were treated with trabectedin for 1 h at the indicated concentrations, washed, and cultured at different time points before testing. Top right, inhibition of proliferation of 402.91 cells in clonogenic assays tested at 15 d. Points, mean of three experiments (five replicates); bars, SD. Left, cell cycle perturbation induced by trabectedin (1 h) in 402.91 cells and performed at different time intervals after drug washout. Bottom right, cell counts over 72 h. B, biparametric BrdUrd/DNA flow cytometric analysis performed in control cells (I) at 0 h, after 24 h (II), and finally, post–trabectedin washout (III). The DNA histograms of the whole population (Full DNA), BrdUrd-negative (G_1 and G_2-M), and BrdUrd-positive (S) are shown. C, left, cell cycle perturbation induced by 24 h of trabectedin exposure on a primary MLS culture at different time intervals after drug washout (24 h T, treatment for 24 h; 24–48–72 h R, times of recovery after drug washout). Right, cell counts over 72 h. D, upregulation of mRNA levels of apoptotic genes at three different time points after drug treatment (5 nmol/L).

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Table 1. TaqMan human inflammation array

(A) mRNA levels of inflammation-related genes in tumor cell lines and surgical tumor samples

Gene	402.91	1765	HT1080	HT1080 FUS-CHOP	Tumor 1	Tumor 2
CCL2	3,329	590	23	178	316	116
CCL3	Und.	Und.	1.2	1.0	2,879	222
CCL5	314	87	44	497	956	174
CXCL8	4,352	3,815	1,975	5,946	1,147	23
CXCL12	Und.	1.82	Und.	Und.	60,965	32,087
CXCR4	0.2	1.4	Und.	Und.	236	76
TNF	4.5	2.7	4.8	12	51	5.8
TNFRS1A	72	41	187	439	9,037	1,508
TNFRS1B	11	3.3	1.6	46	649	103
IL-1β	73	129	656	55	415	68
IL-6	64	58	85	2.6	Und.	Und.
M-CSF	175	88	314	254	1,382	529
MIF	592	451	2,891	6,417	51,408	15,104
PTX3	3,171	717	728	3,438	63,863	7,051
SPARC	2,717	960	1,036	1,582	112,667	19,738
TGFβ	20	34	321	1,349	4,027	613
VEGF	17	13.4	93	192	8,173	1,088
ANGPT1	Und.	Und.	0.6	0.5	19,801	3,820

(B) Effect of trabectedin on TNF-stimulated cells (fold change)

Gene	402.91	1765	HT1080	HT1080 FUS-CHOP
CCL2	0.25	0.21	0.08	0.06
CCL5	0.69	0.32	0.17	0.18
CXCL8	0.81	0.40	0.35	0.53
TNF	4.3	2.1	0.8	0.6
TNFRS1A	0.9	1.1	0.8	1.2
TNFRSF1B	1.6	1.0	0.2	1.0
IL-1β	0.81	0.35	0.39	0.36
IL-6	3.31	1.44	0.02	0.75
M-CSF	1.33	0.90	0.97	1.52
MIF	1.44	1.60	1.01	1.39
PTX3	0.50	0.45	0.66	0.54
SPARC	1.08	1.35	0.95	1.60
TGFβ	1.28	1.48	1.0	1.0

NOTE: A, total RNA was extracted from TNFα-stimulated 402.91 and 1765 MLS cell lines, fibrosarcoma HT1080 cell line and its variant FUS-CHOP–transfected, and from surgical samples from two patients with MLS (tumor 1 and tumor 2). Results are shown as arbitrary units ($2^{-\Delta Ct} \times 10{,}000$) that correlate with transcriptional activity of genes. B, cell lines treated with trabectedin (5 nmol/L). Results are expressed as fold change ($2^{-\Delta\Delta Ct}$) of trabectedin-treated cells compared with untreated. Und.: undetermined (i.e. > 35 cycles).

before therapy and at the indicated time points. Plasma PTX3 levels were measured by ELISA.

Statistical analysis. Prism software (GraphPad) and Microsoft Excel were used for all statistical analyses. Student's *t* tests were used to determine statistically significant differences between experimental groups.

Results

Trabectedin induces cell cycle perturbation in MLS cells and triggers cell death. We first investigated the direct effects of trabectedin on human MLS. A short 1-hour exposure affected the proliferation of the cell line 402.91. Figure 1A (top right) shows the results of a representative clonogenic experiment: a strong dose-dependent effect was observed with 50% reduction at concentration of 1.5 nmol/L. Cell cycle analysis, performed at different time intervals after drug treatment, showed that trabectedin induced a block of cell proliferation and a dose-dependent accumulation of cells in S phase (5 and 10 nmol/L), followed by a G_2-M block (Fig. 1A, left). A strong growth inhibition consequent to G_2-M block is depicted in Fig. 1A (bottom right). Biparametric

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flow cytometric BrdUrd/DNA analysis showed that at 24 hours after drug treatment (1 hour) and washout, both BrdUrd-positive (i.e., cells in S phase during drug treatment) as well as BrdUrd-negative (i.e., cells in G_1 or G_2-M phase during drug treatment) accumulated in G_2-M phases (Fig. 1B). Similar results were observed with primary cultures obtained from a MLS surgical specimen (Fig. 1C). Prolonged drug exposure to trabectedin induced a cytotoxic effect in 402.91 and 1765 cell lines; after 48 hours of continuous exposure to 2.5 nmol/L, cell death was 70% and 50%, respectively. At 24 hours, it was <20% at 5 nmol/L (data not shown). The majority of cells were in necrosis but a proportion of Annexin V+ PI– cells (22%) was also detected; as shown in Fig. 1D (bottom), some apoptosis genes were upregulated at 18 hours: BIK, FAS, TRAILR1 (DR4), and caspase 10. Overall, the results show that MLS cells are highly susceptible to trabectedin within the low nanomolar range.

MLS cells express inflammatory mediators and build up an inflammatory microenvironment. As little is known about the presence of an inflammatory microenvironment in human MLS, we first explored a wide panel of biological mediators with a customized TaqMan Inflammation Array. mRNA was obtained from (*a*) frozen sections of two distinct human MLS surgical samples; (*b*) the MLS cell lines 402.91 and 1765; (*c*) the fibrosarcoma cell line HT1080 and its FUS-CHOP–transduced variant. Table 1 shows that several inflammatory mediators were highly expressed in the tumor milieu of the two surgical MLS samples. For instance, chemokines (*CCL2, CCL3, CCL5, CXCL8,CXCL12, CXCR4*), cytokines and growth factors (*TNF* and receptors, *IL-1β, M-CSF, MIF, TGFβ,VEGF,* and *angiopoietin 1*), and matrix-binding proteins (*PTX3* and *SPARC*). PTX3, originally described by our group, is an inflammatory protein acting as a soluble pattern recognition receptor and is also involved in the organization of the extracellular matrix (ECM; refs. 29, 30). In addition, molecules related to infiltrating myeloid cells (CD68, CD14, M-CSFR; Supplementary Fig. S1) were highly expressed. Some inflammatory mediators were also elevated in the cell lines 402.91 and 1765: *CCL2, CCL5, CXCL8, PTX3, SPARC, M-CSF*, and *MIF*. The FUS-CHOP–transfected HT1080 cell line was tested to investigate the inflammation-related genes activated downstream of the oncogenic transcript distinctive of MLS. HT1080-FUS-CHOP cells expressed higher levels of *CCL2, CCL5, CXCL8, MIF, PTX3, TGFβ,* and *VEGF* compared with HT1080 cells, indicating that the transactivating ability of FUS-CHOP indeed involves genes of the inflammatory response.

Selected factors were confirmed by immunohistochemistry in surgical tumor specimens as well as in MLS xenografts

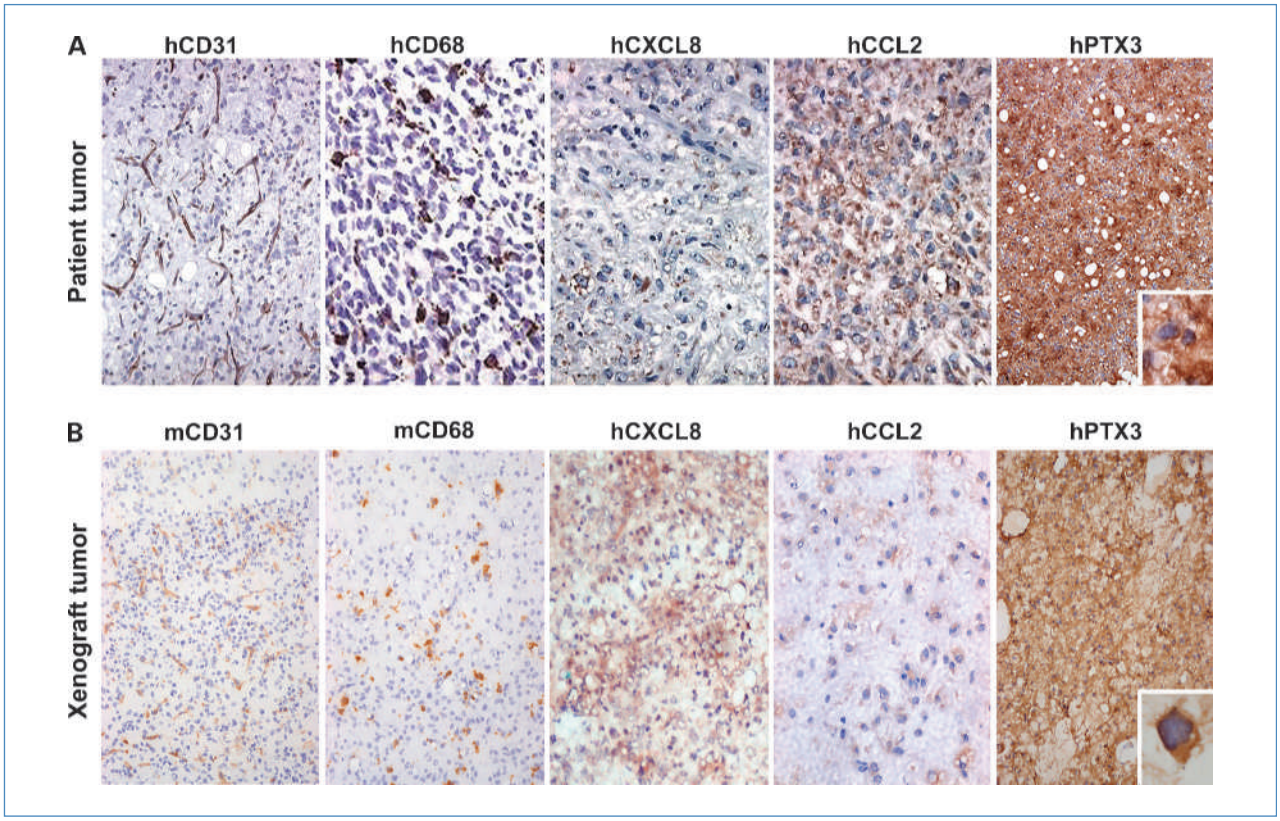

Figure 2. Immunohistochemistry of CD31, CD68, CCL2, CXCL8, and PTX3 in tumor tissues from (A) patient MLS surgical specimens and (B) MLS xenografts established in nude mice (m, antimurine antibodies; h, antihuman antibodies). Original magnifications: anti-CD31, antimouse CD68, and anti-PTX3 (×10); antihuman CD68, anti-CXCL8, and anti-CCL2 (×20); insets (×100).

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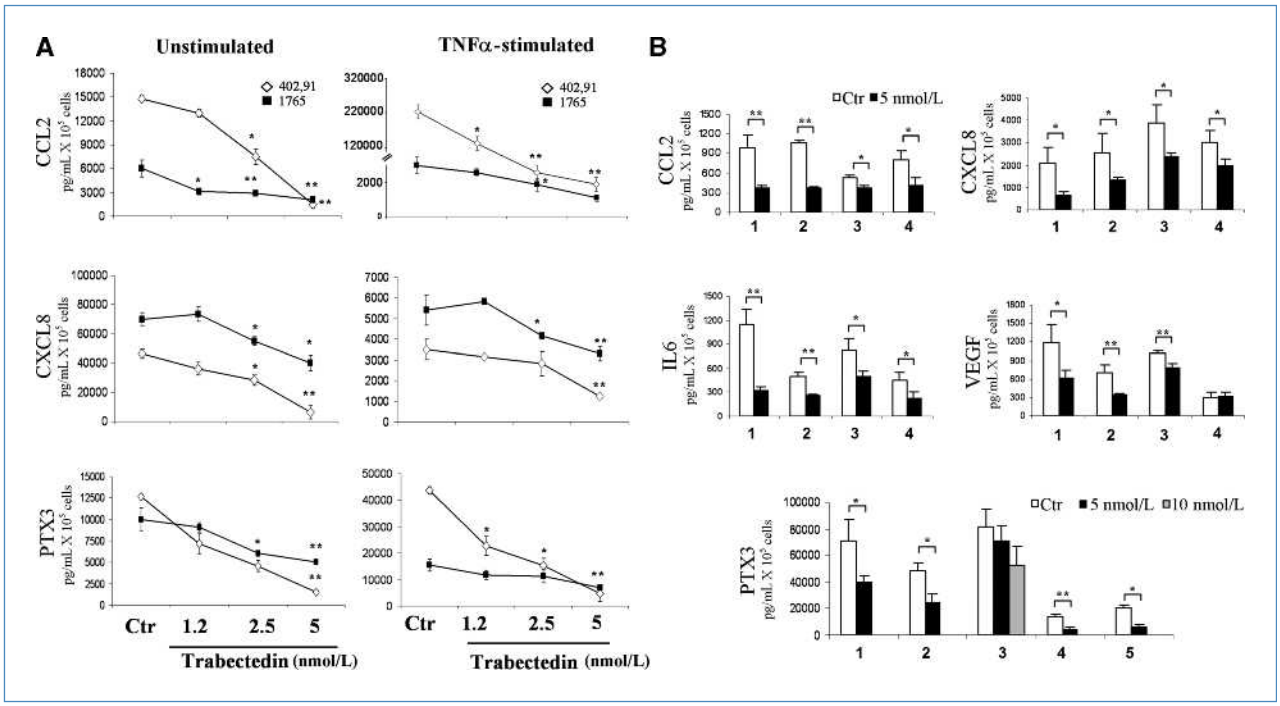

Figure 3. Trabectedin inhibits the production of inflammatory mediators in MLS cells. A, CCL2, PTX3, and CXCL8 protein levels in 402.91 and 1765 cells measured by ELISA. Cells were treated for 4 h with trabectedin, washed and incubated with or without 20 ng/mL of TNFα for 24 h. Results are from a representative experiment (four to eight experiments performed). B, CCL2, CXCL8, IL-6, VEGF, and PTX3 protein levels in primary MLS cultures measured by ELISA. Cells were treated for 4 h with trabectedin, washed and incubated for 24 h. Results are from four different primary MLS cultures (five cultures for PTX3). Trabectedin: 5 nmol/L (black columns), 10 nmol/L (gray column). *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

established in nude mice from patient tumor samples. Genetic analysis of xenografted MLS confirmed that growing tumors retained the original fusion transcript type (data not shown). Staining for CXCL8, CCL2, PTX3, and for macrophage infiltration (CD68) and vessels (CD31) are shown in Fig. 2. Both samples from patient tumors (A) and xenograft tumors (B) have a florid angiogenesis, a remarkable macrophage content, and tumor cells producing CXCL8 and CCL2. The whole sections stained strongly for PTX3, which was secreted by cancer cells (insets). To further control PTX3 origin in xenograft tumors, an antimurine PTX3 was used. Supplementary Fig. S2 shows that tumor cells were negative, only few scattered stromal cells were positive.

Overall, the results highlight that human MLS build up a prominent inflammatory microenvironment. We next determined the effect of trabectedin on this inflammatory network.

***Trabectedin inhibits the production of selected inflammatory mediators* in vitro.** *In vitro* treatment of the two cell lines (402.91 and 1765) with trabectedin reduced mRNA levels of selected mediators: *CCL5, CXCL8, IL1β* in 1765 cells, and *CCL2* and *PTX3* in both cell lines (Table 1). Other expressed genes (*TGFβ, TNFRII, M-CSF, MIF,* and *SPARC*) were unaffected, ruling out the occurrence of a nonspecific cytotoxic effect. In HT1080-FUS-CHOP cells, trabectedin strongly reduced the expression of *CCL2, CXCL8,* as well as *PTX3* (Table 1). Selected mediators were corroborated by real-time PCR in untreated and TNF-stimulated 402.91 and 1765 cells. Inhibition of *CCL2, CXCL8,* and *PTX3* was confirmed after trabectedin treatment (Supplementary Fig. S3)

Determination of protein levels was performed by ELISA in cell supernatants. In the absence of exogenous stimuli, considerable levels of CCL2, CXCL8, and especially of PTX3, were secreted by both cell lines. Lower levels of TNFα, IL-1β, IL-6, IL-10, and VEGF were also detected (Supplementary Fig. S4). To assess the inhibitory effect of trabectedin, cell supernatants were collected after 24 hours of drug treatment (4 hours) and washout. Under these conditions, cell viability was usually >87% for all drug concentrations shown. Both constitutive and TNF-induced production of all three inflammatory mediators was dose-dependently reduced, with a more dramatic effect in TNF-induced production. Figure 3A shows representative experiments. Mean inhibition of CCL2 was 61.2% and 64.6% at 2.5 nmol/L of trabectedin for 402.91 (n = 8 experiments) and 1765 (n = 6), respectively; at 5 nmol/L, a complete block of CCL2 production was observed (>88% for both cell lines). CXCL8 levels were also reduced with 64.9% and 38.8% inhibition at 5 nmol/L (402.91 and 1765, respectively; n = 4). For PTX3, reduction was higher in 402.91 cells (60.5% at 2.5 nmol/L and 79.6% at 5 nmol/L) compared with 1765 cells (39.6% at 2.5 nmol/L and 50% at 5 nmol/L; n = 6). Cell-associated PTX3 was tested by Western blot in 402.91 cells and was also downmodulated by 50% (Supplementary Fig. S5).

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We next investigated cytokine/chemokine and PTX3 production by primary tumor cultures derived from surgical MLS samples. As observed with the cell lines, primary MLS cultures constitutively produced CCL2, CXCL8, IL-6, VEGF, and high levels of PTX3 (Supplementary Fig. S4). Figure 3B shows that CCL2 and CXCL8 were reduced by trabectedin with variable levels of inhibition. The mean decrease of CCL2 was 50.82% (range, 29.4–64.8%), whereas CXCL8 decreased by 48.7% (range, 38.4–68.2%). IL-6 and VEGF were also significantly downmodulated, the mean inhibition of IL-6 was 52% (range, 38.8–72.4%), whereas VEGF was at 40.2% (range, 23.1–50.5%) in three out of four cultures. PTX3 was also reduced, with a mean inhibition of 44.1% (range, 13.3–70%). Culture no. 3 secreted very high levels of PTX3 and a decrease was evident only at 10 nmol/L.

Of interest, in parallel experiments, we noticed that PTX3 levels (mRNA and protein) were not decreased in lipopolysaccharide-stimulated monocytes. We therefore tested other PTX3-producing cells. TNF-stimulated human umbilical vascular endothelial cell and a liposarcoma cell line (SW872) not harboring the FUS-CHOP transcript were not inhibited in their PTX3 production (Supplementary Fig. S6), indicating that trabectedin inhibits PTX3 only in FUS-CHOP–positive cells. Overall, a brief exposure to trabectedin significantly reduced the production of key inflammatory mediators by MLS cell lines and primary cultures.

***Trabectedin reduces the inflammatory microenvironment* in vivo.** To investigate whether a reduction of inflammatory mediators also occurs *in vivo*, mice bearing xenograft tumors were treated with three doses of i.v. trabectedin; tumor samples were excised 48 hours after the third dose and stained for CXCL8, CCL2, PTX3, macrophage infiltration, and vessels. Pictures from untreated (Fig. 4A) and trabectedin-treated animals (Fig. 4B) are shown. The number of CD68+ infiltrating macrophages significantly decreased after trabectedin from 72.4 ±8 (mean ± SD, three tumors) to 30.2 ± 6 ($n = 3$; $P < 0.05$). Tumor vessels were also strongly reduced, from 40.5 ± 7($n = 3$) to 10.2 ± 5 ($n = 3$; $P < 0.05$) in untreated and treated animals, respectively. A clear inhibition of CXCL8 and CCL2 staining and a partial reduction of PTX3 were observed after treatment.

We had the opportunity to test surgical MLS patient samples resected after several cycles of trabectedin therapy, from whom the original bioptic tissue was available. Figure 5A shows tissues from a patient with clinical response to trabectedin. The surgical sample excised after therapy had a marked decrease in CD31+ vessels and CD68+ macrophages; PTX3 positivity was also strongly decreased compared with the sample before therapy (Fig. 5A). These findings indicate that the reduction of inflammatory mediators observed *in vitro* with trabectedin may have *in vivo* relevance.

Our group tested the potential diagnostic and prognostic value of plasma PTX3 levels in different pathologies, including infectious, cardiovascular, and autoimmune diseases (29, 30). As MLS cancer cells are potent producers of PTX3, it was of interest to investigate the circulating levels of PTX3 in

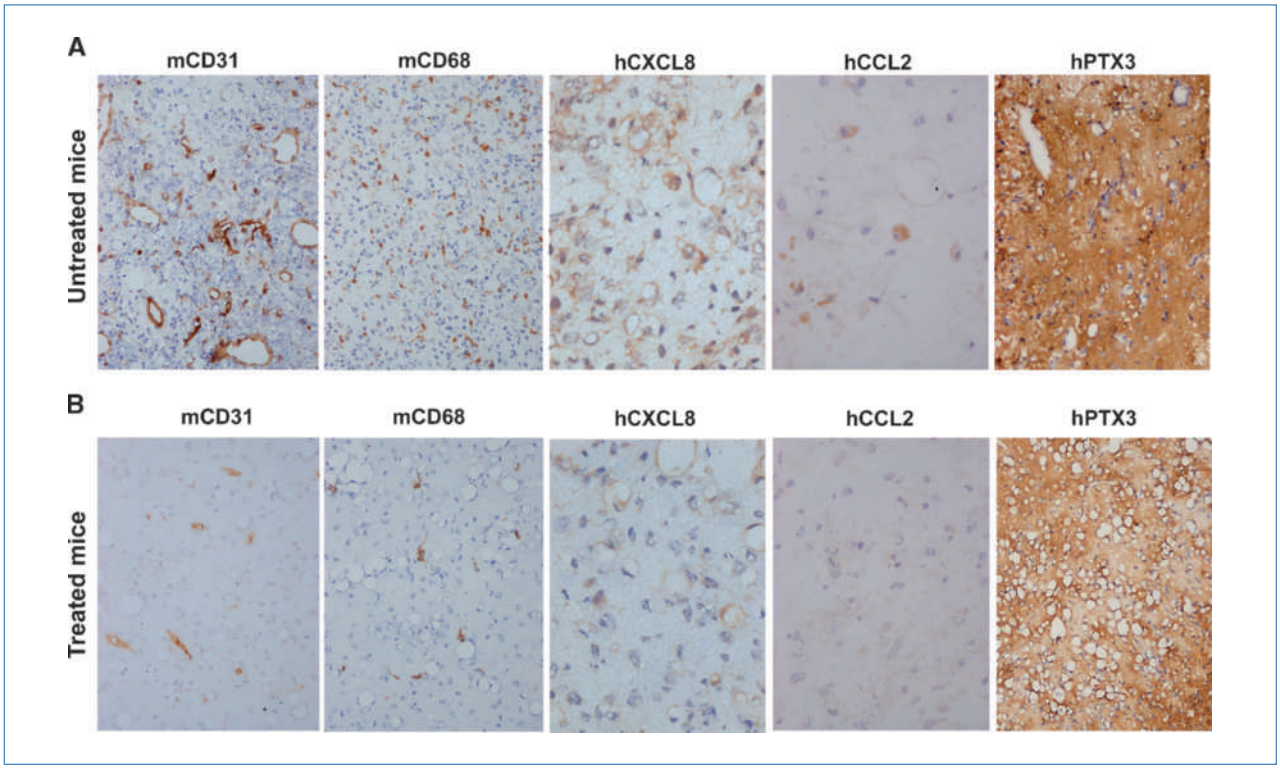

Figure 4. Immunohistochemistry of antimurine CD31 and CD68 and antihuman CCL2, CXCL8 and PTX3 in MLS xenografts before (A) and after (B) trabectedin treatment *in vivo*. Original magnifications: anti-CD31, anti-CD68, and anti-PTX3 (×10); anti-CXCL8 and anti-CCL2 (×20).

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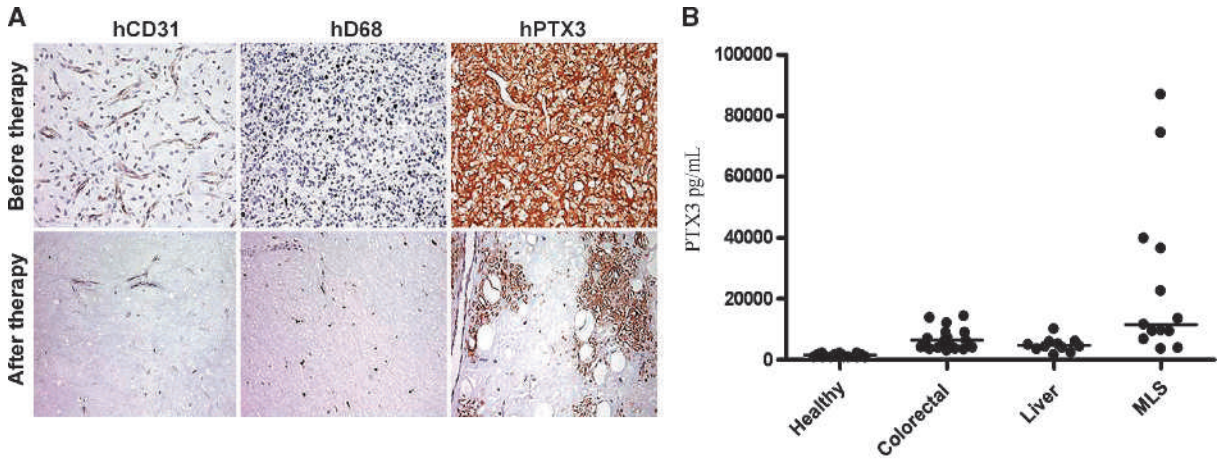

Figure 5. A, immunohistochemistry of CD31, CD68, and PTX3 in the bioptic sample of MLS tissue performed before therapy (top) and on the surgical sample obtained after trabectedin therapy (bottom). B, PTX3 plasma levels in patients with MLS and in patients with colorectal and liver cancer. C, PTX3 plasma levels in patients with MLS receiving trabectedin therapy. PTX3 measurement was performed before therapy (white columns) and at the indicated cell cycles (gray columns). SD, stable disease; PR, partial response; MR, mixed response; TR, tissue response (decreased tissue density); PD, progressive disease.

these patients. Figure 5B shows that median plasma levels of PTX3 were significantly higher (11,540 pg/mL) compared with those of healthy donors (2,000 pg/mL) or of cancer patients with other malignancies (colorectal and liver cancer). Of 11 patients undergoing therapy with trabectedin, it was possible to follow the plasma levels of PTX3 in seven cases. A decrease of circulating PTX3 was observed in four patients experiencing a measurable clinical response according to the Response Evaluation Criteria in Solid Tumors (ref. 10; Fig. 5C). In three other patients (two with low levels of plasma PTX3), no modification was observed in spite of one partial response, one stable disease, and a mixed response. Thus, plasma PTX3 levels in patients with MLS are considerably elevated; the significance of high PTX3 levels and its validity as a biomarker of response to therapy in these patients needs to be further investigated.

Discussion

In this study, we have evaluated multiple effects of trabectedin on MLS cell lines, primary tumor cultures, and MLS xenografts as a direct antiproliferative effect and for the reduction of selected proinflammatory mediators. In MLS cells, this compound induces a marked growth inhibition due to a delay in S phase and a G_2-M block and, in a fraction of cells, subsequent activation of apoptosis. Inhibition of proliferation *in vitro* was observed at very low concentrations (1–3 nmol/L) and short exposure times (1 hour), that are realistically achievable according to pharmacokinetic data (19). Indeed, trabectedin showed high efficacy in advanced MLS (10). This liposarcoma subtype is characterized by the presence of the oncogenic fusion transcript *FUS-CHOP* (16, 17). Whether *FUS-CHOP* is a target of trabectedin is an open issue. Several tumor types which do not express FUS-CHOP are susceptible to the drug (e.g., other sarcomas, breast and ovarian carcinoma). Recent studies indicate that trabectedin has no effect on the transcription of *FUS-CHOP* gene, but blocks its transactivating ability causing the detachment of the FUS-CHOP protein from the promoter of target genes (18).

The mechanism of action of this compound is not limited to binding and damage of DNA but also includes the transcriptional inhibition of selected genes (2). We previously showed that trabectedin reduces the production of CCL2 and IL-6 in activated macrophages (20). These and other mediators of the inflammatory response are frequently expressed in the tumor microenvironment by infiltrating leukocytes, stromal and cancer cells themselves (21–23). It is generally considered that the presence of a

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smoldering inflammation in neoplastic tissues promotes tumor cell survival and proliferation, as well as the maintenance of a reactive stroma with neoangiogenesis and matrix turnover (21–23). Here, we show that MLS cells produce *in vitro* and *in vivo* several cytokines, chemokines, and growth factors, and that the oncogenic transcript FUS-CHOP is involved in the activation of the inflammatory cascade. Trabectedin significantly reduced CCL2, CXCL8, as well as IL-6 and VEGF in MLS cells and xenografts. Reduction of these key inflammatory mediators may represent an important mechanism of its antitumor efficacy.

CCL2 is a major chemokine promoting monocyte recruitment at sites of inflammation and tumors, and its expression significantly correlates with macrophage accumulation (31, 32). In most studies, CCL2-mediated attraction of macrophages is associated with the angiogenic switch and poor prognosis (22, 33–36). The chemokine CXCL8 is a potent mediator of angiogenesis enhancing endothelial cell proliferation, chemotaxis, survival, and protease activation (32, 37). Moreover, CXCL8 is directly mitogenic for some cancer cells and plays a critical role in tumor growth at metastatic sites (32, 37–39). In xenograft-bearing mice treated with trabectedin therapy, the reduced expression of CCL2 and CXCL8 was paralleled by a marked decrease in the number of infiltrating macrophages and vessels. In primary MLS cultures, IL-6 and VEGF were also downmodulated by trabectedin. VEGF is a key angiogenic factor, which certainly cooperates with the florid vessel network of MLS. IL-6 is a growth-promoting and antiapoptotic inflammatory cytokine (21, 40–43) and a major effector signal of activated nuclear factor κB in the promotion of neoplasia (44, 45).

In a previous collaborative study, we reported that PTX3 was one of the most expressed genes in MLS (46). We found that trabectedin caused a marked downmodulation of PTX3 mRNA and protein. PTX3 belongs to the family of acute phase proteins (which also includes CRP and SAP) and plays an important role in innate resistance to pathogens (30). In addition, PTX3 has been shown to be essential for the construction of the ECM by forming complexes with the TNF-regulated protein TSG6 which, in turn, combines with hyaluronan (47). In the tumor microenvironment, the constitution of a dynamic stroma is of overriding importance for the enlarging tumor mass. Our data also show that trabectedin affects the transcription and production of PTX3 only in the presence of the *FUS-CHOP* transcript. No inhibition was observed in a FUS/CHOP-negative liposarcoma cell line, nor in monocytes and endothelial cells. Forni and colleagues recently observed by chromatin immunoprecipitation experiments that the *FUS-CHOP* transcript is bound to the PTX3 promoter in the 402.91 cell line, providing a possible mechanistic explanation for this selectivity (18). Immature adipocytes produce PTX3 in response to TNF and IL-1, and PTX3 mRNA is reduced during terminal differentiation by long-chain fatty acids (48). Thus, PTX3 is transiently expressed during normal adipocyte differentiation (48) and the high levels in MLS may reflect a "frozen" undifferentiated state.

Whether a decrease in PTX3 levels is relevant for the antitumor activity of trabectedin in MLS is unknown. We attempted to study *in vitro* the effect of PTX3 on MLS cell lines 402.91 and 1765. PTX3 inhibition with blocking antibodies, or its silencing in 402.91 cells with short interfering RNA did not significantly impair tumor proliferation, nor susceptibility to trabectedin (data not shown). More in general, the role of PTX3 in cancer in unclear and its involvement, particularly in MLS biology, deserves further investigation. Of note, MLS cell lines also expressed high levels of SPARC, another matricellular protein that has been investigated in several tumor types (49, 50). Unlike PTX3, SPARC was not modulated by treatment with trabectedin.

In conclusion, we have examined the possible mechanisms of action of trabectedin in human MLS. Beyond a direct and strong growth-inhibitory effect on cancer cells, this drug significantly hampered the production of selected proinflammatory cytokines such as IL-6, CCL2, CXCL8, VEGF, and PTX3. The inhibition of some mediators was confirmed *in vivo* in animals bearing MLS xenografts, paralleled by a marked decrease of infiltrating macrophages and tumor vessels. Downregulation of key inflammatory molecules in the tumor microenvironment might be of benefit in light of the increasingly clear relationship between persistent inflammation and cancer progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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